Advances in Pharmacological Sciences

Nature-Inspired Drugs: Expanding Horizons of Contemporary Therapeutics

Lead Guest Editor: Azhar Rasul
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Editorial Board

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Editorial

Nature-Inspired Drugs: Expanding Horizons of Contemporary Therapeutics

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In this special issue, a total of 61 articles were received and 20 of them were approved for publication. This special issue demonstrated the ever-growing role of natural products as lead structures for the treatment of cancer, microbial infections, oxidative stress-associated health ailments, neurodevelopmental disorders, and polycystic ovary syndromes. Phytochemical investigations of natural products for the exploration of bioactive entities, eco-friendly, rapid, and cost-effective synthesis of drugs from natural products, utilization of green chemistry approaches, and optimization of nature-derived compounds for the synthesis of potent derivatives can build up stronger foundation of nature-inspired drug discovery.

This special issue has successfully attracted various interesting research articles and reviews addressing several aspects of natural product-based drug discovery, nature-inspired synthesis of nanoparticles, synthetic analogues of natural products as novel anticancer agents, cost-effective green chemistry approaches, and nanocarriers for enhancing the natural drug delivery system. For example, M. Azeemuddin et al. have attempted to explore the role of a polyherbal formulation, DXB-2030, to reverse the TP-induced polycystic ovary syndrome in rat models and demonstrated that DXB-2030 has a potential ability to enhance GLUT4 expression, to downregulate testosterone and cystic follicles, thus, recommending its usage for the treatment of polycystic ovary syndromes and inviting other researchers to explore more about DXB-2030's mechanism of action. Cost of the drug should be a key consideration while working on drug discovery. D. A. Jamdade et al. have reported an eco-friendly and low-cost synthesis method of copper nanoparticles from medicinal plants which have capability to inhibit porcine pancreatic α-amylase activity and α-glucosidase activity, thus, opening up avenues for development of antidiabetic nanomedicine. S. Biswas et al. found 3-hydroxyflavone analogue as a novel inhibitor of epigenetic enzyme, histone deacetylase 8 (HDAC8), which is a therapeutic drug target for cancer. This study also paves a way for further investigations on 3-hydroxyflavone analogue in *in vivo* studies.

This issue also gathered several studies that have explored the capability of various plants for their antimicrobial, antidiabetic, and antimutagenic potential. This screening has identified various novel plants and described their mechanism of action.

Furthermore, this special issue has also published few interesting review articles addressing various aspects of phytochemicals such as pharmacological profile, therapeutic potential, current status in drug discovery, and efficient drug delivery by plant-based nanocarriers. For example, S. Chanda et al. reviewed various nutraceuticals having therapeutic potential and provided the classification of nutraceuticals based upon their mechanism of action, chemical nature, and food availability. However, M. Gharbavi et al. have discussed diversified structures, synthesis approaches, techniques for characterization, and routes of administration of noisome to overcome the blood-brain barrier for the development of efficient drug delivery systems.

Therefore, this issue will hopefully pave a way for researchers and encourage the scientific research community for further research in this field for the development of safer, selective, and cost-effective drugs from natural products.

Conflicts of Interest

The editors declare that there are no conflicts of interest regarding the publication of this article.

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Research Article

Gnidia glauca- and *Plumbago zeylanica*-Mediated Synthesis of Novel Copper Nanoparticles as Promising Antidiabetic Agents

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Guest Editor: Ghulam Hussain

Rapid, eco-friendly, and cost-effective one-pot synthesis of copper nanoparticles is reported here using medicinal plants like *Gnidia glauca* and *Plumbago zeylanica*. Aqueous extracts of flower, leaf, and stem of *G. glauca* and leaves of *P. zeylanica* were prepared which could effectively reduce Cu^{2+} ions to CuNPs within 5 h at 100°C which were further characterized using UV-visible spectroscopy, field emission scanning electron microscopy, high-resolution transmission electron microscopy, energy dispersive spectroscopy, dynamic light scattering, X-ray diffraction, and Fourier-transform infrared spectroscopy. Further, the CuNPs were checked for antidiabetic activity using porcine pancreatic α-amylase and α-glucosidase inhibition followed by evaluation of mechanism using circular dichroism spectroscopy. CuNPs were found to be predominantly spherical in nature with a diameter ranging from 1 to 5 nm. The phenolics and flavonoids in the extracts might play a critical role in the synthesis and stabilization process. Significant change in the peak at ~1095 cm^{-1} corresponding to C-O-C bond in ether was observed. CuNPs could inhibit porcine pancreatic α-amylase up to 30% to 50%, while they exhibited a more significant inhibition of α-glucosidase from 70% to 88%. The mechanism of enzyme inhibition was attributed due to the conformational change owing to drastic alteration of secondary structure by CuNPs. This is the first study of its kind that provides a strong scientific rationale that phytogenic CuNPs synthesized using *G. glauca* and *P. zeylanica* can be considered to develop candidate antidiabetic nanomedicine.

1. Introduction

Nature has an infinite collection of medicinal plants which serve as repository of bioactive principles that are considered as complementary and alternative medicine. Combinatorial chemistry, nanotechnology, and cutting edge research on nutraceuticals have helped to expand the horizons beyond contemporary therapeutics [1, 2]. Interdisciplinary research has enabled to exploit the medicinal plants which are storehouses of diverse groups of phytochemicals for fabrication of novel nanomedicine with broad-spectrum therapeutic applications [2–5]. Numerous medicinal plants like *Dioscorea bulbifera*, *Dioscorea oppositifolia*, *Gloriosa superba*, *Barleria prionitis*, and *Litchi chinensis* are used for synthesis of gold, silver, platinum, and palladium nanoparticles with antimicrobial, antibiofilm, and anticancer activities [6–12]. However, there is a lacuna in the area of synthesis of copper nanoparticles (CuNPs) using medicinal plants. Hereby, synthesis of therapeutic CuNPs using medicinal plants has drawn considerable attention recently. Among various nanoparticles, CuNPs have gained wide applications in photothermal ablation, photoacoustic

imaging, drug delivery, theranostics, electrical conductors, biochemical sensors, electrocatalysis, photocatalysis, and catalytic organic transformations [13, 14]. Although there are various physical and chemical routes for synthesis of CuNPs, the involvement of hazardous and toxic chemicals poses a threat to the environment and compromises with the biocompatibility [15]. Hence, there is a growing need to develop the green synthesis approach for fabrication of stable CuNPs with therapeutic significance.

Gnidia glauca is also known to be of utmost medicinal importance as it is used for treatment of cancers, burns, wounds, abdominal pain, snake bites, and sore throat. Similarly, leaves are applied to treat back ache, joint aches, contusions, and swellings [16, 17]. Another medicinal plant, *Plumbago zeylanica*, has exhibited carminative, anthelmintic, anti-inflammatory, antiplasmodial, antimicrobial, antifungal, antihyperglycemic, hypolipidaemic, and antiatherosclerotic activities [18]. Further, it is used in the treatment of piles, rheumatic pain, diarrhoea, dysmenorrheal, anemia, contusion of the extremities, leprosy, ulcers, and furunculous scabies [19]. It is rich in coumarins like seselin, 5-methoxyseselin, suberosin, xanthyletin, and xanthoxyletin [19]. Alkaloids, glycoside, reducing sugars, simple phenolics, tannins, lignin, saponins, and flavonoids are found in the leaves of *P. zeylanica* which are caustic, vesicant, and aphrodisiac [20]. Thus, from the above information, it is evident that both *G. glauca* and *P. zeylanica* can be used for synthesis of metal nanoparticles since they are treasure house of both reducing as well as capping agents. However, there are no reports of synthesis and therapeutic applications of CuNPs from *G. glauca* or *P. zeylanica*. Hereby, there is a huge scope to design novel routes for synthesis of therapeutically active CuNPs using both the plants.

Development of antidiabetic nanomedicine is one of the thrust areas of nanotechnology as it is estimated that, by 2030, diabetes mellitus-afflicted population will shoot up to 366 million. Type II diabetes mellitus (T2DM) is the most prevalent ailment both globally as well as in the Indian subcontinent. Hereby, there is a continuous need to develop and screen novel antidiabetic agents that can target elevated postprandial hyperglycemia [17]. Herein, we report for the first time on the fabrication of CuNPs using *G. glauca* and *P. zeylanica* followed by characterization and evaluation of antidiabetic and antioxidant activity.

2. Materials and Methods

2.1. Chemicals. α-Glucosidase and 4-nitrophenyl α-D-glucopyranoside were obtained from Sigma Aldrich, USA. DNSA (3,5-dinitrosalicylic acid) was obtained from SRL Pvt. Ltd. (Mumbai, India). Copper sulphate, dipotassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), methanol, sodium potassium tartarate, and sodium hydroxide (NaOH) were procured from Qualigens, Mumbai, India. Porcine pancreatic α-amylase and sodium chloride (NaCl) were obtained from HiMedia Laboratories, Mumbai, India. Acarbose was obtained from Bayer Pharmaceuticals Pvt. Ltd. (Mumbai, India). All the chemicals and reagents procured were of AR grade.

2.2. Plant Material and Preparation of Extract. *G. glauca* flowers, leaves, and stems and *P. zeylanica* leaves were collected from the Western Ghats of Maharashtra and shade-dried for 2-3 days at room temperature. The dried plant materials were reduced to fine powder using an electric blender. *G. glauca* flower extract (GGFE), leaf extract (GGLE), and stem extract (GGSE) were prepared by adding 5 g of the powdered plant material in 100 mL distilled water in a 250 mL Erlenmeyer flask, followed by boiling at 100°C for 5 min. Similarly, *P. zeylanica* leaf extract (PZLE) was prepared. After filtering the extract through a Whatman No.1 filter paper, the filtrate was collected and stored at 4°C for further use [21].

2.3. Synthesis and Characterization of Copper Nanoparticles. Synthesis of CuNPs was initiated by addition of 5 ml of GGFE, GGLE, GGSE, and PZLE separately to 95 ml of 1 mM aqueous $CuSO_4{\cdot}5H_2O$ solution and incubated in darkness at 100°C. UV-visible spectra were recorded at regular intervals on a spectrophotometer (SpectraMax M5, Molecular Devices Corporation, Sunnyvale, CA) operated at a resolution of 1 nm; also, visible colour change was monitored to confirm the reduction of Cu^{2+} ions to CuNPs. Bioreduced CuNPs were characterized by employing a field emission scanning electron microscope (FESEM), high-resolution transmission electron microscope (HRTEM), energy dispersive spectroscopy (EDS), dynamic light scattering (DLS), X-ray diffraction (XRD), and Fourier-transform infrared spectroscopy (FTIR) as per our earlier reports [22].

3. Glycosidase Inhibitory Activity

3.1. Porcine Pancreatic Amylase Inhibition Assay. In order to study the antidiabetic activity of the bioreduced CuNPs, α-amylase inhibitory activity was checked using the chromogenic 3,5-dinitrosalicylic acid (DNSA) method as per our earlier report [17]. In short, 10 μg/mL CuNPs and porcine pancreatic α-amylase (50 μg mL^{-1}) were mixed and incubated for 10 min at 37°C. 1% starch was used as substrate which was added thereafter. DNSA assay was used to estimate the reducing sugar by recording the absorbance at 540 nm. Inhibitory activity was calculated by using the following formula:

$$\%\ \text{inhibition} = \frac{\left(\text{A540}_{\text{control}} - \text{A540}_{\text{test}}\right)}{\left(\text{A540}_{\text{control}}\right)} \times 100. \quad (1)$$

3.2. α-Glucosidase Inhibition Assays. Inhibition of α-glucosidase activity in presence of CuNPs was checked by mixing 100 μL of α-glucosidase (0.1 unit/mL) with 200 μL of CuNPs (100 μg/mL) followed by incubation for 1 h at 37°C [1]. 10 mM *p*-nitrophenyl-α-D-glucopyranoside in 100 mM phosphate buffer of pH 6.8 was added to initiate the enzyme activity which was incubated for 10 min at 37°C and eventually stopped by addition of 2 mL Na_2CO_3 (0.1 M). Absorbance of the *p*-nitrophenol released from *p*NPG was recorded at 420 nm and percentage of glucosidase inhibition was evaluated using the following formula:

$$\% \text{ inhibition} = \frac{\left(\text{A420}_{\text{control}} - \text{A420}_{\text{test}}\right)}{\left(\text{A420}_{\text{control}}\right)} \times 100. \quad (2)$$

3.3. Circular Dichroism (CD) Spectrometry. CuNPs were incubated with porcine pancreatic α-amylase and α-glucosidase separately at 37°C followed to which CD spectra were recorded on a Jasco J-1500 spectropolarimeter at a scan speed of 40 nm/min with a response time of 1 s and a slit width of 1 nm, as reported earlier. The measurements were recorded in a range from 190 to 300 nm in a Quartz cell of 2 mm path length using a reaction mixture comprising 0.1 unit/mL of enzyme and CuNPs in phosphate-buffered saline [23].

4. Results

4.1. UV-Visible Spectroscopy. Synthesis of CuNPs was confirmed by the visible colour change from pale blue to yellow and finally to dark brown. The intensity of the spectra increased up to 5 h followed by which no significant increase was noticed which indicated the completion of the bioreduction process in 5 h. A similar pattern in the enhancement of the spectral intensity was observed in all the cases where CuNPs were synthesized using GGFE, GGLE, GGSE, and PZLE at 100°C (Figure 1). This synthesis was found to be both rapid and efficient. The yield was found to be ~62.66% from 100 mL reaction mixture.

4.2. HRTEM, EDS, DLS, and XRD Analysis. Morphological features of the bioreduced CuNPs were evaluated using HRTEM analysis as FESEM failed to show high-resolution images since the CuNPs were very small in size (Figure S1). CuNPs were found to be embedded in the biological matrix which might play a critical role in synthesis and stabilization. It was observed that very small spherical nanoparticles were successfully fabricated which were found to be of 5 nm in size, when synthesized using GGFE (Figure 2(a)). However, the size of the CuNPs increased when synthesized using GGLE which was in a range between 70 and 93 nm, as found in case of the irregular brush border rods apart from the spherical ones (Figure 2(b)). In case of spherical CuNPs synthesized using GGSE, the particles were found to be monodispersed and discretely placed without any sign of aggregation or agglomeration indicating the high stability (Figure 2(c)). However, it is important to note that CuNPs synthesized using PZLE were found to be smaller with their size in a range between 1 and 5 nm (Figure 2(d)). EDS analysis revealed and confirmed that the nanostructures were composed of elemental copper (Figure 3). The high intensity peak of Si could be observed in EDS as particles were drop casted on silicon wafers and dried to do the analysis. Other peaks were due to sulphur and oxygen that might be an integral part of the biomolecule skeleton responsible for synthesis and capping of the CuNPs. Particle size analysis as evident from the DLS data can be closely correlated with the observed dimension recorded in HRTEM analysis (Figure 4). Increase in the particle diameter as observed in DLS might be attributed due to the close association of biological matrix around the CuNPs. XRD analysis is included in the supplementary information document (Figure S2). Although the nanoparticulate nature of the samples was observed through HRTEM, XRD data did not show distinct characteristic peaks of metallic CuNPs. There could be several reasons to this, including oxidation of copper when exposed to air during characterization and excess of plant extracts on the nanoparticles. Nevertheless, the EDS data confirmed the presence of CuNPs.

4.3. FTIR Analysis. The role of the extracts and the corresponding functional groups towards synthesis and stabilization of CuNPs was studied by recording the FTIR spectra of GGFE, GGLE, GGSE, and PZLE before and after synthesis of CuNPs (Figure 5). The plant extracts used for synthesis were recovered from the completed reaction mixtures and were independently added to KBr powder in order to record FTIR data. It is evident that all the four extracts showed similar characteristic peaks, before synthesis, indicating similar functional groups, irrespective of the plant part from which they are extracted. The variation in the intensity suggests variation in the concentration of the corresponding functional groups. All the four extracts exhibited strong characteristic peak at ~3400–3420 cm^{-1} which is attributed to the hydroxyl group in alcoholic and phenolic compounds. However, no significant change was observed in the peaks after synthesis of CuNPs. The other significant and predominant peaks which were noticed before synthesis were not much altered after synthesis. The peaks at ~1215 and ~1624, 1365–1370, and ~1740 cm^{-1} can be attributed to the unassigned amide mode, CH_3 bend, and stretching of C=O bond, respectively. Remarkable change was observed in the peak at ~1095 cm^{-1}, which corresponds to C-O-C bond in ether. In case of GGFE, this peak was diminished significantly, while in rest of the extracts, there was notable change in the peak, after synthesis. This suggests that C-O-C bond is utilized during conversion of the Cu^{2+} to CuNPs. Moreover, minor change was also observed in the amide bond intensity, located at ~1624 in case of GGSE, indicating its role in CuNPs. No prominent change in the rest of the peaks indicates that the corresponding functional groups help in stabilizing the as synthesized CuNPs.

4.4. Porcine Pancreatic α-Amylase Inhibition Assay. Bioreduced CuNPs showed promising inhibition against porcine pancreatic α-amylase (Figure 6). Among the various tested CuNPs samples, GGLE synthesized CuNPs showed the highest inhibition of porcine pancreatic α-amylase up to 50.99 ± 4.27% followed by GGFE-CuNPs that showed 50.01 ± 4.19%. CuNPs synthesized using GGSE (32.36 ± 2.71%) and PZLE (33.34 ± 2.79%) showed an inhibition equivalent to the standard drug acarbose (35.30 ± 2.95%).

4.5. α-Glucosidase Inhibition Assay. Among different CuNPs synthesized by 4 plant extracts, GGSE-synthesized CuNPs exhibited highest α-glucosidase inhibition up to 88.60 ± 0.78% followed by GGLE-synthesized CuNPs (86.58 ± 3.26%).

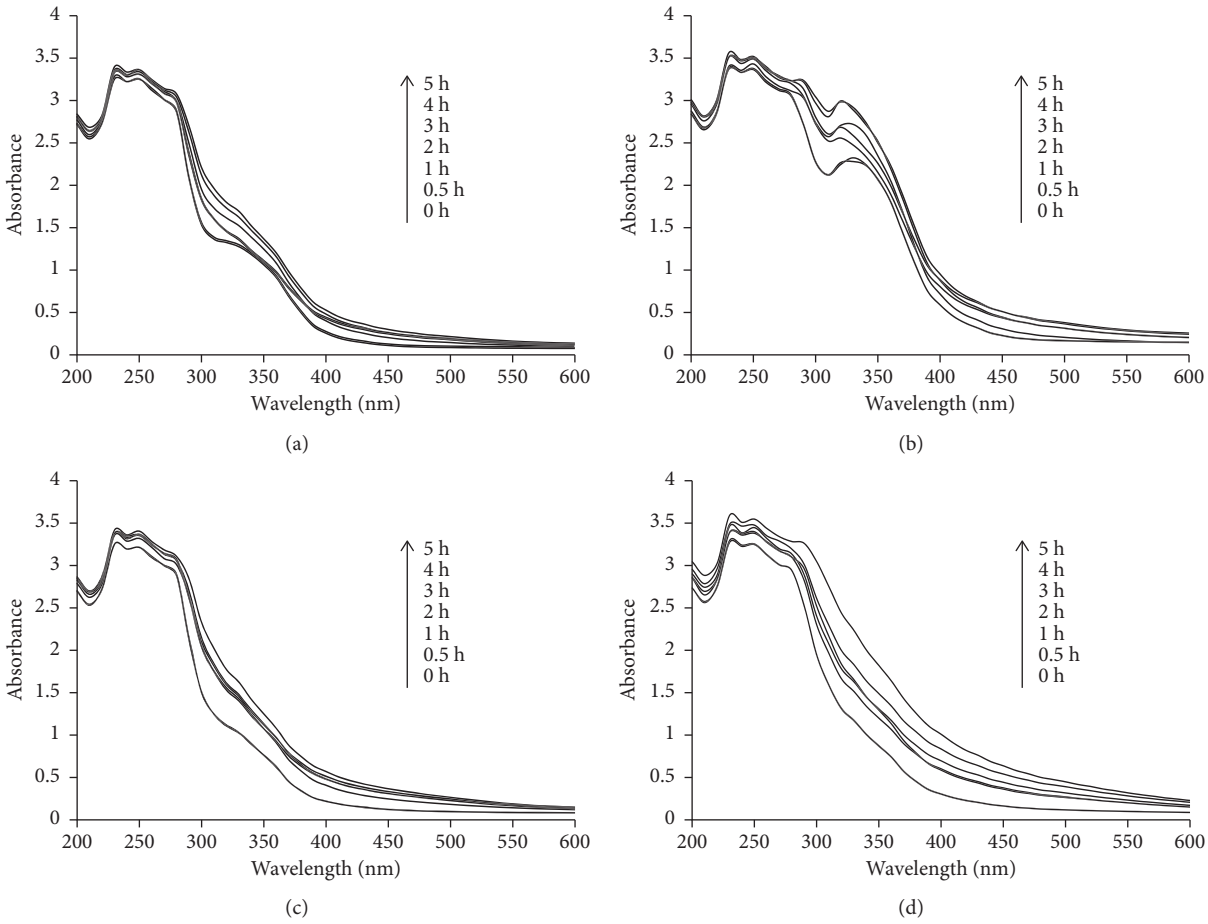

Figure 1: UV-visible spectra of CuNPs synthesized by plant extracts using 10^{-3} M aqueous $CuSO_4{\cdot}5H_2O$ solution in the dark at 100°C: (a) GGFE, (b) GGLE, (c) GGSE, and (d) PZLE.

Standard drug acarbose also showed similar inhibitory potential (81.09 ± 2.82%). CuNPs synthsized by GGFE and PZLE showed comparatively lower inhibitory potential up to 76.20 ± 1.14% and 73.29 ± 0.96%, respectively (Figure 7).

4.6. Circular Dichroism Analysis. Circular dichroism spectra confirmed the structural and conformational alteration in the enzymes in presence of CuNPs (Figures 8 and 9). Circular dichroism (CD) spectroscopy revealed the nature of interaction of porcine pancreatic α-amylase and α-glucosidase with CuNPs. As indicated by CD spectroscopy, the secondary structure of the enzymes was seen to be altered in presence of the CuNPs. Commonly, α-helical content of an enzyme shows two characteristic minima at 208 and 222 nm which were further compared after inhibition with CuNPs. The variation at 208 nm in the presence of CuNPs when compared with the control enzymes provided strong evidence that the interaction of CuNPs with the α-helix of both the enzymes resulted in a conformational change in the secondary structure of the enzymes.

5. Discussion

Metal nanoparticles have got wide applications in optoelectronics, semiconductors, sensors, and biomedical applications as well. Medicinal plants are widely explored to synthesize metal nanoparticles. In this study, we found that *G. glauca* and *P. zeylanica* have tremendous potential to synthesize and stabilize metallic CuNPs. In our previous studies, we have reported *G. glauca* flower-, leaf-, and stem-mediated synthesis of AuNPs and AgNPs [24–26]. However, there are no reports till date on their potential to synthesize bioactive CuNPs. Hereby, we have used three parts of *G. glauca*. Similarly, earlier, we could find that only *P. zeylanica* leaf can synthesize AuNPs, AgNPs, and bimetallic nanoparticles most effectively. But, till date, there are no reports of synthesis of CuNPs using *P. zeylanica* leaf extract [10]. In our present study, synthesis of CuNPs was found to be rapid and efficient which is well in agreement with our previous reports where AuNPs and AgNPs were synthesized using the aforementioned plants. The parts of the plants used in this study are reported to contain coumarins like seselin, 5-methoxyseselin, suberosin, xanthyletin, and xanthoxyletin apart from alkaloids, glycoside, reducing sugars, simple phenolics, tannins, lignin, saponins, and flavonoids which have a high potential to synthesize and stabilize nanoparticles [16, 17, 19]. Absorption bands of CuNPs are in the range between 550 and 600 nm. However, in our phytogenic approach, no sharp peak attributed to the surface plasmon resonance was observed which is well established by the earlier reports where similar

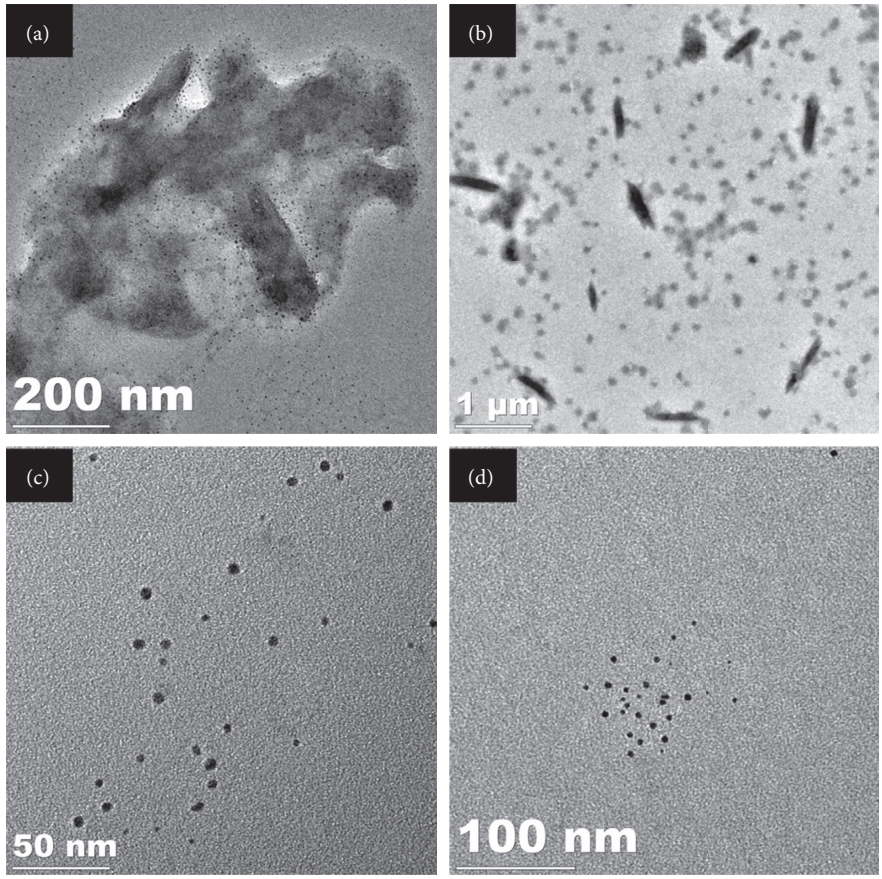

Figure 2: HRTEM micrographs of CuNPs synthesized by plant extracts using 10^{-3} M aqueous $CuSO_4{\cdot}5H_2O$ solution in the dark at 100°C: (a) GGFE, (b) GGLE, (c) GGSE, and (d) PZLE.

observations were made for CuNPs coated with biomolecules [27]. Intensity of the UV-visible spectra progressively increases similar to the synthesis of CuNPs by hydroxyl ion-assisted alcohol reduction [28]. It can be rationalized by the earlier observations where freshly synthesized CuNPs (size, <5 nm diameter) at lower copper ion concentration demonstrated a featureless Mie scattering profile without the appearance of an apparent surface plasmon band which is in close agreement with our observation in the present study. This featureless broad peak may be attributed to the small size of bioreduced CuNPs [29]. Likewise, CuNPs synthesized using L-ascorbic acid were found to be less than 4 nm in diameter that exhibited a broadened peak and featureless absorbance, which increased monotonically towards higher energies. In our study as well, the bioreduced CuNPs did not show a plasmon peak at around 570 nm but rather displayed a broadened peak indicating the presence of a very small dimension of CuNPs which can be rationalized by the presence of ascorbic acid in the plant extracts that can lead to efficient reduction of Cu^{2+} to Cu^0 and further more effective capping capacity [30–33]. High temperature was found to be suitable for synthesis of CuNPs which was evident from the visible colour change. Enhancement of the rate of synthesis of metal nanoparticles with rise in temperature is in close correlation with previous reports where the rate of synthesis of AgNPs using the *Lippia citriodora* leaf aqueous extract could be enhanced by increasing the temperature from 25°C to 95°C resulting in average particle size of 15–30 nm [34]. It is important to note that temperature plays a very critical role in the size and shape of the synthesized nanoparticles. Owing to the higher rate of reduction at higher temperatures, the copper ions could be consumed mainly on the formation of nuclei, whereas the secondary reduction process which takes place on the surface of the preformed nuclei might be hindered. This phenomenon is well documented during synthesis of AgNPs and AuNPs using *Lippia citriodora* and lemon grass, respectively [34, 35]. The rate of synthesis of AgNPs using aqueous extract of the leaves of *Mimosa pudica* could be effectively enhanced by heating the reaction mixture from ambient (29 ± 3°C) to 70°C. Moreover, increase in the reaction temperature evidently led to the synthesis of larger quantities of nanoparticles and, simultaneously, reduction in the size of the nanoparticles [36].

Synthesis and stabilization of the CuNPs by the both the plants may be attributed due to their rich phytochemistry. *G. glauca* is reported to have potent antioxidant activity owing to its high phenolic and flavonoid content [37]. Although mechanisms for synthesis of phytogenic nanoparticles are under exhaustive research, it is difficult to generalize a single factor for reduction of metal ions to their respective nanoparticles. Thus, in the light of previous reports, it may be hypothesized that multiple factors underlying the rapid

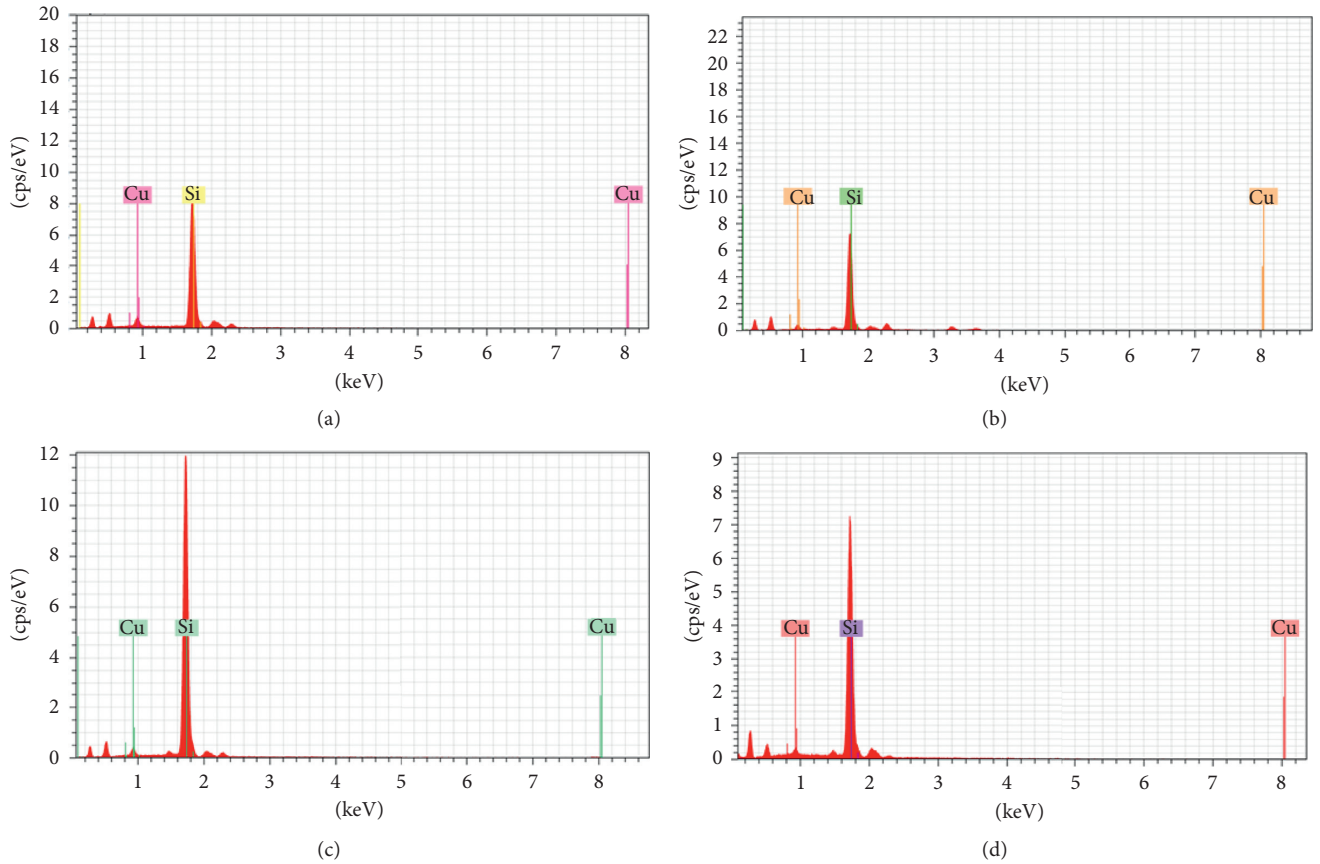

Figure 3: Spot EDS profile of CuNPs synthesized by plant extracts using 10^{-3} M aqueous $CuSO_4 \cdot 5H_2O$ solution in the dark at 100°C: (a) GGFE, (b) GGLE, (c) GGSE, and (d) PZLE.

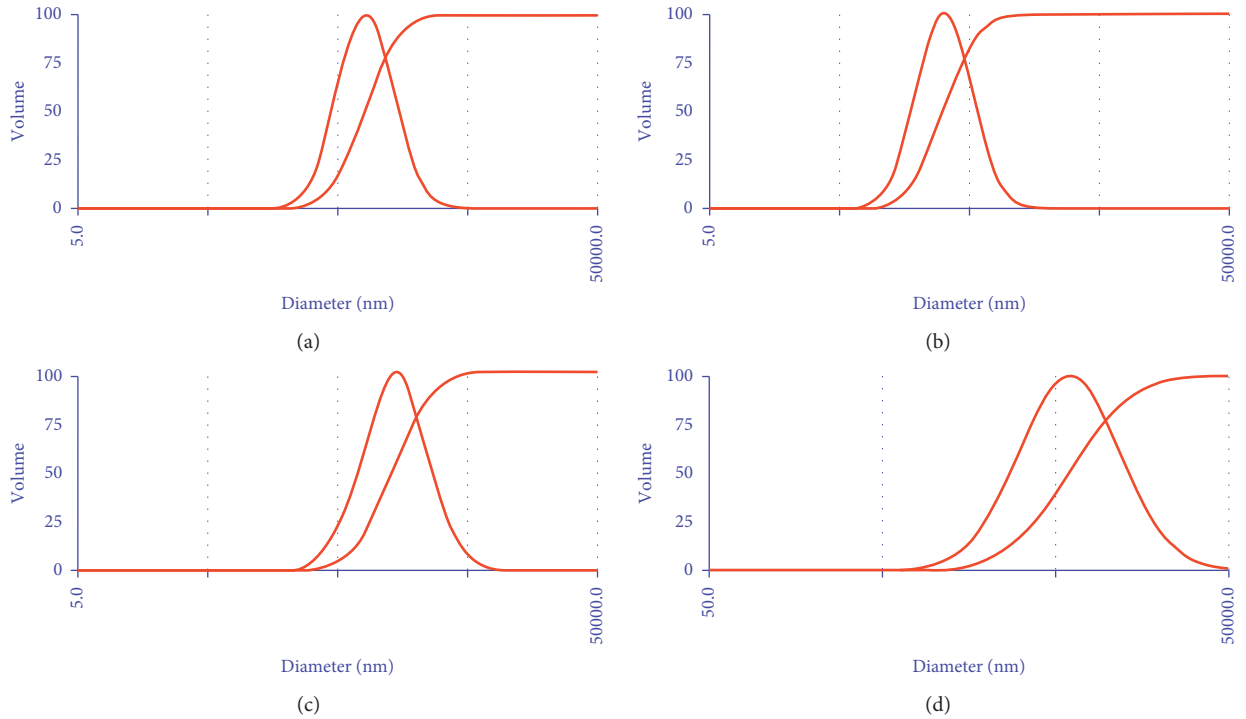

Figure 4: Particle size analysis using dynamic light scattering for CuNPs synthesized by plant extracts using 10^{-3} M aqueous $CuSO_4 \cdot 5H_2O$ solution in the dark at 100°C: (a) GGFE, (b) GGLE, (c) GGSE, and (d) PZLE.

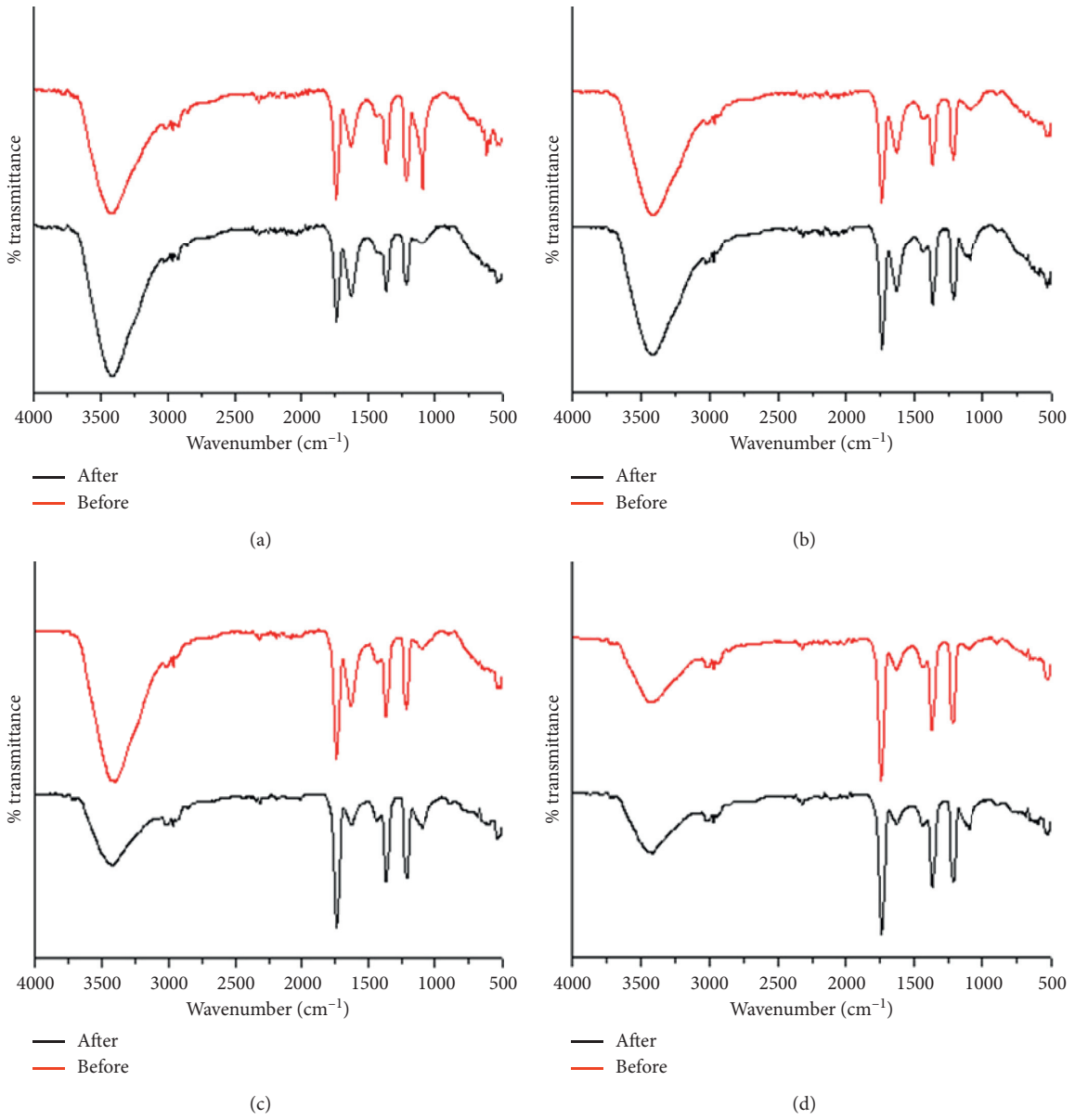

Figure 5: FTIR spectra of plant extracts before and after synthesis of CuNPs: (a) GGFE, (b) GGLE, (c) GGSE, and (d) PZLE.

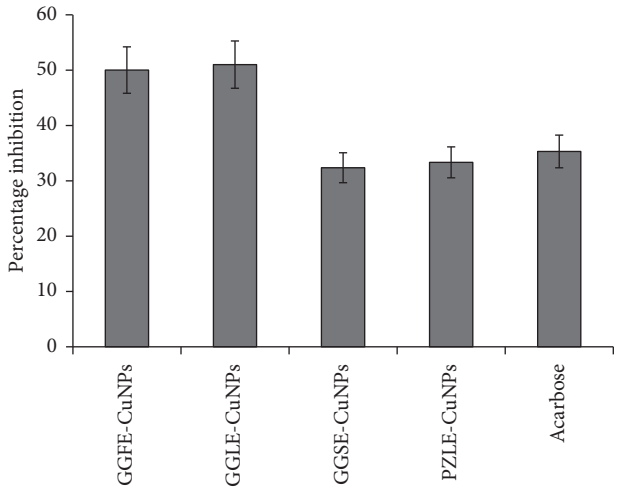

Figure 6: Porcine pancreatic α-amylase inhibition by CuNPs synthesized by different plant extracts: (a) GGFE, (b) GGLE, (c) GGSE, and (d) PZLE.

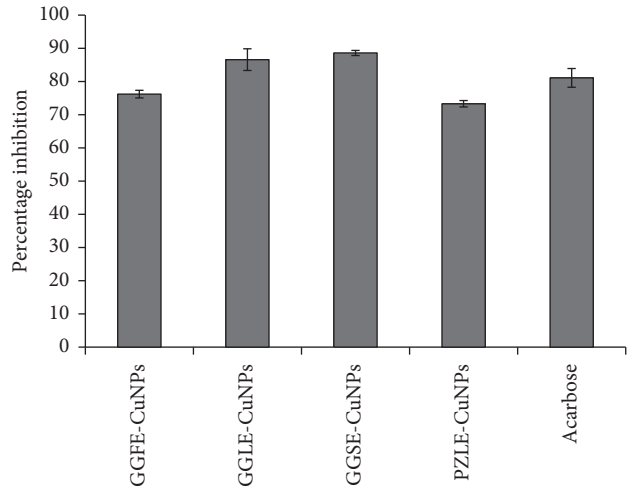

Figure 7: α-Glucosidase inhibition by CuNPs synthesized by different plant extracts: (a) GGFE, (b) GGLE, (c) GGSE, and (d) PZLE.

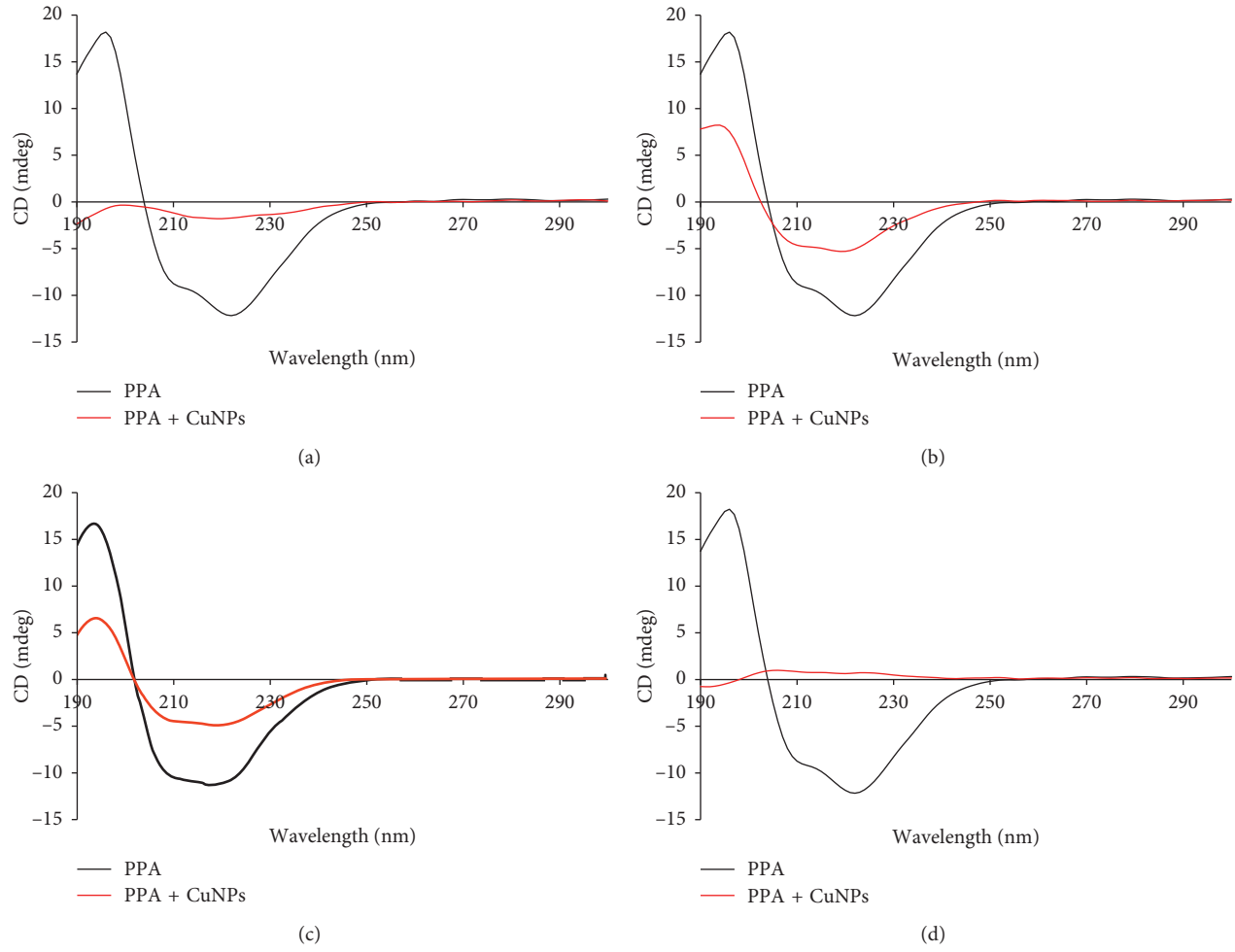

Figure 8: CD spectra of porcine pancreatic α-amylase inhibited by CuNPs synthesized by different plant extracts: (a) GGFE, (b) GGLE, (c) GGSE, and (d) PZLE.

synthesis of CuNPs in the present study may include reducing sugars (aldoses) and ketones, biomolecules with functional groups like–C–O–C, –C–O–, –C=C–, and–C=O–, derived from several heterocyclics, polyol components, flavonoids, and hydroxyls in the terpenoids. Further, peptides may also play a dual role for simultaneous reduction and capping. Earlier reports also rationalize the probability that quasi-spherical-shaped nanoparticles within

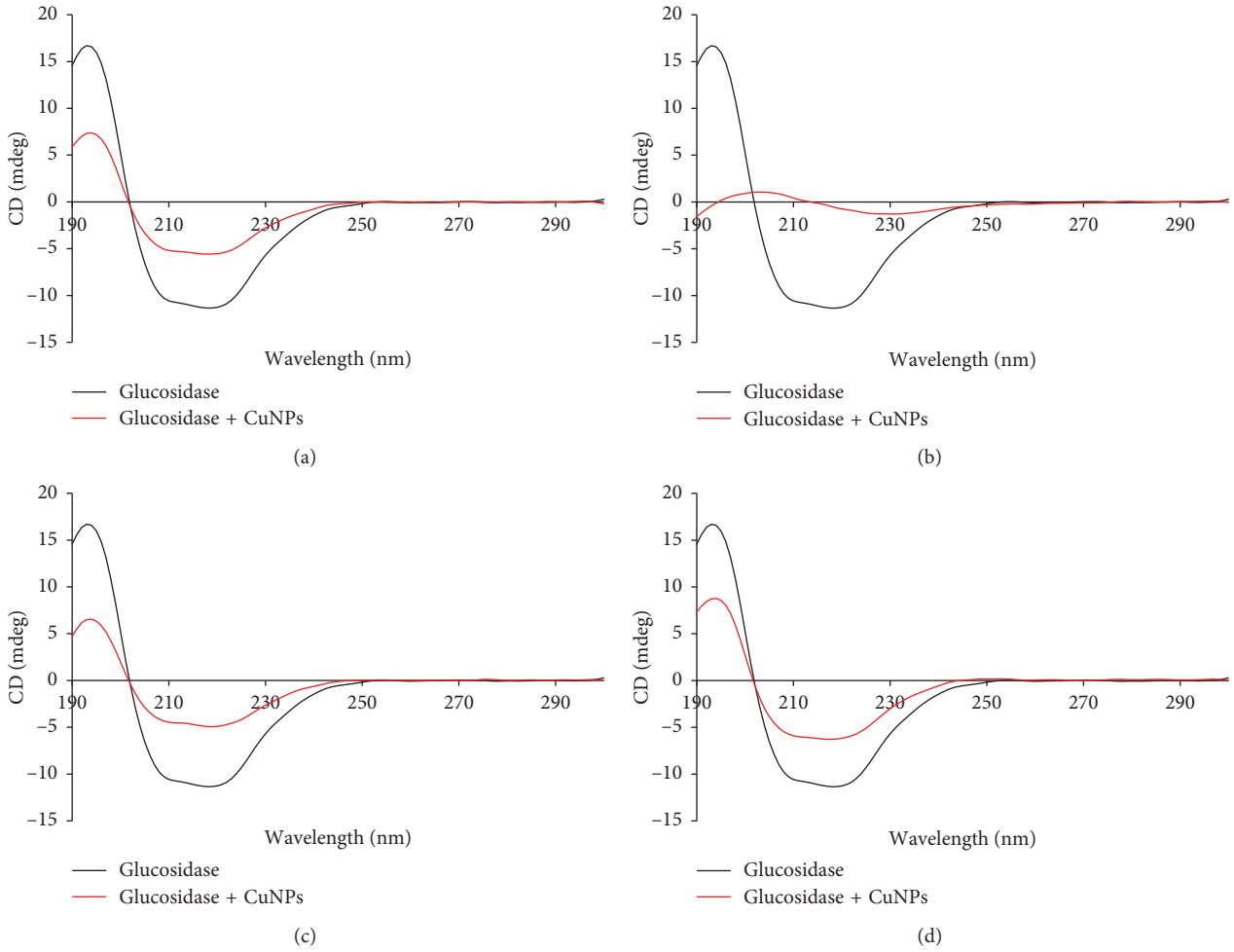

Figure 9: CD spectra of α-glucosidase inhibited by CuNPs synthesized by different plant extracts: (a) GGFE, (b) GGLE, (c) GGSE, and (d) PZLE.

a range between 10 and 30 nm may be synthesized by reductants like oxalic acid and aldehyde groups present in the plant extract [38]. In our previous studies, we have demonstrated that *P. zeylanica* has high concentration of phenolics, flavonoids, reducing sugar, citric acid, and plumbagin which might play a significant role in the process of bioreduction and capping [10]. FTIR analysis strongly rationalizes the interrelationship and interdependence of the phytochemical diversity and its role towards reduction and capping.

The phytogenic CuNPs could efficiently inhibit both α-amylase and α-glucosidase, which are key enzymes of carbohydrate metabolism. The variation in the extent of inhibition may be attributed due to the variation in size and shape. This fact may be evident from the observation where GGLE-synthesized CuNPs showed the highest inhibition of porcine pancreatic α-amylase while GGSE-synthesized CuNPs exhibited highest α-glucosidase inhibition. Type II diabetes mellitus (T2DM) is associated with postprandial hyperglycemia which can be effectively controlled using α-amylase and α-glucosidase inhibitors. However, the adverse effects associated with the available drugs like biguanides, thiazolidinediones, sulphonylureas, meglitinides, and α-glucosidase inhibitors in addition to insulin includes hepatotoxicity, abdominal pain, flatulence, diarrhoea, and hypoglycaemia [17, 39–42]. In this study, we found that, in the presence of CuNPs, the structural and/or conformational change in both α-amylase and α-glucosidase may be the most predominant mechanism of inhibition. Hereby, these phytogenic CuNPs may serve as novel complementary and alternative antidiabetic nanomedicine for the effective treatment and management of T2DM. Our findings are in close agreement with the previous reports where CuNPs and associated copper complexes were proved to be antidiabetic in nature. Recently, our findings about CuNPs, synthesized by *D. bulbifera* tuber extract, *B. prionitis* leaf extract, *L. chinensis* peel extract, and *P. orientalis* leaf extract, have shown spectacular success towards considering phytogenic CuNPs as potential candidate for designing antidiabetic nanomedicine [43]. Similarly, *Calotropis procera* L. latex is reported to produce highly biocompatible CuNPs which did not show any toxicity even at a concentration as high as 120 μM which substantiates the biocompatibility of phytogenic CuNPs [44]. The yield of phytogenic CuNPs (62.66%) was found to be higher as compared to the chemical synthesis involving micelles of dodecylamine (yield 49%) while lower

compared to CuNPs obtained with Triton X-100 (yield 99%) [45].

6. Conclusion

G. glauca- and *P. zeylanica*-mediated synthesis of CuNPs may prove to be a novel, rapid, and efficient route to fabricate spherical nanoparicles of smaller dimensions. The extracts rich in diverse phytochemicals not only bioreduce but also stabilize the nanoparticles as well. Elevated temperature was found to be suitable for synthesis. Phenolics and flavonoids might play a key role in the synthesis process. CuNPs could effectively inhibit α-amylase and α-glucosidase. The mechanism of enzyme inhibition was established to be alteration of the secondary structures in the enzyme leading to conformational change. In view of the background, it can be concluded that phytogenic CuNPs reported herein may lead to development of environmentally benign route for rational designing of safe and effective antidiabetic nanomedicine.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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Supplementary Materials

Figure S1: the FESEM micrographs of CuNPs synthesized by plant extracts using 10–3 M aqueous $CuSO_4{\cdot}5H_2O$ solution in the dark at 100°C. Figure S2: the X-ray diffraction profile of CuNPs. (*Supplementary Materials*)

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Research Article

Effect of "DXB-2030," a Polyherbal Formulation, on Experimental Polycystic Ovary Syndrome Associated with Hyperandrogenism

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The objective of the present study was to evaluate "DXB-2030," a polyherbal combination of *Trigonella foenum-graecum*, *Aloe vera*, *Sphaeranthus indicus*, *Nardostachys jatamansi*, and *Symplocos racemosa* extracts in an experimental model of testosterone propionate (TP), induced polycystic ovary syndrome (PCOS) in female rats. Thirty animals were divided into 3 groups of 10 each; group 1 served as normal control; group 2 was administered with TP and served as positive control; along with TP, group 3 was treated with "DXB-2030" at a dose of 100 mg/kg p.o., for 60 days. At the end of the study period, the animals were subjected for the estimation of serum testosterone levels, oral glucose tolerance test (OGTT), weight of the ovaries, estrous cycle, and histopathological evaluation. An in vitro assay on GLUT4 expression was carried out to understand the effect of "DXB-2030" on insulin resistance. Results showed that treatment with "DXB-2030" reversed the TP-induced changes by increasing the GLUT4 expression and decreasing the body weight, testosterone levels, AUC of glucose in OGTT, and the cystic follicles of the ovaries, thus indicating its beneficial effect in PCOS by ameliorating the metabolic dysfunction and reproductive impairment, which are the pathophysiological conditions associated with PCOS. From the results obtained, it can be concluded that "DXB-2030" was effective in the management of experimental PCOS and hence may be recommended in the treatment of PCOS.

1. Introduction

Polycystic ovary syndrome (PCOS) has become a major area of concern as it is affecting 12–21% of reproductive-aged women causing infertility [1]. This syndrome is characterized by multiple disorders such as hyperandrogenism, hyperinsulinemia, dyslipidemia, obesity, insulin resistance, anovulation, and cystic follicles in the ovary [2]. Almost 30–40% of women affected with PCOS have impaired glucose tolerance [3]. If early intervention is ignored, it may lead to other serious consequences such as type II diabetes mellitus (DM), cardiovascular disorders, and ovarian cancer [4]. According to the widely accepted Rotterdam Consensus, women can be diagnosed with PCOS when they exhibit at least any two of three features: androgen excess, ovulatory dysfunction, and polycystic ovary appearance on ultrasound after excluding other causes of these abnormalities [5].

Due to complex nature, complete understanding of pathogenesis of PCOS is still lacking. There are multiple pathophysiological mechanisms and various theories which have been proposed to explain the pathogenesis of PCOS. Some of the mechanisms are an alteration in gonadotropin releasing hormone (GnRH) secretion, increase of luteinizing hormone (LH) secretion, and alteration in insulin secretion which leads to hyperinsulinemia and insulin resistance (IR). Insulin resistance is one of the key players in the

pathophysiology of PCOS. Insulin resistance and high insulin levels directly and indirectly stimulate ovarian theca cells to secrete androgens, and these result in an increase of androgen levels. The defect in androgen synthesis leads to increase in ovarian androgen production and ultimately to PCOS [6–8].

Currently, many of the treatments for PCOS primarily depend on desired clinical effect and include insulin sensitizers, infertility treatment, regulation of menstrual disturbances, antiandrogens, and hormonal therapies. But, all these treatments or treatment regimens have their own side effects. Either they are associated with substantial cost or may cause various side effects, such as irregular menstruation, gastrointestinal disturbances, weight gain, and increased insulin resistance [9–11]. The side effects of these medicines and their identification have significant importance in PCOS management. Many studies including randomized controlled trials, case studies, and animal experiments are focused on investigation of herbal drugs in this condition. Hence, exploration of potential herbs and their combination needs to be evaluated for the treatment or to have a check on all the aspects of pathogenesis of PCOS [11–13].

Based on the etiology and pathogenesis of PCOS, a polyherbal formulation "DXB-2030," which is a combination of *Trigonella foenum-graecum*, *Aloe vera*, *Sphaeranthus indicus*, *Nardostachys jatamansi*, and *Symplocos racemosa* was prepared. The herbs are mixed in a right proportion as per the ayurvedic criteria to obtain a blend which can be targeted against multiple ramifications of PCOS in a holistic approach. The individual herbs used in this combination are reported to have significant relevance to the pathogenesis of PCOS. *Trigonella foenum-graecum* is used as insulin sensitizer in diabetes mellitus and also used in female reproductive disorders [14–17]. *Aloe vera* is known to bring estrus cyclicity to normalcy by controlling hyperglycemic conditions and modulating steroidogenesis and thus is a potential candidate for the maintenance of PCOS [18, 19]. *Sphaeranthus indicus* is reported to possess anxiolytic, central nervous system depressant and anticonvulsant activities, thus supporting its use in the management of anxiety related to PCOS [20, 21]. *Nardostachys jatamansi* is used in the management of stress causes due to various etiologies [22]. Research has shown its usefulness in the treatment of PCOS due to its antiandrogenic activity [23]. *Symplocos racemosa* is used in menorrhagia and other female reproductive dysfunctions which are some of the symptoms of PCOS [24, 25].

Some of the known actives identified in this combination "DXB-2030" are saponins, flavonoids, alkaloids, volatile oil, and polyphenols. The herbs used in this combination were carefully selected to balance the reproductive and metabolic aspects of PCOS. Based on the reported pharmacological properties of the herbs, the present study was designed to explore the effect of "DXB-2030" in the experimental model of PCOS in rats.

2. Materials and Methods

2.1. Chemicals. Testosterone propionate (TP), olive oil, and D glucose were obtained from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Glucose oxidase kit was procured from Erba Mannheim (Transasia), Mumbai, India. Testosterone kit was purchased from Diagnostics Biochem Canada (DBC), Ontario, Canada.

2.2. Experimental Animals. Inhouse-bred female Wistar rats (9 days old) were housed in standard conditions of temperature ($22 \pm 3^{\circ}$C), relative humidity ($55 \pm 5\%$), and light (12 hr light/dark cycles) before and during the study. Animals were fed with standard pellet diet (Provimi Animal Nutrition India Pvt. Ltd.) and water *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) of The Himalaya Drug Company, Bangalore, and the animals received humane care as per the guidelines prescribed by the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), The Ministry of Environment & Forests, Government of India.

2.3. In Vitro Studies

2.3.1. GLUT4 Expression Study

(1) Cell Culture and Treatment. Differentiated C2C12 myotubes (1.2×10^5 cells/well in a 6-well plate, procured from ATCC) were incubated with 500 μM palmitate for 24 hours in DMEM high-glucose media. After the incubation, the cells were washed with sterile PBS and further incubated with the "DXB-2030" sample at nontoxic concentration of 200 μg/ml (toxicity as determined from MTT assay) with and without 100 nM insulin in a 37°C incubator with 5% CO_2 for 24 h. After incubation, media were removed, and the resulting adherent cells were subjected for total RNA isolation for further experimentation.

(2) Gene Expression. Total RNA was extracted from treated myotubes ($n = 3$) using RNA isolation kit (Krishgen Biosystem). The isolated RNA was quantified on agarose gel. Total RNA and random primers were used for the first-strand cDNA synthesis by reverse transcriptase. The PCR amplification was carried out in a reaction volume of 20 μl containing 2 μl of cDNA and 10 μl of SYBR Green Supermix (BioRad, USA). The GLUT4 expression levels were normalized to that of 18s rRNA expression, and control was normalized to 1.

2.4. In Vivo Studies

2.4.1. Testosterone Propionate-Induced PCOS. PCOS was induced in 20 animals and 10 remained as normal control. On the 9th day of birth, testosterone propionate (TP) was administered at a dose of 1.25 mg/pup s.c. (1.25 mg TP in 0.02 ml of olive oil) to induce PCOS in 20 female pups, and olive oil 0.02 ml/pup was administered to 10 pups which served as normal controls. Pups were kept with respective mother until weaning; after that, they were housed in respective groups. After 70 days of age, vaginal smear was monitored daily to confirm the development of PCOS [26, 27].

Thirty animals were divided into 3 groups of ten each. Groups 1 and 2 were animals administered with demineralized water at a dose of 10 ml/kg b.wt. and served as normal and PCOS control, respectively, and group 3 was PCOS animals treated with "DXB-2030" at a dose of 100 mg/kg b.wt.p.o. for 60 days.

2.4.2. Oral Glucose Tolerance Test (OGTT). OGTT was performed one day before the terminal sacrifice. Glucose (2 g/kg) was administered to overnight-fasted rats to perform OGTT, and blood samples were collected from the retro-orbital plexus at 0 (before glucose load), 30, 60, and 120 min after glucose administration. Serum was separated, and serum glucose was estimated by the enzymatic glucose oxidase method.

2.4.3. Estrous Cycle. Vaginal smear of all the animals were monitored daily in the last week of treatment and observed under microscope for the presence of different stages (proestrus, estrus, metestrus, and diestrus) of estrous cycle.

2.4.4. Serum Testosterone Estimation. Two hours after the last dose of treatment, blood was collected from retro-orbital sinus under isoflurane anesthesia for the estimation of total testosterone (TT), and it was quantified using the ELISA method.

2.4.5. Histopathological Evaluation. Briefly, after the blood collection, animals were euthanized using excess of anesthesia, and ovaries were excised, weighed, and fixed in 10% neutral buffered formalin and embedded into paraffin blocks. Tissue sections of 5 μm were cut and stained with hematoxylin and eosin and subjected for histopathological evaluation. The slides were evaluated using the microscope (Olympus, Nikon Eclipse E-400, Japan). The change in ovary like corpus luteum (CL), atretic follicle (AF), and cystic follicle (CF) was evaluated.

2.5. Statistical Analysis. All values are expressed as the mean ± standard error of the mean (SEM). The results were statistically analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's comparison using Prism GraphPad 6.07 software, San Diego, CA, USA. A p value < 0.05 was considered statistically significant.

3. Results and Discussion

3.1. In Vitro Studies

3.1.1. Effect of "DXB-2030" on GLUT4 Expression. Palmitic acid is known to induce the insulin-resistance condition in the myotubes which results in decreased glucose uptake. In our assay (which measures the GLUT4 expression levels), it was observed that the GLUT4 levels were very low even in the presence of insulin. When these cells were treated with "DXB-2030," it resulted in increased expression of GLUT4 levels indicating an increased glucose uptake and hence increased insulin sensitivity (Figure 1).

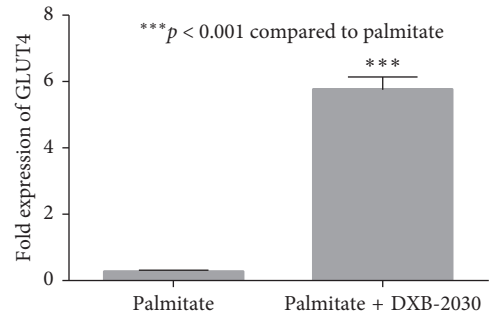

Figure 1: Effect of "DXB-2030" on GLUT4 gene expression: cells were treated with insulin under indicated conditions, total RNA was isolated, and the expression level of GLUT4 was analyzed by quantitative real-time PCR, using 18s RNA as internal control. Expression levels in control were normalized to 1. The level of significance is denoted as *** $p < 0.001$ compared to the palmitate group. The unpaired t-test was used for statistical comparison.

3.2. In Vivo Studies. After 70 days of age, vaginal smear was monitored daily for 10 consecutive days, and the animals which exhibited irregular estrous cycle were considered as PCOS-positive animals and used for the study. All the animals administered with TP showed irregular estrus cycle and used for further evaluation.

3.2.1. "DXB-2030" Reduces Body Weight. All animals were weighed weekly once in the study period till the end of the study. The percentage increase in the body weight was mentioned in the data. The data represent the increase in percentage body weight on the last day of treatment. An overall increase in body weight in all the group of animals over the experimental period was observed. A significant increase in the body weight was observed in the PCOS control group compared to the normal control group, whereas treatment with "DXB-2030" showed a significant decrease in body weight compared to the PCOS control group (Figure 2).

3.2.2. "DXB-2030" Ameliorates Glucose Intolerance. In the last week of the treatment, OGTT was performed, and intragastric administration of glucose did not produce many changes in normal control and showed the normal profile to glucose tolerance, whereas the PCOS control animals showed the increase in the glucose intolerance when compared to normal control. Blood glucose levels at 30, 60, and 120 min were higher in the PCOS group compared to the control group. Further "DXB-2030-treated" animals showed significant reduction in glucose levels at different time points over the period of 120 min when compared to the PCOS control group (Figure 3).

3.2.3. "DXB-2030" Decreases Ovary Weight. TP injection showed bilateral polycystic ovaries, increase in the ovary weight, and irregularity in the estrus cycle when compared to normal control, whereas treatment with "DXB-2030" showed decrease in the ovary weight and normalization of

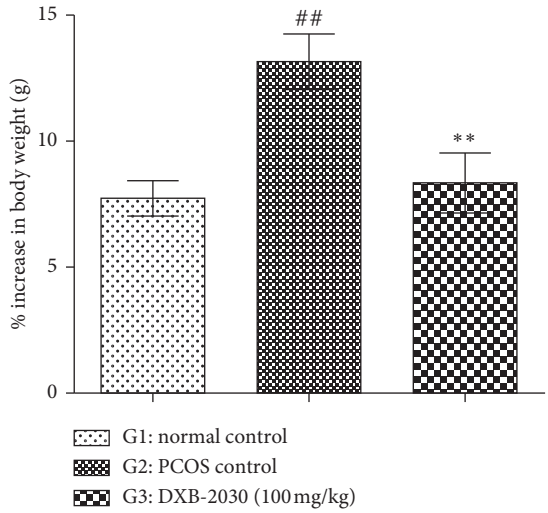

Figure 2: Effect of DXB-2030 on the percentage increase in body weight of rats: a significant increase in % body weight in PCOS control rats ($^{\#\#}p < 0.01$) compared to normal controls and significant decrease in % body weight in "DXB-2030" treatment group ($^{**}p < 0.01$) compared to PCOS controls were observed. One-way ANOVA was used for statistical comparison.

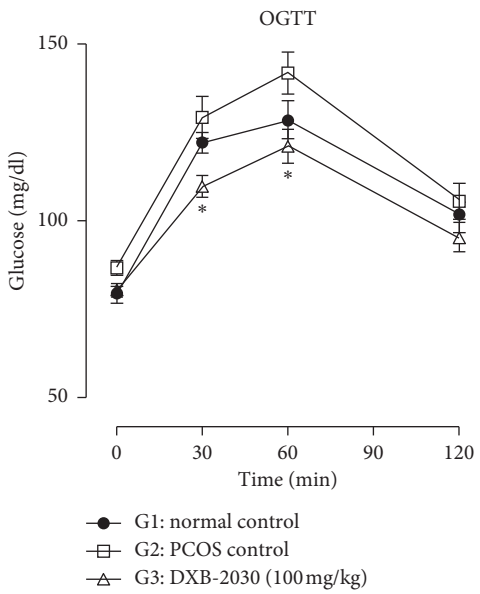

Figure 3: Effect of "DXB-2030" on the oral glucose tolerance test (OGTT) of rats: the glucose levels were estimated at 0, 30, 60, and 120 minutes. The "DXB-2030-treated" group showed a significant decrease in glucose levels at 30 ($^{*}p < 0.05$) and 60 ($^{*}p < 0.05$) minutes compared to the PCOS control group. One-way ANOVA was used for statistical comparison.

irregular estrus cycle (data not shown), when compared with the PCOS control group (Figure 4).

3.2.4. "DXB-2030" Decreases Serum Testosterone Level. The serum testosterone level was quantified by ELISA assay. Significant increase in the testosterone level of the PCOS control group was observed compared to the normal control

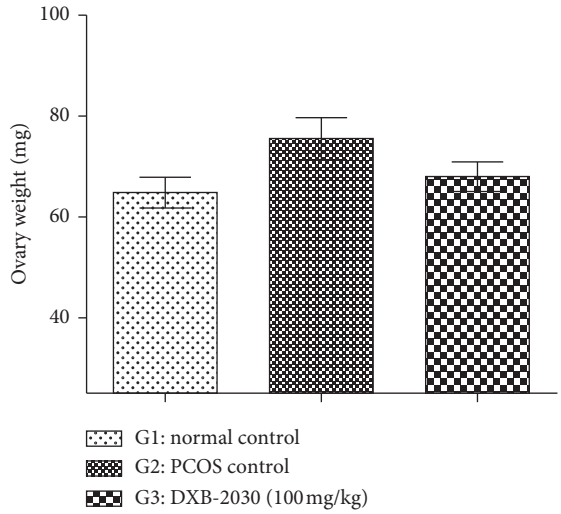

Figure 4: Effect of "DXB-2030" on ovary weight of rats: an increase in ovary weight in PCOS control rats and a decrease in ovary weight in "DXB-2030" rats were observed. The changes in ovary weight were not found to be statistically significant.

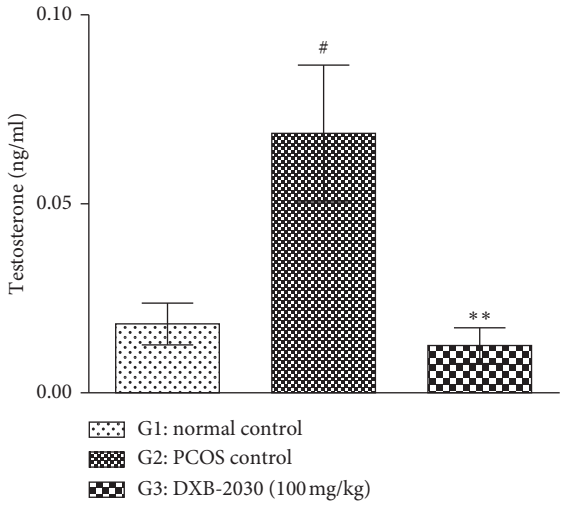

Figure 5: Effect of "DXB-2030" on serum testosterone of rats: a significant increase in serum testosterone levels in PCOS control group ($^{\#}p < 0.05$) and significant decrease in serum testosterone in the "DXB-2030" treatment group ($^{**}p < 0.01$) compared to PCOS control were observed. One-way ANOVA was used for statistical comparison.

group. Treatment with "DXB-2030" significantly suppressed the elevation of the testosterone level compared to the PCOS control group (Figure 5).

3.2.5. Histopathological Changes in "DXB-2030" Treated Rats. The histoarchitecture of ovaries got disrupted due to the administration of TP. Animals showed an increase in cystic follicle and atrophic changes in the PCOS control group compared to normal control. Treatment with "DXB-2030" showed decrease in cystic follicle formation and atrophic changes when compared to PCOS control and an overall recovery in the histoarchitecture of ovaries was recorded (Table 1).

Table 1: Histopathological evaluation of ovaries in testosterone propionate-induced PCOS rats: increase in cystic follicles and atrophic changes were observed in PCOS control rats, and these changes were reversed with the treatment of "DXB-2030."

Groups	Corpus luteum (%)	Cystic follicle (%)	Atrophic changes (%)
G1: normal control	95	10	0
G2: PCOS control	70	75	80
G3: DXB-2030 (100 mg/kg b.wt.)	78	63	57

PCOS is a heterogeneous disorder linked with both reproductive and metabolic dysfunction. The etiology of PCOS is complex and multifactorial. Women with PCOS are usually diagnosed with irregular menstrual cycles, altered hormone levels, and also presence of ovarian cysts [28]. Further evidence also suggests that overweight or obesity with decreased glucose tolerance is a common feature of metabolic dysfunction which plays an important role in development of PCOS [29]. Multiple mechanisms are involved in the pathogenesis of the PCOS. Mainly polycystic ovaries develop when the ovaries are stimulated to produce a large amount of male hormones/androgens mainly testosterone. This stimulation may be due to the release of excessive luteinizing hormone (LH) by the anterior pituitary gland or by high levels of insulin in the blood of women whose ovaries are sensitive to this stimulus or due to reduced levels of sex hormone-binding globulin (SHBG) resulting in increased free androgens [30]. In some cases, women with PCOS will have total testosterone levels within the normal range but will be clinically hyperandrogenic; this is due to elevated free testosterone levels [31].

Insulin regulates the glucose homeostasis by enhancing the glucose uptake by muscle and adipose tissue while suppressing the glucose output by the liver cells. C2C12 myotubes when incubated in the presence of insulin show increased glucose uptake, and this can be indirectly measured by the level of expression of GLUT4. This assay is employed to measure the insulin sensitivity [32]. In this study, the C2C12 myotubes were employed to study the effect of different phytoactives on insulin-mediated glucose uptake. GLUT4 expression levels were measured by qPCR as a surrogate for glucose uptake. Various combinations were subjected for this assay, and based on the outcome and the Ayurvedic wisdom, "DXB-2030" was finalized for further evaluation.

Based on the understanding of pathophysiology mentioned above, the experimental model of TP-induced PCOS was selected to evaluate "DXB-2030" for its beneficial effect in PCOS. This model was found to interfere with the reproductive and metabolic function of the female rats. It causes a change in normal morphology of the reproductive tract and disturbance in the duration of the particular phase of the estrous cycle. The changes in the estrous cycle, hyperandrogenism, hormonal imbalance, and presence of peripheral cysts in the ovaries due to TP administration are some of the symptoms comparable to reproductive anomalies of human PCOS [33, 34].

"DXB-2030" is prepared based on the Ayurvedic relevance, published literature, and in vitro efficacy studies performed on the use of individual herbs in the various symptoms of PCOS. These herbs have shown the activity on reproductive disorders, improved glycemic control, and decrease in insulin resistance, androgen receptor inhibition and anxiolytic effect. The individual herbs present in this combination are reported of exerting their beneficial effects on the female reproductive system. *Trigonella foenum-graecum* seed extract showed encouraging results in 94% of patients, and surprisingly, 12% of study population got pregnant and showed significant improvement in regulating the menstrual cycle [35]. In another clinical study, *Trigonella foenum-graecum* seed extract showed significant reduction in ovary volume and size of the cyst. It also showed significant increase in luteinizing hormone (LH) and follicular stimulating hormone (FSH) levels compared to the baseline values [17]. *Aloe vera* leads to reversion of estrus cyclicity to normal by controlling hyperglycemic conditions and modulating steroidogenesis, and thus, it is the potential candidate for the maintenance of PCOS, which was supported by many preclinical studies [18, 19]. *Sphaeranthus indicus* is reported to be used in the management of anxiety and stress related to PCOS [20, 21]. *Nardostachys jatamansi* showed its usefulness in the treatment of PCOS by its antiandrogenic effect [22, 23]. *Symplocos racemosa* bark is given in menorrhagia and other female reproductive dysfunctions which are some of the symptoms of PCOS. Experimental studies show that *S. racemosa* treatment significantly decreased the elevated testosterone levels and restored estrogen, progesterone, and cholesterol levels. It also restored the normal weight and histology of ovarian tissue. These effects of *S. racemosa* were found to be comparable with clomiphene citrate [24, 25].

The possible mechanism of "DXB-2030" may be due to the inhibition of androgen receptors which aggravated due to the administration of TP, which further reduces the testosterone concentration which is responsible for the development of PCOS. The metabolic dysfunction which is associated with PCOS due to glucose intolerance and decreased glucose uptake was corrected with the treatment of "DXB-2030" may be by the upregulation of GLUT4 expression and increasing glucose tolerance, thus increasing the insulin sensitivity.

4. Conclusion

Intervention with "DXB-2030" reverses the pathophysiological changes caused due to the administration of TP in immature female rats. The beneficial effect of "DXB-2030" on PCOS may be due to the synergistic effect of the individual herbs which are known to exert their effect on the abnormal female reproductive system by various mechanisms like, reversion of estrus cyclicity, reduction in ovary volume and size of the cyst, antiandrogenic effect, decreased testosterone levels and restoration of the histology of ovarian tissue. Based on the outcome of the study, it can be inferred "DXB-2030" was found to be useful in the treatment of PCOS. However, further experimental and clinical studies

are required to confirm the same and to derive the exact mechanism of action of "DXB-2030."

Data Availability

The experimental data used to support the findings of this study are included within the article.

Conflicts of Interest

All the authors are the employees of The Himalaya Drug Company, Bangalore, and declare no conflicts of interest and guarantee no further ethical conflicts among both the authors and the experimental methodology.

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Research Article

Novelty of Bioengineered Iron Nanoparticles in Nanocoated Surgical Cotton: A Green Chemistry

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The current focus of nanotechnology is to develop environmentally safe methodologies for the formulation of nanoparticles. The phytochemistry of *Zingiber officinale* inspired us to utilize it for the synthesis of iron nanoparticles. GC-MS analysis revealed the phytochemical profile of ginger. Out of 20 different chemicals, gingerol was found to be the most potent phytochemical with a retention time of 40.48 min. The present study reports a rapid synthesis method for the formation of iron nanoparticles and its potential efficacy as an antibacterial agent and an antioxidant. Because of its antibacterial property, ginger extract was used to coat surgical cotton. Synthesized ginger root iron nanoparticles (GR-FeNPs) were characterized by UV-visible spectroscopy, Fourier-transform infrared spectroscopy (FT-IR), X-ray diffraction analysis, and particle size analysis. XRD confirmed the crystalline structure of iron oxide nanoparticles as it showed the crystal plane (2 2 0), (3 1 1), (2 2 2), and (4 0 0). The particle size analyzer (PSA) showed the average size of the particles, 56.2 nm. The antimicrobial activity of the FeNPs was tested against different Gram-positive and Gram-negative bacteria. *E. coli* showed maximum inhibition as compared with the other organisms. Antioxidant activity proved the maximum rate of free radicals at 160 μg/mL produced by nanoparticles. In addition, the antimicrobial activity of nanocoated surgical cotton was evaluated on the first day and 30th day after coating, which clearly showed excellent growth inhibition of organisms, setting a new path in the field of medical microbiology. Hence, iron-nanocoated surgical cotton synthesized using green chemistry, which is antimicrobial and cost effective, might be economically helpful and provide insights to the medical field, replacing conventional wound healing treatments, for better prognosis.

1. Introduction

In the modern era, it is crucial to find a substitute for conventional antibiotics because of the emergence of new multidrug-resistant bacterial strains that are able to form biofilms, decreasing the action of antibiotics [1]. The recent advancements in the field of nanotechnology include the preparation of nanoparticles of specific size and shape that exhibit antimicrobial properties. The antimicrobial activity of the nanoparticles can be determined by the size, physicochemical properties, and surface area-to-volume ratio [2–5]. It is reported that nanoparticles of smaller size tend to exhibit excellent antimicrobial activity. Various polyphenols and antioxidants present in the *Z. officinale* root play an important role in the field of medicine, and interaction of these with the metallic surface of the nanoparticles exhibits a possible antioxidant activity [4]. Because of the growing concerns of society regarding health issues, consumers are paying attention before they pick any product. That is the reason for the demand of antimicrobial agents in the market. The essential oil of *Z. officinale* has antimicrobial, antioxidant, antifungal, insecticidal, and anti-inflammatory properties [5]. It created a special interest for choosing ginger as a plant for the preparation of iron nanoparticles. Various chemical and biological methods can be used to prepare nanoparticles, but synthesis via a green approach using *Z. officinale* root extract is ecofriendly, cost effective, easy, and less hazardous [6–8].

In comparison with the previous reports, few studies have reported on the synthesis of iron oxide nanoparticles from *Z. officinale* and its antimicrobial evaluation on surgical cotton. Iron is a cost-effective alternative compared

with other expensive metals reported earlier as antimicrobial agents. This study's focus is to synthesize iron nanoparticles (FeNPs) from *Z. officinale*; confirm the formation of FeNPs by different characterization methods such as UV-visible spectroscopy, Fourier transform infrared spectroscopy, and X-ray diffraction; check the bactericidal activity of FeNPs; and coat the FeNPs on surgical cotton. Because of antibiotic-resistant bacterial strains, wound dressing of patients is difficult. Thus, the study may provide insight into a new path which may be an alternative for antibiotic use in the near future.

2. Materials and Methods

2.1. Materials and Reagents. All the chemicals including ferric chloride [$FeCl_3$], isopropyl alcohol, DPPH [2, 2-diphenyl-1-picrylhydrazyl], ascorbic acid, methanol, and antibiotic disks were of analytical reagent grade and used directly without any further purification. Ingredients for media preparation were from HiMedia. Ginger was collected from a local market in Gandhidham, Gujarat, India. Distilled water was used in all experiments.

2.2. GC-MS Analysis of Zingiber officinale Root Extract. The root extract of Z. officinale was analyzed using HP5 Agilent Technology 5977B (Santa Clara, US) model no. 7820A MS coupled to a 5977B equipped with a HP-5 fused silica capillary column (30 m × 0.320 mm × 0.25 μm film thickness). Helium gas was used as the carrier gas. GC-MS programme was set as per the method described by Dhalani et al. [9].

2.3. Preparation of the Plant Extract. Ginger was collected from the local market and washed thoroughly with distilled water to eliminate dust on the surface, chopped into small pieces, sundried, and powdered. The extract was prepared by mixing 12 grams of dried ginger powder in 200 mL of isopropyl alcohol, and the mixture was stirred on a magnetic stirrer at 80°C for 1h; thereafter, the extract was filtered carefully, and the supernatant collected and stored at room temperature for further use [9].

2.4. Green Synthesis of Iron Nanoparticles. GR-FeNPs were synthesized by adding the equivalent extract to 0.01 M·$FeCl_3$ at room temperature and constantly stirring for 10 min. Immediate appearance of black brown color showed the reduction of Fe^{+3} ions, which is the first indication of the formation of iron nanoparticles. Afterwards, the liquid was poured into large Petri plates and dried at 100°C for 24 h in a hot-air oven and cooled down the next day. The upper layer of the plate was scraped out carefully using a spatula. The fine-dried black powder of ginger iron nanoparticles was kept ready for further characterization. All nanoparticle preparations were performed according to our previous study [10, 11].

2.5. Characterization

2.5.1. UV Visible Spectroscopy. UV visible spectroscopic analysis of the synthesized FeNPs was done by using 0.1 ml of sample diluted in 2 ml of deionized water. Absorbance was measured with the help of ABTRONICS Model No. LT-2900 in the range of 200–700 nm [12, 13].

2.5.2. FT-IR Analysis (Fourier Transform Infrared Spectrophotometer). FT-IR spectra of dried FeNPs and plant extract were determined using a Fourier transform infrared spectroscope. The synthesized nanoparticles were lyophilized and mixed with KBr pellets and further processed. An average of 9 scans were collected for each measurement with a resolution of 4 cm^{-1} at a range of 4000–650 cm^{-1} [14].

2.5.3. Particle Size Analyzer (PSA). The synthesized nanoparticles were analyzed by using a particle size analyzer, which measures the particle size by its flow through a beam of light producing a size distribution from the smallest to largest dimensions. When the particles are dissolved in water, they stay in a colloidal form and flow with a velocity that depends on their size and zeta potential (Brownian movement) [15].

2.5.4. X-Ray Crystallography. The crystalline structure of the synthesized nanoparticles was analyzed by powder X-ray crystallography (XRD).

2.6. Antimicrobial Activity. Gram-negative bacterial strains such as *Escherichia coli* MCC 2246 and *Klebsiella pneumoniae* MCC 2716 and Gram-positive strains such as *Staphylococcus aureus* MCC 2408 and *Bacillus subtilis* MCC 2244 were cultured overnight in nutrient broth media at 37°C with continuous agitation on an orbital shaker platform at 180 rpm. Simultaneously, nutrient agar media was dispersed into sterile petri plates and incubated for 24 hours at 37°C for sterility check. The overnight broth cultures of the test organisms (*E. coli*, *K. pneumoniae*, *S. aureus*, and *B. subtilis*) were used as inoculum. Antimicrobial activity was analyzed using the agar well diffusion method. The test culture of each organism (100 μl) was applied to each plate. The nanoparticles (30 μg/ml) were tested against kanamycin antibiotic (30 μg/disk), distilled water, ginger root extract(control), and $FeCl_3$ (0.01 M). The plates were incubated for 24 hrs at 37°C [16–18]. The zone of inhibition was observed and calculated.

2.7. Antimicrobial Activity of Iron Nanoparticle-Coated Surgical Cotton. FeNPs (30 μg/ml) were dissolved in methanol and sonicated for 10 minutes in an ultra sonicator. Dip coating is the precision- controlled immersion and withdrawal of a substrate into a reservoir of liquid for the deposition of a layer of material over it. A cotton piece of size 0.5 cm × 0.5 cm (substrate) was fixed to the head portion of the dip coater, which has a mechanical body that moves up and down. A small beaker with diluted NPs sample is placed

below the body, and the dip coating process was performed three times at a particular pressure and speed. The coated cotton was carefully removed using sterile forceps, dried using an air dryer, and stored in a ziplock bag for further use. Antimicrobial activity was analyzed using the agar well diffusion method. 100 μl of the test culture for each microorganism was spread using a sterile glass spreader on the nutrient agar plate and left for 10 minutes for the inoculum to get absorbed into the agar medium. A small piece of FeNPs-coated surgical cotton was placed in the middle of the plate and incubated for 48 hours, and the results noted [19, 20].

2.8. Antioxidant Activity of Iron Nanoparticles. Removal of free radicals using iron nanoparticles was performed using the DPPH [1, 1-diphenyl-2-picrylhydrazyl] assay. DPPH is considered a stable radical based on electron transfer (delocalization of the spare electron over the molecule as a whole) and produces a violet solution in methanol, showing a strong absorption band at 517 nm. The mixtures of different concentrations (20 μg/ml, 40 μg/ml, 60 μg/ml, 80 μg/ml, 100 μg/ml, 120 μg/ml, 140 μg/ml, and 180 μg/ml) of ginger powder, iron nanoparticles, $FeCl_3$, and ascorbic acid were prepared in absolute methanol. A 0.2 mM solution of DPPH was prepared by adding 0.007 g of DPPH in 100 mL of methanol. 2 mL of DPPH solution was added to all the test tubes. After incubation of all the test tubes for 30 min, absorbance was measured at 517 nm by using a UV-vis spectrophotometer [21–23]. The experiment was performed in triplicate and % inhibition was calculated using the following equation:

$$\%\ \text{inhibition} = \frac{\text{OD of control} - \text{OD of sample}}{\text{OD of sample}} \times 100. \quad (1)$$

3. Results and Discussion

3.1. GC-MS Analysis of Zingiber officinale Root Extract. The GC-MS profile of *Z. officinale* root extract is shown in Figure 1. The retention time and area (%) of each compound are given in Table 1. Out of 20 different chemical compounds, gingerol was found to be the major component with the highest retention time of 40.368 min. The presence of gingerol might be the reason for the reduction of metal ions and bactericidal activity of the nanoparticles.

3.2. Mechanism of FeNPs Formation from Z. officinale Extract. The process of synthesizing nanoparticles from plant extracts has been proven to be one of the most reliable, nontoxic, and eco-friendly approaches towards green chemistry and plant biotechnology. Plant extract is mixed with 0.01 M·$FeCl_3$ at a ratio of 2:3. The color change is due to the presence of polyphenols present in the plant extract which act as a reducing and capping agent, lowering the valency of Fe^{+3} to Fe^0, as shown in Figure 2.

Earlier studies reported that the color transformation from yellow to reddish black is the primary indication of the formation of iron oxide nanoparticles [24]. The aldehyde and polyphenol groups present in the leaf extract are responsible for the reduction of ferric chloride [12, 25].

3.3. UV-Visible Spectroscopy (UV-Vis). Spectroscopy is an analytical technique concerned with the measurement of absorption of electromagnetic radiation. UV-Vis spectroscopy is one of the oldest methods in molecular spectroscopy. It refers to absorption spectroscopy in the UV-visible spectral region. This means it uses light in the visible and adjacent ranges. The absorption varies with the difference in color in the chemicals in the given samples. The UV-visible spectra of iron nanoparticles in the aqueous ginger extract are shown in Figure 3. The absorption peak at a wavelength between 200 and 260 nm indicates the formation of iron nanoparticles (Figure 3). The sharp and intense peak is attributed to the uniform size of the particles [26, 27].

3.4. FT-IR Analysis. FT-IR analysis was carried out to evaluate the possible interaction between the biomolecules and Fe^{3+} during the biogenic reduction reaction. The FT-IR data for FeNPs containing *Z. officinale* root extract are given in Table 2. The bond stretching at 2927.8 cm^{-1} is attributed to the C-H bond, 1638.3 cm^{-1} to the C=O bond, 1517 cm^{-1} to the C-C amide group at 861 cm^{-1}, and C-N to 1075.3 cm^{-1} and 760 cm^{-1}, which was found to be very close to 688 cm^{-1}, which was attributed to the presence of zero valent FeNPs as shown in Figure 4. We can observe the FT-IR data of FeNPs with a plant sample [Figure 4] by analyzing the shift in bond stretching of the C-H bond from 2922 cm^{-1} to 2927 cm^{-1}, C-C bond from 1514 cm^{-1} to 1517 cm^{-1}, and C-N bond from 1037 cm^{-1} to 1075.5 cm^{-1}. This shift in bond stretching indicates the presence of FeNPs [28–30].

3.5. Particle Size Analysis. It is evident from the particle size analysis that smaller nanoparticles below 100 nm were synthesized, which might have agglomerated and resulted in larger nanoparticles. Furthermore, because of the magnetic property, the particles might have agglomerated, producing larger dimensions as depicted in Figure 5.

3.6. X-Ray Crystallography. To confirm the crystal structure of synthesized FeNPs, powder crystallography is an effective tool. Figure 6 represents the diffraction peak at 2θ values of 31.01°, 36.25°, 38.62°, and 42.2° corresponding to the crystal plane; (2 2 0), (3 1 1), (2 2 2), and (4 0 0) represent the crystalline structure of iron oxide nanoparticles.

3.7. Antimicrobial Activity. Various Gram-positive and Gram-negative bacterial strains were used to check the bactericidal activity of FeNPs synthesized via green chemistry. The excessive use of antibiotics has led to the emergence of new multidrug-resistant strains. It is necessary to find an alternative to antibiotics. Earlier studies reported the use of expensive metal nanoparticles as antimicrobial agents

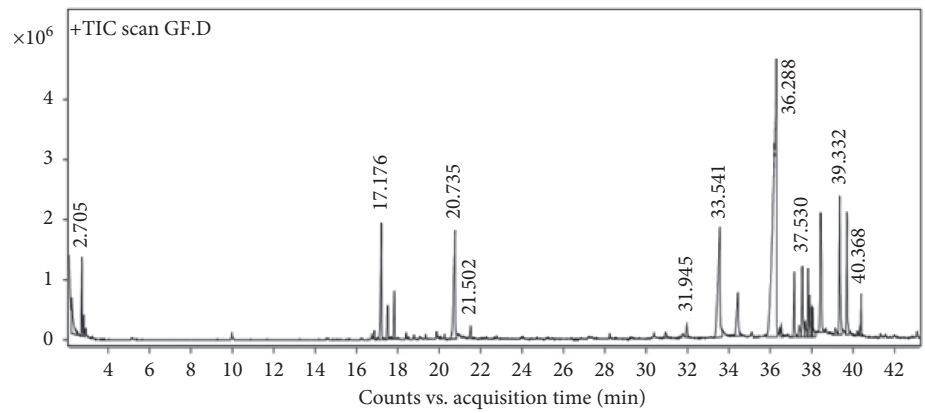


Figure 1: GC-MS profile of *Zingiber officinale* root extract.

Table 1: Phytochemical GC-MS analysis of *Zingiber officinale* root extract.

Peak	Compounds	Start	RT (min)	End	Area (%)
1	Propanol	2.196	2.213	2.459	6.25
2	2-Hexanol, 2-methyl	2.654	2.705	2.768	5.34
3	Benzene, 1,3-dimethyl(1S,5S)-2-methyl-5-((R)-6-methylhept-5-en-2-yl)bicyclo[3.1.0]hex-2-ene	2.768	2.797	2.865	1.34
4	(1S,5S)-2-methyl-5-((R)-6-methylhept-5-en-2-yl) bicyclo[3.1.0]hex-2-ene	17.062	17.176	17.279	11.84
5	Alpha.-farnesene	17.388	17.48	17.542	4.19
6	Beta.-bisabolene	17.731	17.806	17.889	4.11
7	Gingerol	20.529	20.735	20.81	18.15
8	Trans-sesquisabinene hydrate	33.281	33.541	33.65	27.63
9	Gingerol	34.222	34.411	34.611	8.79
10	3-Decanone, 1-(4-hydroxy-3-methoxyphenyl)	35.767	36.288	36.328	100
11	7-Oxabicyclo[4.1.0]heptane, 1-(2,3-dimethyl-1,3-butadienyl)-2,2,6-trimethyl-, (E)	37.055	37.14	37.249	6.34
12	Gingerol	37.455	37.53	37.627	7.83
13	1-(4-Hydroxy-3-methoxyphenyl)dec-4-en-3-one	37.747	37.804	37.839	5.26
14	Cedrol	37.839	37.879	37.947	4.22
15	Gingerol	37.947	37.982	38.01	2
16	Gingerol	38.01	38.045	38.176	2.97
17	1-(4-Hydroxy-3-methoxyphenyl)tetradec-4-en-3-one	38.302	38.411	38.625	18.28
18	Limonen-6-ol, pivalate	39.258	39.332	39.446	14
19	Oligandrol	39.612	39.687	39.795	10.59
20	Gingerol	40.328	40.368	40.419	3.06

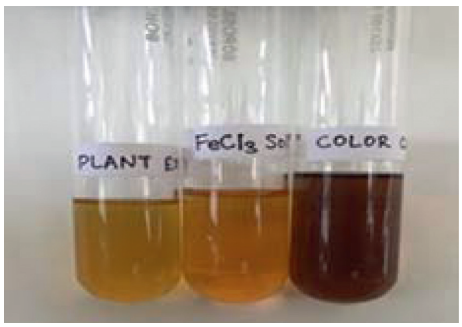


Figure 2: Primary indication of formation of nanoparticles—color change from yellow to brown.

[31–33]. To overcome this problem, the current study's focus was to design an eco-friendly and cost-effective method. The results are shown in Table 3. *E. coli* and *K. pneumoniae* show more sensitivity than Gram-positive bacteria. Compared with Gram-positive bacteria, Gram-negative bacteria have a thin layer of peptidoglycan. Hence, FeNPs can easily

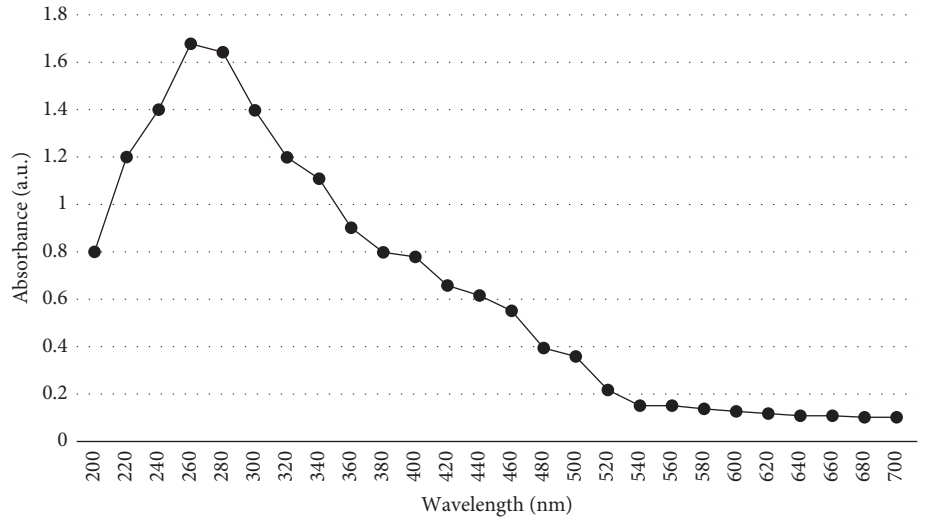


Figure 3: UV-visible spectroscopy analysis of synthesized GR-FeNPs.

Table 2: Comparison of bond stretching of synthesized FeNPs with *Z. officinale* root extract.

Samples	C-H bond stretching	C-C bond stretching	C-N bond stretching
Zingiber officinale root extract	2857 cm^{-1}	1514 cm^{-1}	1037 cm^{-1}
FeNPs synthesized from *Zingiber officinale* root extract	2927 cm^{-1}	1636 cm^{-1}	1075 cm^{-1}

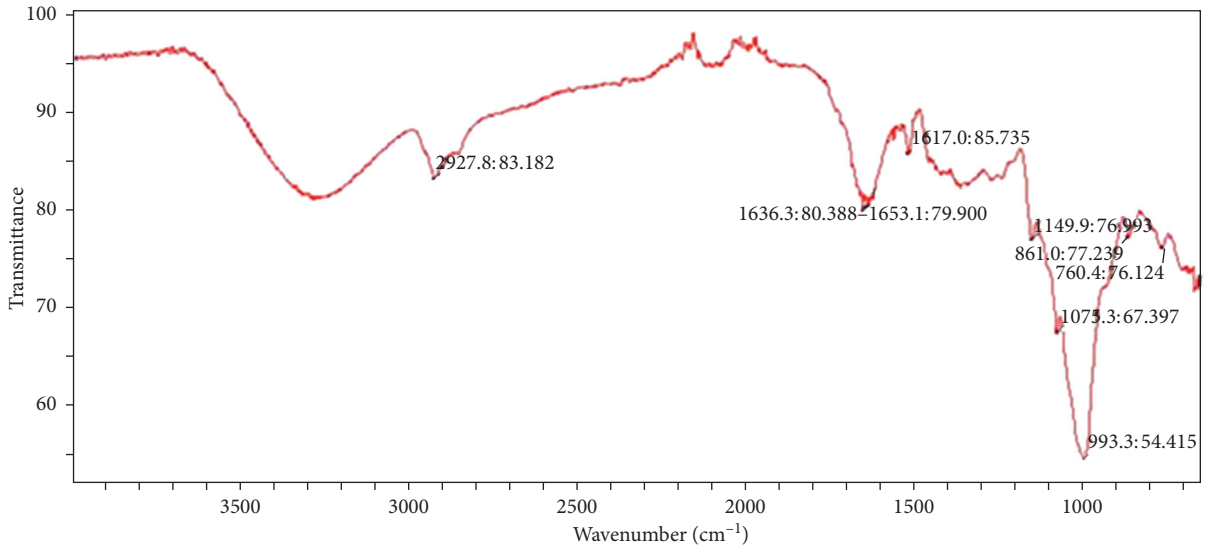


Figure 4: FT-IR analysis of GR-FeNPs.

penetrate the cell wall of Gram-negative bacteria (Figure 7). Our results support the notion of earlier studies that *E. coli* and *K. pneumoniae* showed higher zones of inhibition than *B. subtilis* and *S. aureus* [26, 27, 34].

3.8. Antimicrobial Activity of Iron Nanoparticle-Coated Surgical Cotton. The key outcome of the present study is the FeNPs-coated surgical cotton. The bactericidal activity of FeNPs extended to the surgical cotton, and it can be used further in wound healing, tissue therapy, and other medicinal applications. 10 μg/ml of FeNPs was used to coat the surgical cotton with the help of a dip coater. The antimicrobial activity was evaluated on Gram-positive *B. subtilis*, *S. aureus*, and Gram-negative *E. coli*, by the standard disc diffusion method. The results are given in Table 4. The results showed the radial diameter of the inhibiting zones of *B. subtilis*, *S. aureus*, and *E. coli* after 24 hours. The clear inhibition zones made by the FeNPs-coated surgical cotton obtained in the present study are shown in Figure 8. Antimicrobial activity was evaluated on day zero and 30 days after coating. Initially, the particles showed higher

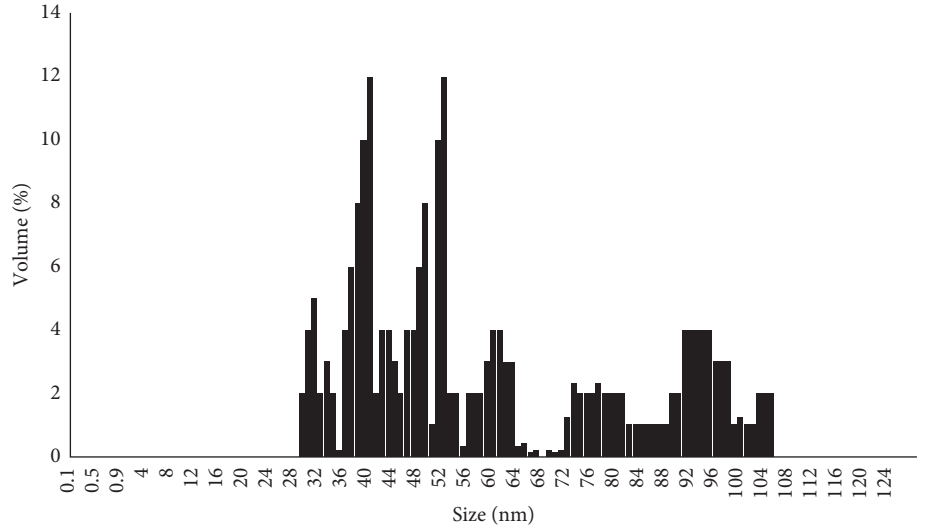


Figure 5: Particle size analysis of GR-FeNPs.

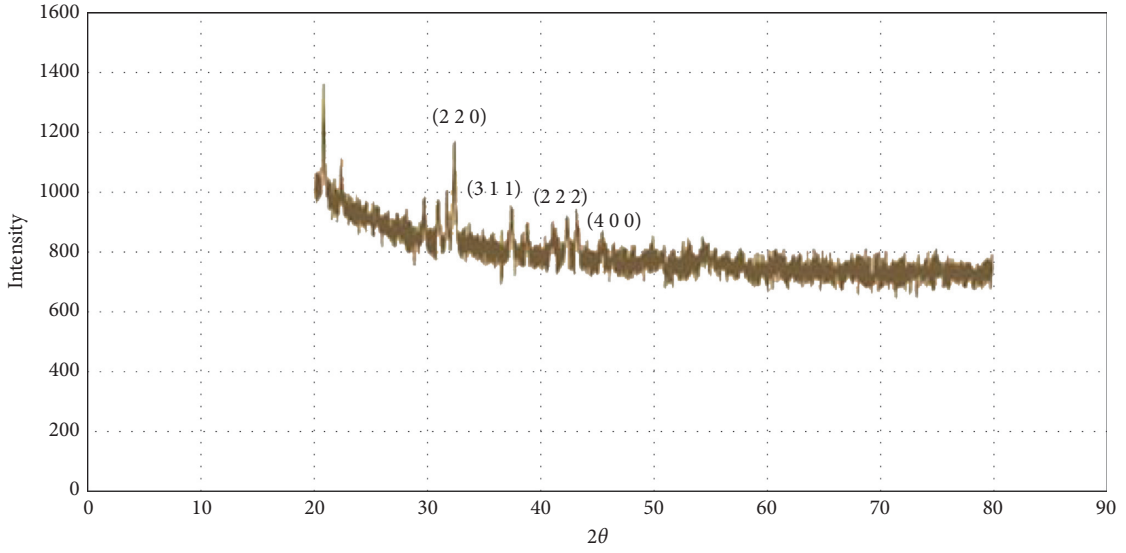


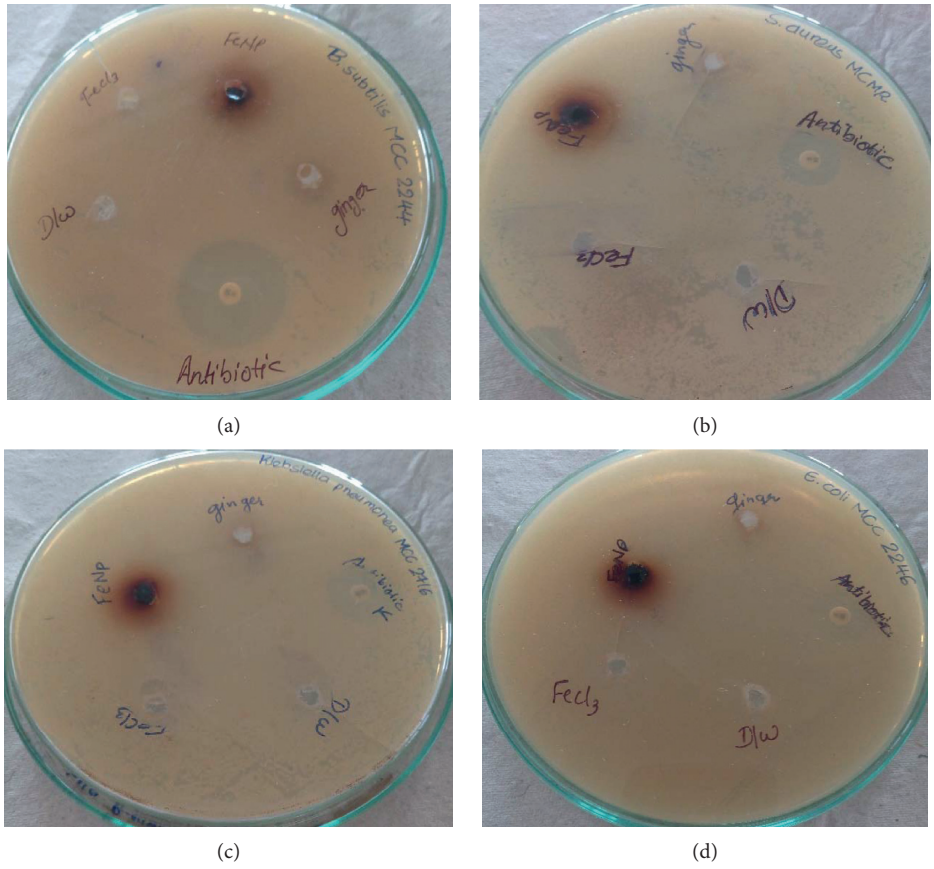
Figure 6: Powder X-ray diffraction pattern of GR-FeNPs.

Table 3: Diameter of the zone of inhibition of GR-FeNPs against Gram-positive and Gram-negative bacteria.

Pathogens	Diameter of the zone of inhibition (mm)				
	GR-FeNPs (30 μg/ml)	Antibiotic (kanamycin 30 μg/disk)	$FeCl_3$ (0.01 M)	Plant extract	Distilled water
Bacillus subtilis MCC 2244	10 ± 0.2	20 ± 0.4	5 ± 0.3	3 ± 0.2	NO
Escherichia coli MCC 2246	18 ± 0.34	13 ± 0.4	8 ± 0.2	2 ± 0.1	NO
Klebsiella pneumoniae MCC 2716	16 ± 0.4	12 ± 0.3	7 ± 0.3	2 ± 0.2	NO
Staphylococcs aureus MCC 2408	13 ± 0.23	15 ± 0.3	5 ± 0.4	3 ± 0.1	NO

NO: not observed

antimicrobial activity, which diminished in terms of zone diameter due to the development of resistance in the microbial culture used for the study. The clear zone even after 30 days indicates bacterial growth restriction by the diffused FeNPs over the surgical cotton. Furthermore, the green approach for synthesis of FeNPs can be applied on cotton fabric which could have good bactericidal activity in wound dressing [30, 35–38].



(a) (b)

(c) (d)

Figure 7: Antimicrobial activity of GR-FeNPs against (a) *Bacillus subtilis*, (b) *Staphylococcs aureus*, (c) *Klebsiella pneumoniae*, and (d) *Escherichia coli*.

Table 4: Antimicrobial activity of GR-FeNPs-coated surgical cotton.

Pathogens	Diameter of zone of inhibition (24 hours)	Diameter of zone of inhibition (30 days)
Bacillus subtilis (MCC 2244)	9 mm ± 0.24 mm	5 mm ± 0.4 mm
Staphylococcus aureus (MCC 2408)	12 mm ± 0.28 mm	11 mm ± 0.29 mm
Escherichia coli (MCC 2246)	14 mm ± 0.3 mm	6 mm ± 0.3 mm

3.9. Antioxidant Activity. *Z. officinale* is well known for its herbal properties as it is used in Chinese and Indian medicine since ancient times. The antioxidant property of ginger is due to the presence of phytochemicals such as gingerol, vitamin C, ß carotene, flavonoids, and tannins. Life on Earth without oxygen is impossible, and oxygen is also a well-known reactive molecule that causes damage to living organisms by producing reactive oxygen species. Our body is a complex network of antioxidant metabolites and enzymes that work together to prevent oxidative damage to cellular components. The main function of reactive oxygen species is signalling redox reactions. Thus, the function of an antioxidant system is not to remove oxidants entirely but instead to maintain an optimum level inside the body. Ascorbic acid has high amounts of antioxidants; thus, it was used as standard (Figure 9). By using the DPPH assay at different sets of concentrations, antimicrobial activity was evaluated in triplicate.

The total antioxidant capacity of *Z. officinale* was expressed as the number of equivalents of ascorbic acid. The color of the DPPH solution in the presence of the GR-FeNPs changes gradually from deep violet to pale yellow, which allowed the visual monitoring of the antioxidant activity of the nanoparticles. The observed effect of FeNPs is in the following order: ascorbic acid > FeNPs > $FeCl_3$ > plant extract [Figure 8]. The study revealed that the antioxidant activity of the extract follows an increasing trend with the increase in concentration of the GR-FeNPs. DPPH activity results showed the highest free radical % scavenging potentials of 0.01 M $FeCl_3$, GR extract, GR-Fe NPs, and ascorbic acid to be 74%, 71%, 89%, and 92%, respectively, at a concentration of 160 μg/mL [39, 40].

4. Conclusion

The present work highlighted the green chemistry of synthesizing iron nanoparticles from the root extract of *Z.*

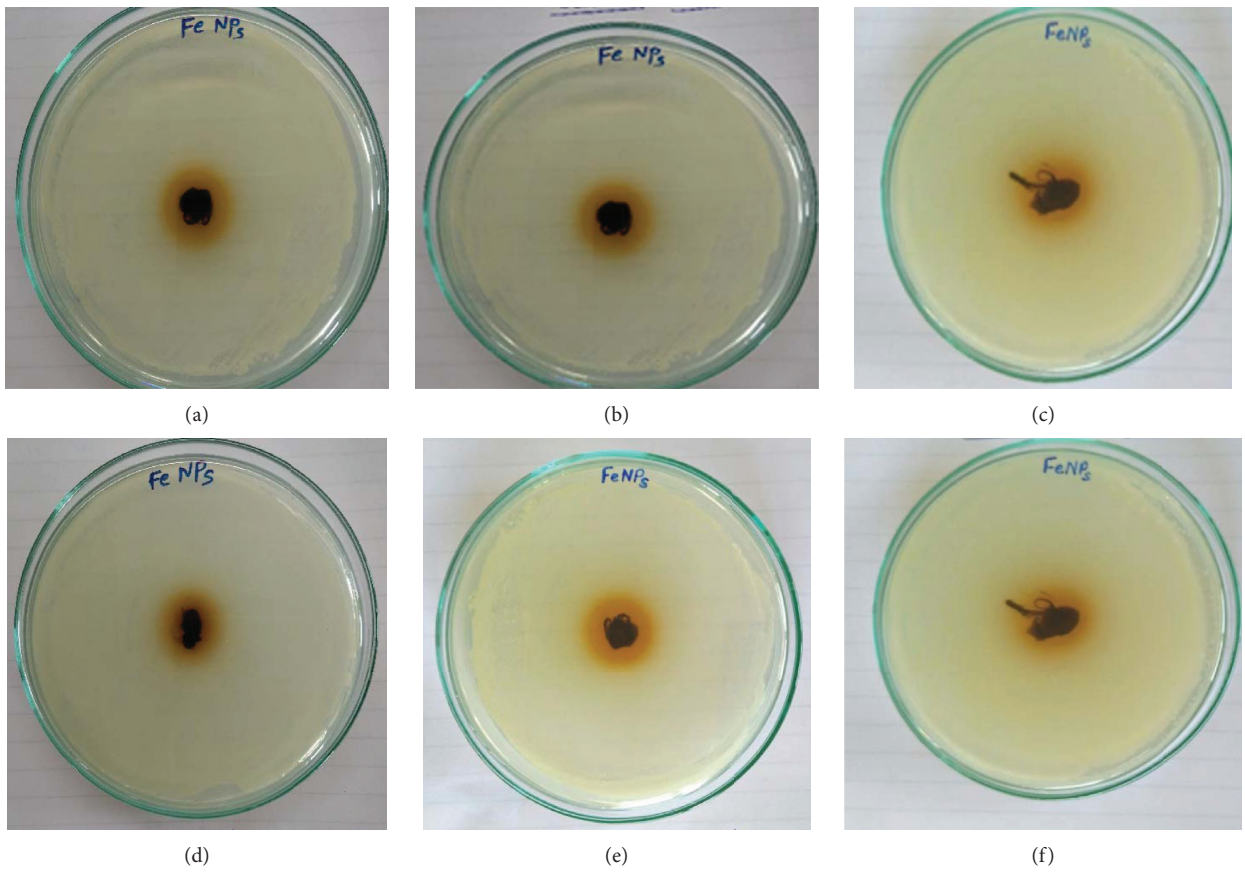


Figure 8: Antimicrobial activity of GR-FeNPs-coated surgical cotton after 24 hrs against (a) *Bacillus subtilis*, (b) *Staphylococcs aureus*, and (c) *Escherichia coli*, and after 30 days against (d) *Bacillus subtilis*, (e) *Staphylococcs aureus*, and (f) *Escherichia coli*.

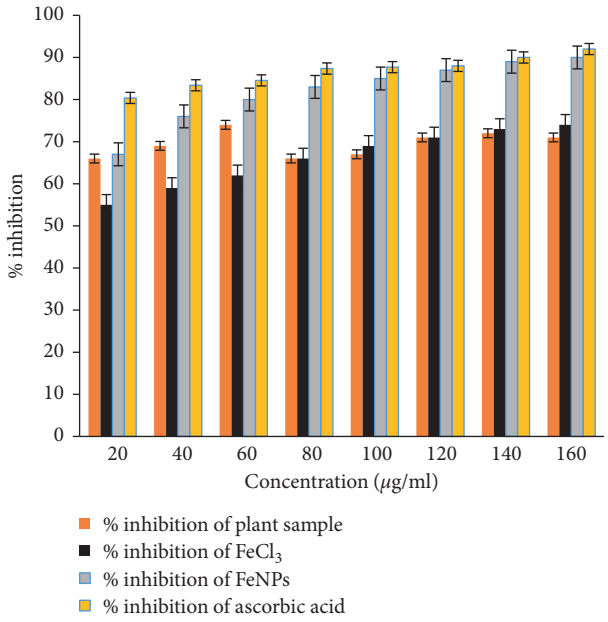


Figure 9: Antioxidant activity of GR-FeNPs.

officinale. The production proved to be easy, cost effective, and eco-friendly with natural reagents and less harsh chemicals. The color change was also remarkable when ferric chloride solution was mixed with the reducing agent of the plant extract. The biosynthesized FeNPs were characterized by UV-Vis spectroscopy that showed a surface plasmon resonance behaviour. The antimicrobial activity reported using green approach-synthesized nanoparticles may be further beneficial for various applications for better prognosis of several diseases, and the antioxidant activity of *Z. officinale* root extract has shown tremendous results. Iron nanocoated surgical cotton would have great role for distinguished health applications by creating new gadgets such as biosensors which can be implemented for the study may enhance the effects of conventional antimicrobials, which will probably decrease costs and improve the treatment quality. Thus, nanocoated surgical cotton obtained using a green synthesis approach might be a promising path in the field of medical microbiology. Future studies are still needed to design nanochips having an antimicrobial property which can replace antibiotics in our lives.

Data Availability

GC/MS analysis data is available in supplementary files, rest of the data are available within manuscript.

Conflicts of Interest

The authors declared no conflicts of interest.

Acknowledgments

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Supplementary Materials

The supplementary material includes detailed GC/MS chromatograph and structure of phytochemicals present in the root extract of *Z. officinale*. Based upon the supplementary data, the retention time of each phytochemical has been included in the current study. (*Supplementary Materials*)

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Research Article

Antimutagenic and Synergistic Cytotoxic Effect of Cisplatin and Honey Bee Venom on 4T1 Invasive Mammary Carcinoma Cell Line

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Introduction. Honey bee venom (HBV) has various biological activities such as the inhibitory effect on several types of cancer. Cisplatin is an old and potent drug to treat most of the cancers. Our aim in the present study was to determine antimutagenic and cytotoxic effects of HBV on mammary carcinoma, exclusively and in combination with cisplatin. *Methods*. In this study, 4T1 cell line was cultured in RPMI-1640 with 10% fetal bovine serum (FBS), at 37°C in humidified CO_2 incubator. The cell viabilities were examined by the MTT assay. Also, HBV was screenedþ for its antimutagenic activity via the Ames test. The results were assessed by SPSS software version 19 and one-way ANOVA method considering $p < 0.05$ level of significance. *Results*. The results showed that 6 mg/ml of HBV, 20 μg/ml of cisplatin, and 6 mg/ml HBV with 10 μg/ml cisplatin could induce approximately 50% of 4T1 cell death. The concentration 7 mg/ml of HBV with of 62.76% inhibitory rate showed the highest antimutagenic activity in comparison with other treatment groups. *Conclusions*. The MTT assay demonstrated that HBV and cisplatin could cause cell death in a dose-dependent manner. The cytotoxic effect of cisplatin also promoted by HBV. Ames test outcomes indicated that HBV could act as a significant mutagenic agent. The antimutagenic activity of HBV was increased considerably in the presence of S9 mix. Therefore, our findings have revealed that HBV can enhance the cytotoxic effect of cisplatin drug and its cancer-preventing effects.

1. Introduction

Breast carcinoma is a common cancer and the most common malignancy among women in the industrialized countries for the last decades. Breast cancer originates from the breast tissue and uncontrolled growth of the inner lining of the milk ducts or lobules [1]. Breast cancer is the most commonly diagnosed cancer and the primary cause of cancer death among worldwide females [2]. Several studies have shown that breast carcinoma mostly occurs in similar structures of the breast, and these structures are the origin site of ductal carcinoma. Approximately 1-2 years after the onset of the first menstrual period, lobule formation is started. There is a gradual process for mammary gland, for which it needs several years. Parous women, particularly women who have full-term pregnancy experience at young age, have full lobular differentiation in breast structure [3]. The most risk factors for breast cancer have been estimated and studied such as diet, oral contraception, and post-menopausal substituent treatment with estrogen, breast irradiation, and environment [4].

The 4T1 mouse mammary tumour cell line is a model of breast cancer, which is able to metastasize to site affected in human breast cancer. In 1983, Fred Miller and coworkers isolated the 4T1 mammary mouse carcinoma cell line from BALB/c mammary tumour. This cell line has the potential to

metastasize to bone and several organs affected by breast cancer consist of the lung, liver, and brain. Hence in recent years, usage of this cell line has increased [5].

One of the most important causes of spontaneous breast cancer is genetic mutations. The molecular studies of mammary tumours indicated that the amplification of oncogenic genes such as erb-B1, erb-B2, c-myc, and int-2 is common [6]. About 5–10% of all breast cancer cases are hereditary breast cancer. BRCA1, tumour suppressor gene, is mutated in the most hereditary breast and ovarian cancer [7].

Recently, the treatment of cancer becomes a global concern. The various types of cancer treatment ways, such as surgery, the anticancer drug (chemotherapy), irradiation, hormone therapy, and nutritional supplementation are used. Chemotherapy is a systematic therapy in which all of the body cells are exposed to chemotropic agents [8].

Using metals as medicine backs to 5000 years ago. The investigations on minerals resulted in developing modern medicine with metal components for the treatment of some diseases such as cancer. Cisplatin (*cis*-dichlorodiammineplatinum (II)) is the first stage of chemotherapy for most cancers, including testicular, ovarian, cervical, small lung-cell, and also breast cancer [9].

The cytotoxic effect of cisplatin depends on dosage, and its high dosage has improved effects on cancerous cells. However, the application of cisplatin is subject to some restrictions as it has several potential side effects, special nephrotoxicity, and neurotoxicity effects [10].

Honey bee venom (HBV) is an active product which is produced by the venom glands associated with the sting apparatus of honey bee workers and their queen. The history of apitherapy returns to 3000 to 5000 years ago [11]. HBV is a complex mixture of active enzymes, peptides, and amines. Its most important components are melittin and phospholipase A2, adolapin, and mast cell degranulating peptide. Several in vitro and in vivo studies revealed that HBV shows anti-inflammatory, cytotoxic, and antibacterial effects, and also it can cause a severe allergic reaction [12]. HBV as an old medicine has been used to treat arthritis, rheumatism, back pain, cancerous tumours, and skin diseases [13].

According to anticancer effects of HBV, the aim of this study was to investigate the cytotoxic effect of honey bee venom on the 4T1 cell line, lonely and in combination with Cisplatin. Also, antimutagenic activity and anti-cancerous effects of bee venom were studied by Ames test.

2. Materials and Methods

2.1. Cell Culture. The mouse mammary carcinoma 4T1 cell line was purchased from cell bank of Pasteur Institute of Iran. Cells were cultured in RPMI-1640 (Gibco-Invitrogen) with 10% fetal bovine serum (FBS) (Gibco-Invitrogen) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) at 37°C in a 5% CO_2 and 95% O_2 humidified incubator. The culture medium was changed every 24 h.

2.2. HBV Preparation. Honey bee venom was mustered from *Apis mellifera* using an electric shocker apparatus composed of a shocker and collector unit. The shocker unit produces a light electric shock once every few seconds. Honey bees were incited through light electric shock and sting. The collector device is a network of wires with small gaps and a glass plane between them. Every 25 minutes, the shocker unit turned off, and the dried bee venom material on the collector panel was collected by scraping.

HBV was stored in powder form at −20°C and dark condition. The main stock solution of HBV was prepared with 1 mg of HBV and 1 ml phosphate-buffered saline (PBS). In the end, to obtain a homogenous and sterile solution, the solution was passed through a 0.2 μm filter. For every assay, this solution was prepared freshly. For the Ames assay, the concentration of the primary stock solution was 10 mg/ml (HBV + PBS) and other interested concentrations were obtained by making dilution from the main solution.

2.3. Cisplatin Preparation. Cisplatin was purchased from Sobhan Oncology Company of Iran with 50 mg/ml concentration, and it was stored in the 4°C and dark situation. Interested concentrations were obtained by diluted the main solution.

2.4. MTT Assay. The MTT assay is one of the basis reductions of yellow MTT-dimethylthiazol diphenyl tetrazolium bromide (tetrazole) to the purple formazan crystal by mitochondrial dehydrogenase in living cells. Adherent 4T1 cells were trypsinized by trypsin-EDTA 0.25% (Gibco-Invitrogen). Then, the cells seeded in 24-well plate and cultured overnight in order to fully adhere the cells to the plate. The 4T1 cells were treated with different concentrations of honey bee venom as follows: zero as the control, 2, 4, 6, 8, and 10 mg/ml, and cisplatin, 0 as a control, 5, 10, 15, 20, 25, and 30 μg/ml, and also cisplatin and HBV together, 0 as control, 2 + 10, 4 + 10, 6 + 10, 8 + 10, and 10 + 10 mg/ml, for 24 h. Cell viability was measured using the MTT assay (Sigma-America). MTT solution was prepared (5 mg MTT powder in 1 ml PBS), and then it was filtered via 0.2 μm micropore filter. After 24 h incubation of treated cells, 50 ml MTT solution was added to each well, and the plate was incubated at 37°C for 4 hours and dark situation. Subsequently, the supernatant liquid was removed, 1 ml dimethylsulfoxide (DMSO) (Merck, Germany) was added to each well, and the plate was kept at room temperature for 15 minutes. Finally, the absorbance was measured at 570 nm wavelength by a spectrophotometer (Milton Roy-Spectronic 2ID- America). The viability percent was calculated as follows:

$$\text{viability percent} = \frac{\text{optical density of experimental group}}{\text{optical density of control group}} \times 100. \quad (1)$$

2.5. MIC Assay. To determine the minimum inhibitory concentration of honey bee venom, the MIC assay was performed. Salmonella TA100 suspension was cultured in nutrient broth medium and justified by comparison with 0.5 Mc-Farland turbidity standard tube (1.5 × 108 organisms/

ml). The main stock solution of HBV with 10 mg/ml concentration was prepared. Then the main stock was diluted, and 1 mg/ml to 10 mg/ml concentrations were obtained. Finally, each tube received a specific level of HBV. Test tubes were incubated at 37°C for 24 h. Distilled water was used as a negative control. The growth of bacteria in control and test tubes were investigated after 24 h.

2.6. Ames Test. The Ames test is designed for analysis of mutagenic and antimutagenic factors [14]. Salmonella TA100, which is used in this test, has various mutations in histidine operon genes. Therefore, in the absence of histidine, the bacteria are not able to grow and create a colony. In the presence of mutagen factors, reverse mutation is occurring, so the bacteria are able to grow and form colonies [15]. Histidine-dependent strain of *Salmonella typhimurium* TA100 used for the Ames test. *Salmonella typhimurium* TA100 developed by Dr Ames of the University of California, Berkeley, USA, was cultured in nutrient broth (Sigma, America). The bacterial suspension was prepared 1-2 $\times 10^9$ cells/ml fresh cultures.

To prepare of rat microsomal liver enzyme (S9), the mature male rats (about 200 g body weights) were deprived of food for 48 h to achieve high-level hepatic enzymes. Then, the rats were killed and the livers were removed. After washing with PBS solution, the livers were cut into small pieces and homogenized by 1M KCl solution. Finally, this solution was centrifuged for 10 min at 8700 rpm. The supernatant was isolated and stored at −80°C.

(i) Test groups: 100 μl bacteria suspension, 100 μl histidine-biotin solution (24 mg biotin + 31 mg histidine in 250 ml distilled water), and 100 μl sodium azide solution (10 ml distilled water plus 0.015 g sodium azide) were added to the test tube containing Top Agar (0.6 g agar plus 0.5 g NaCl plus 100 ml distilled water), and finally the test tubes were incubated with 1–7 mg/ml concentrations of HBV.

(ii) Positive control: 100 μl bacteria suspension, 100 μl histidine-biotin solution, and 100 μl sodium azide solution were combined to a tube contain Top Agar.

(iii) Negative control: 100 μl bacteria suspension, 100 μl histidine-biotin solution, and 100 μl distilled water were combined to a test tube contain Top Agar.

Finally, the content of these tubes after 3-second shaking was distributed on the top of the minimum medium of glucose agar (% 40 glucose). The plates were incubated at 37°C for 48 hours. All of these antimutagenic assays were performed in the absence and presence of S9, and for each test, three repeats were considered. Finally, reversed colonies were counted and inhibition percentage was calculated by this formula:

$$\text{inhibition percentage} = \left[\frac{1-T}{M}\right] \times 100, \quad (2)$$

where T is the number of revertants per plate in the presence of mutagen and test sample and M is the number of revertants per plate in the positive control.

2.7. Statistical Analysis. The results were assessed by the one-way ANOVA method and also in combination with the Tukey test for pairwise comparison. p values less than 0.05 were considered significant. Statistical analysis was performed by SPSS 22.0, and the charts were drawn by Excel software.

3. Results and Discussion

3.1. MTT Assay. For investigating the cytotoxic effect of HBV and cisplatin on the 4T1 cell line, the cells were treated with various concentrations of HBV and cisplatin alone and in combination (HBV/cisplatin). Also, to determine cell viability, the MTT assay was performed. The MTT assay revealed that cisplatin and HBV have a cytotoxic effect on 4T1 cell line, and they can reduce the cell viability in a dose-dependent manner. As shown in Figure 1(a), by increasing of HBV concentrations, the cell viability has been reduced. Also, the treated group in comparison with the control group has a significant reduction of viability in a dose-dependent manner ($p < 0.05$) (Figure 1(a)).

On the other hand, cisplatin has a cytotoxic effect on the 4T1 cell line. High concentrations of cisplatin have shown more effective cytotoxicity in comparison with the control group ($p < 0.001$) (Figure 1(b)). Combination treatment of HBV and cisplatin on the 4T1 cell line showed that HBV could promote the cytotoxic effect of cisplatin in a dose-dependent manner (Figure 1(c)). Treatment with 6 μg/ml HBV and 25 μg/ml with cisplatin for 24 h can cause an approximately 50% 4T1 cell death. In combination, cisplatin and HBV, 6 μg/ml + 10 μg/ml can cause about 50% cell death.

3.2. MIC Assay. The minimum inhibitory concentration assay for serial dilution concentrations of HBV was performed. The survey of results indicated that HBV can cause death in salmonella TA100 with dosages more than 8 mg/ml. The tubes with 1 mg/ml to 7 mg/ml concentrations showed turbidity, so the bacteria were able to grow. Hence, the MIC of HBV on *Salmonella* TA100 was determined to be 8 mg/ml.

3.3. Ames Assay. To examine the antimutagenic and anticancerous activities of HBV, the Ames test was performed with 1 to 7 mg/ml concentrations of HBV (less than MIC) in the presence and absence of S9 fraction. After 48 hours, reversed colonies were counted (Figure 2). The plates with different concentrations of HBV have shown reduced colonies in a dose-dependent manner. Comparison between the test and positive control groups has demonstrated significant differences ($p < 0.001$) (Figure 3(a)). Also, the Ames assay was performed in the presence of S9, and the result indicated that antimutagenic activity was improved with S9 (Figure 3(b)). The inhibition percentages of HBV in the presence and absence of S9 were obtained, 62.76 and 56.17 (Figure 4).

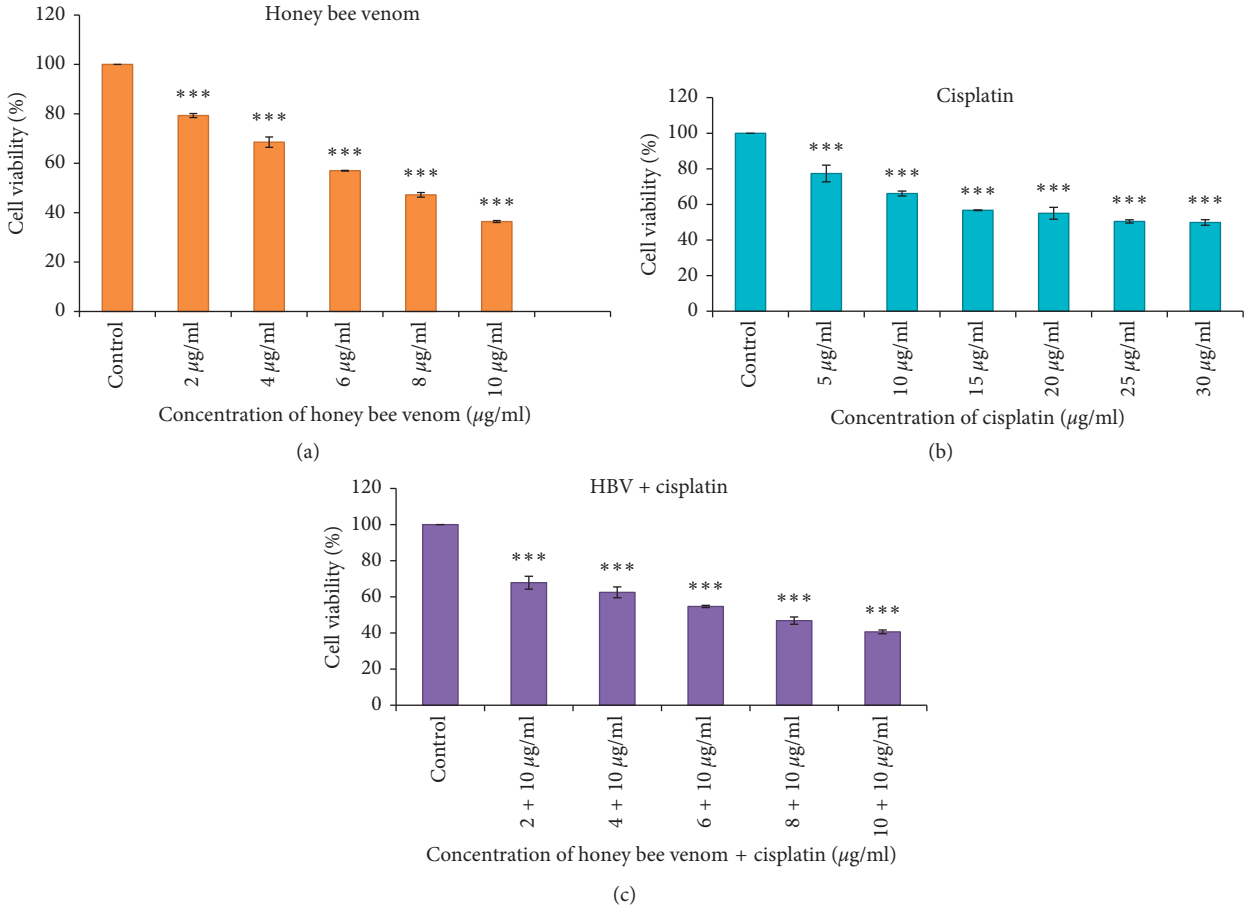

Figure 1: The cell viability percentages of a treated 4T1 cell line with various concentrations of HBV (a), cisplatin (b), and HBV/cisplatin (c) after 24 h by MTT staining (mean ± SEM, $^{***}p < 0.001$). HBV: honey bee venom.

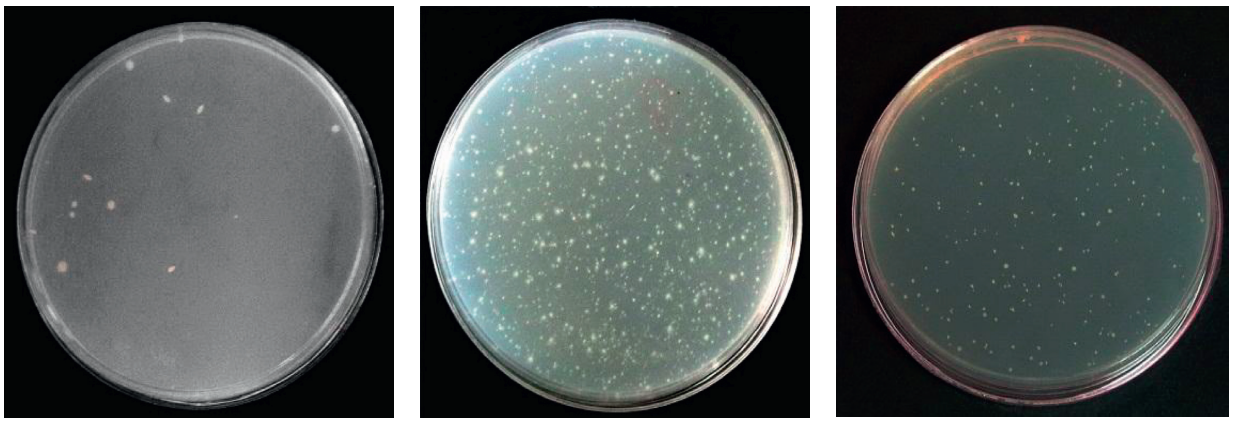

Figure 2: The revertants colonies in the negative test (a), positive test (b), and 7 mg/ml concentration of HBV (c).

4. Discussion

Breast cancer is the most prevalent cancer among women of developed countries, and its incidence has been expanding worldwide [16]. The cytotoxic chemotherapy is used to cure the early and late stages of most cancers in the recent decade [17]. In the 1970s, findings about the anticancer effect of Cisplatin lead to a revolution in the clinical chemotropic agent [18]. Our objectives included examining the cytotoxic and antimutagenic effects of HBV and cisplatin on the mouse mammary carcinoma 4T1 cell.

Our results revealed the cytotoxic activity of cisplatin on 4T1 cell line. Determined IC50 value for cisplatin was 25 μg/ml after 24 hr. Cisplatin, *cis*-diamino-dichloro-platinum (II)

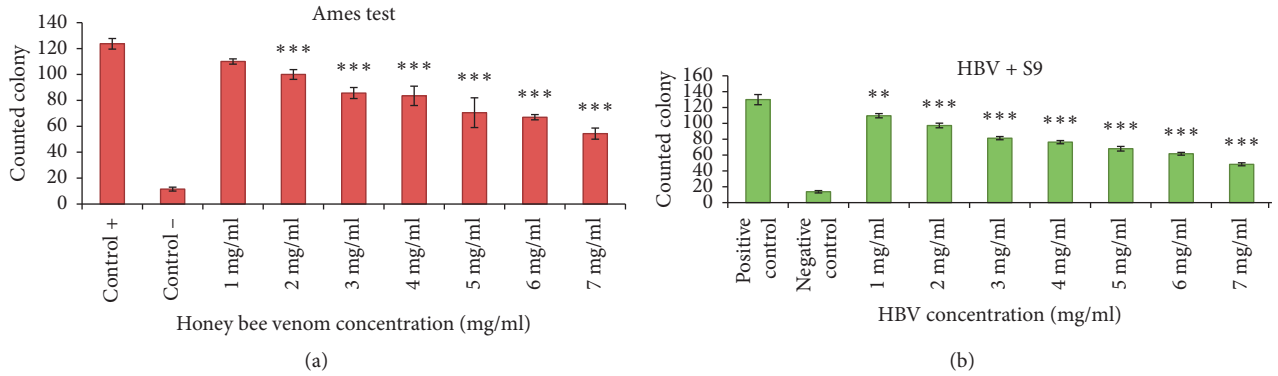

Figure 3: Reverted *Salmonella* TA100 colonies counts in compression with a positive control group with (a) and without S9 (b) by the Ames test (mean ± SEM, $^{***}p < 0.001$, $^{**}p < 0.01$).

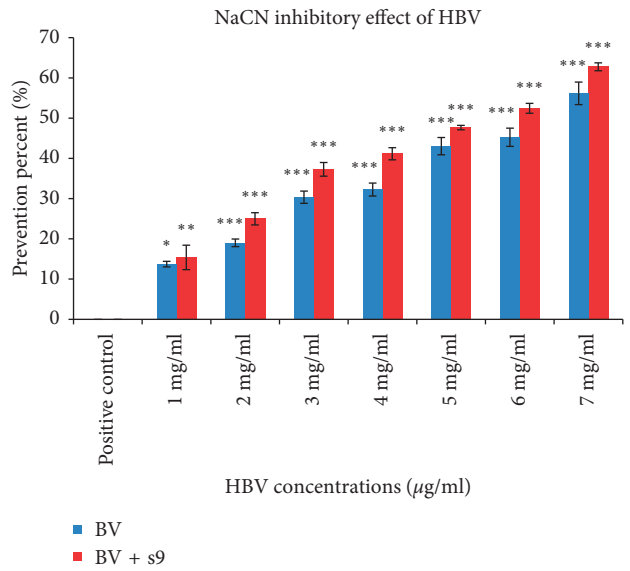

Figure 4: Sodium azide inhibitory effect of HBV in comparison with the positive control group with and without S9 fraction by the Ames test. S9 fraction promoted the prevention effect in a dose-dependent manner (mean ± SEM, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$).

(CDDP), a complex containing a central atom of platinum to which two ammonium ions and two chlorine ions are bonded in the *cis* position with respect to the horizontal plane of the molecule [19].

Positively charged metals are able to bind to the negatively charged molecules such as protein and nucleic acid [20]. The evidence suggests that the membrane proteins such as the copper transporter 1 (CTR1) accumulate cisplatin in the cells. Meanwhile, replacement of water molecules with chloride ligands activates the cisplatin molecule. The equated forms of cisplatin can bind to DNA at the N7 position of purine bases and form primarily 1,2-intrastrand adducts between adjacent guanosine residues [21]. This cross-linking with DNA and the resulting DNA bending disrupt the replication and transcription process. Hence, the cell cycle is arrested or the apoptosis process will be activated [18]. Cisplatin and other platinum complexes such as carboplatin and oxaliplatin are more useful and effective for treating the most of cancers; however, these components have several severe side effects which affect other healthy organs. Therefore, these side effects result in some limitations of using these chemodrugs in cancer treatment [21].

Most of the new small molecules discovered in cancer treatment are natural products and their derivatives [22]. HBV as a natural product has been used for medicinal applications for thousands of years. HBV contains at least 18 pharmacologically active components, including melittin, phospholipase A2, histamine, and adolapin. The outcomes of various studies on the biological activity of HBV suggest that it is highly effective in several diseases such as arthritis, MS, and back pain [11].

In the present experiment, the concentration of HBV that inhibited growth 4T1 cell line by 50% after 24 hr (IC50) was 6 μg/ml. Our result is different in comparison with the lethal dosage in other cell lines. As illustrated, this level was 1.43 g/ml for mammary carcinoma MCa [23]), 2 μg/ml for human leukemic U937 cell line [12]), 8 μg/ml for A2780cp [21]), 2 μg/ml for human melanoma A2058 cell lines [24], and 10 μg/mlþ for the human lung cancer NCI-H1299 cell line [25].

Hait et al. [26] demonstrated that melittin is one of the most potent inhibitors of calmodulin activity and also a potent inhibitor of cell growth and clonogenicity. The calcium binding protein, which is named calmodulin, plays a vital role in cellular proliferation [26]. In 2003, Oroli et al. investigated the effect of HBV on MCa in both in vitro and in vivo environments. His results showed that HBV exerts direct (inhibition of calmodulin and prevention of cell growth) and indirect (the stimulation of macrophages and cytotoxic T lymphocytes) effect on MCa tumour cells [27]. Furthermore, Moon et al. [12] studied the key regulators in HBV-induced apoptosis in human leukemic U937 cells. Their outcomes confirmed that HBV inhibits cell proliferation via inducing apoptosis in U937 cells through downregulation of Bcl-2 and upregulation of caspase-3. They demonstrated that HBV increases Fas/Fas ligand levels and decreases Cox-2 and hTERT [12]. According to their study, it can be presumed that HBV induces apoptosis through the extrinsic pathway.

In 2008, Siu-Wan Ip indicated that HBV induces the mitochondria-dependent pathway of apoptosis in human breast cancer MCF7 cells. Their results confirmed that HBV induces DNA strand breaks and promoted P53 and P21 factors. HBV also affects the ratio of Bax/Bcl-2 level, leading to releasing cytochrome c and finally triggering of the mitochondrial apoptosis pathway [28].

Accordingly, our findings are in agreement with other reports about the cytotoxic effect of HBV on cancerous cells. While HBV is probably targeting the DNA molecule and also inhibits calmodulin protein, it is probable that HBV exerts its cytotoxic and growth prevention effects on 4T1 cell line through the intrinsic/extrinsic apoptosis pathway or cell cycle arrest.

Our findings revealed that the cytotoxic effect of cisplatin is risen by a combination of nonlethal concentrations of HBV. In 2009, Orsolic investigated the cytotoxic effect of HBV in combination with Bleomycin in Hela and V79 cell lines. The nonlethal dosage of HBV with bleomycin cause increase death cell in a dose-depended manner. He suggested that HBV inhibits DNA repair, and this may be the mechanism by which it increases bleomycin lethality and inhibits recovery from bleomycin-induced damage [23].

On the other hand, Gajski and Garaj-Vrhovac [29] demonstrated that HBV induces single- and double-strand DNA breaks in human lymphocytes [29]. According to these findings, since cisplatin targets the DNA molecule, it is probable that HBV is able to promote the cytotoxic effect of cisplatin via this mechanism. Our data in the present investigation are similar to Alizadeh's study on the investigation of the synergistic effect of BV and cisplatin on human ovarian cancer cell line A2780cp. He showed that HBV boosts the cytotoxic effect of cisplatin [21].

A mutation, which is a natural process that changes a DNA sequence, is one of the most important causes of cancer. Also, changes in the structure of chromosomes have a critical role in creating most of malignancies [30]. In this study, HBV inhibited reverse mutation in *Salmonella typhimurium* TA100 in a dose-dependent manner. According to Ames theory, the number of revertants colony on the positive control plate (with carcinogen substrate) should be 2 times more than test plates. Also, mutagen prevention percentage contains three classes including inhibitory percent more than 40% which shows high prevention potential, between 25 and 40% which shows medium potential and less than 25% which shows negative prevention [30].

The outcomes of the present investigation showed that the number of reversed colonies in the treated plate with 7 mg/ml concentration of HBV (with S9 and without S9) is less than half of colonies in positive control plate. On the other hand, the inhibitory percentage for 5 mg/ml, 6 mg/ml, and 7 mg/ml concentrations of HBV was 43.05%, 45.26%, and 56.17% in the absence of S9, respectively. Furthermore, the S9 solution improved the HBV inhibitory effect. The S9 fraction is prepared from the liver of rats, and it contains a hepatic enzyme which effectively converts bioactive promutagens to mutagens.

This metabolic activation of mutagens is considered a vital step for carcinogenesis because most of the carcinogen must enzymatically transform to electrophilic specious. This activated mutagen can covalently bind to DNA molecule leading to mutation [31, 32]. Other studies were performed to investigate the antimutagenic potential of some natural products. In 2011, Ghazali et al. confirmed that extract of *M. speciosa* indicates antimutagenic activity [33]. Also, Issazadeh and Aliabadi [34] concluded that olive leaf shows antimutagenic and anticarcinogenic effects [18]. Also, our data demonstrated that, in the presence of S9 fraction, the mutagen prevention percentage of HBV was promoted. Since S9 fraction activates sodium azide mutagen, it is likely that HBV prevents the mutagenic effects of sodium azide. Finally, our result is in agreement with previous studies and its interpretation confirmed that HBV has antimutagenic and anticancerous effect in a dose-independent manner.

5. Conclusions

According to our results in the present research, we assessed that HBV as a natural product has cytotoxic effects on the mouse mammary carcinoma 4T1 cell line. Cisplatin as a chemotropic drug to cure several types of cancer has cytotoxic effects on the 4T1 cell line. However, in combination with HBV, its cytotoxic effect is promoted, and it can be more effective in nonlethal dosages. Furthermore, antimutagenic and anticancer activities of HBV were seen in the presence of the S9 metabolic activation system in all concentrations of HBV. It will be an excellent perspective to innovate approaches to prevent and treat some features of cancer.

Abbreviations

Cisplatin: *cis*-Diamino-dichloro-platinum (II)
CTR1: Copper transporter 1
DMSO: Dimethylsulfoxide
FBS: Fetal Bovine Serum
HBV: Honey Bee Venom
KCl: Potassium Chloride
MIC: Minimum Inhibitory Concentration
MTT: Dimethylthiazol Diphenyl Tetrazolium Bromide
NaCl: Sodium chloride
PBS: Phosphate Buffer Saline
S9: Supernatant fraction.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

In this research, Faranak Shiassi Arani performed the cell culture, MTT assay, and statistical analysis, and wrote the manuscript. Seyed Mohammad Ghafoori provided *Salmonella typhimurium* TA100 and performed the Ames test. Mohammad Nabiuni was responsible for designing the experiments and supervision of work. Latifeh Karimzadeh was responsible for providing 4T1 cell line and rats, performing cell culture, and also editing the manuscript.

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Research Article

Antipathogenic Potential of a Polyherbal Wound-Care Formulation (Herboheal) against Certain Wound-Infective Gram-Negative Bacteria

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This study investigated antipathogenic efficacy of a polyherbal wound-healing formulation Herboheal against three multidrug-resistant strains of gram-negative bacterial pathogens associated with wound infections. Herboheal was evaluated for its quorum-modulatory potential against three different human-pathogenic bacteria, first *in vitro* through the broth dilution assay and then *in vivo* in the model host *Caenorhabditis elegans*. Herboheal at ≥0.1% v/v was able to inhibit (19–55%) *in vitro* production of quorum sensing-regulated pigments in all these bacteria and seemed to interfere with bacterial quorum sensing by acting as a signal-response inhibitor. This formulation could compromise haemolytic activity of all three bacteria by ~18–69% and induced their catalase activity by ~8–21%. Herboheal inhibited *P. aeruginosa* biofilm formation up to 40%, reduced surface hydrophobicity of *P. aeruginosa* cells by ~9%, and also made them (25%) more susceptible to lysis by human serum. Antibiotic susceptibility of all three bacteria was modulated owing to pretreatment with Herboheal. Exposure of these test pathogens to Herboheal (≥0.025% v/v) effectively reduced their virulence towards the nematode *Caenorhabditis elegans*. Repeated subculturing of *P. aeruginosa* on the Herboheal-supplemented growth medium did not induce resistance to Herboheal in this mischievous pathogen, and this polyherbal extract was also found to exert a post-extract effect on *P. aeruginosa*, wherein virulence of the Herboheal-unexposed daughter cultures, of the Herboheal-exposed parent culture, was also found to be attenuated. Overall, this study indicates Herboheal formulation to be an effective antipathogenic preparation and validates its indicated traditional therapeutic use as a wound-care formulation.

1. Introduction

Wound refers to a form of physical injury where the skin is torn, cut, or burned, and it can serve as a site of entry for the pathogenic microbes. Although small, a wound always carries the probability of serving as an entry port for microorganisms, some of whom can complicate the situation further by their biofilm-forming ability. Biofilm formation in chronic wounds presents a difficult challenge in wound management [1]. For effective and rapid healing of a wound, preventing infection in it is essential [2]. Advanced wound care is a big market globally, and many of the leading pharmaceutical firms are actively engaged in this area [3].

Traditional medicines from different geographic locations prescribe a variety of wound-healing formulations, many of which are based on plant extracts and/or oils. A search for plants useful in wound care in the IMPPAT database returns 40 plants (https://cb.imsc.res.in/imppat/Therapeuticsplants/WOUNDS). Since preventing infection is an essential requirement for effective wound healing, we undertook this study to investigate the possible anti-infective potential of a polyherbal formulation indicated for wound healing, namely, "Herboheal," wherein we studied the effect of this formulation on three different gram-negative bacteria's growth and quorum sensing (QS), besides few other virulence traits. QS is a chemical signal-based process of intercellular communication among bacteria, which regulates expression of many genes including those associated with virulence. Recently, QS is being considered as an important novel target for development of new

antipathogenic agents, which in gram-negative bacteria usually employs AHLs (acyl homoserine lactones) as the chemical signal [4]. Pigment production in all three bacteria (*Chromobacterium violaceum*, *Serratia marcescens*, and *Pseudomonas aeruginosa*) used in this study is believed to be regulated by QS [5–8], and association of each of them with wound infections has also been reported. Fatal wound infection caused by *Chromobacterium violaceum* in Vietnam was reported in [9]. Traumatically injured tissues are prone to *P. aeruginosa* wound infection [10]. Involvement of *S. marcescens* in wound and soft tissue infections in patients receiving wound care has also been reported [11]. These pathogens have also been reported to possess a variety of immune evasion mechanisms. Virulence factors like exotoxin A, haemolysin, alkaline protease, elastase, phospholipase C, and biofilm formation enable *P. aeruginosa* to evade the hose immune response. Formation of the alginate layer by this bacterium limits the accessibility of host plasma factors [12]. *S. marcescens* has also been reported to suppress host innate immunity by inducing apoptosis of host immune cells via flagella- and lipopolysaccharide-dependent motility [13]. Ishii et al. showed suppression of host cellular immunity by this pathogen via production of an adhesion-inhibitory factor against immunosurveillance cells [14]. *C. violaceum* exoproteome has also been shown to contain a protein involved in cell adhesion, namely, EF-Tu, which in *P. aeruginosa* allows it to evade the immune system and invade the hose [15]. In face of the multiple immune evasion strategies adopted by these pathogens, investigation on novel formulations capable of curbing their virulence becomes even more important.

2. Materials and Methods

2.1. Test Formulation. Herboheal formulation (License no. GA/1616) was procured from the SRISTI organization, Ahmedabad. Brief notes on this formulation can be seen at http://www.sristiinnovation.com/human-products-best-for-your-skin.html#herboheal-herbal-wound-healing-ointment. Further details on this formulation including its composition have been provided in the supplementary file (Appendix A). For the purpose of our assays, we took this formulation without one of its bulking agents, i.e., bee wax, as the whole formulation (along with bee wax) was not soluble in the assay media. Bee wax was separately confirmed to have no effect on bacterial growth and pigment production. Before being used for experiments, the test formulation was filtered through a 0.45 μm PVDF membrane filter (Axiva, Haryana).

2.2. Test Organisms. *C. violaceum* (MTCC 2656) and *S. marcescens* (MTCC 97) were procured from MTCC (Microbial Type Culture Collection, Chandigarh). *P. aeruginosa* was taken from our own lab's culture collection, whose identity has been confirmed through biochemical tests, and earlier, we had subjected this culture to whole transcriptome sequencing (Bioproject no. PRJNA386078) too. Pseudomonas agar (HiMedia, Mumbai) was used for the maintenance of this culture. *C. violaceum* and *S. marcescens* were grown in the nutrient broth (HiMedia, Mumbai). Incubation temperature for *C. violaceum* and *P. aeruginosa* was 37°C, and for *S. marcescens*, it was 28°C. Incubation time for all three bacteria was kept as 22–24 h. The antibiotic susceptibility profile of the bacterial strains used in this study was generated using the antibiotic discs—Dodeca Universal-I, Dodeca G-III-Plus, and Icosa Universal-2 (HiMedia, Mumbai). *C. violaceum* and *S. marcescens* were found to be resistant to cefadroxil (30 μg), ampicillin (10 μg), cloxacillin (1 μg), and penicillin (10 μg). *S. marcescens* showed resistance against vancomycin (30 μg) too. The strain of *P. aeruginosa* was found to be resistant to amoxicillin (30 μg), cefadroxil (30 μg), ampicillin (10 μg), cloxacillin (1 μg), penicillin (10 μg), chloramphenicol (30 μg), cefixime (5 μg), clindamycin (2 μg), and nitrofurantoin (300 μg).

Two probiotic strains were also used in this study for assessing the prebiotic potential of the test formulation. Of them, *Lactobacillus plantarum* (MTCC 2621) was grown in the Lactobacillus MRS medium (HiMedia, Mumbai), and *Bifidobacterium bifidum* (NCDC 255), procured from the National Collection of Dairy Cultures, Karnal, was grown on MRS agar with 0.05% cysteine.

2.3. Broth Dilution Assay. Assessment of QS-regulated pigment production by test pathogens in presence or absence of the test formulation was done using the broth dilution assay [16]. Organisms were challenged with different concentrations (0.05–1% v/v) of Herboheal formulation (HF). Nutrient broth or pseudomonas broth (peptic digest of animal tissue 20 g/L, potassium sulphate 10 g/L, and magnesium chloride 1.4 g/L; pH 7.0 ± 0.2) was used as a growth medium. Inoculum standardized to 0.5 McFarland turbidity standard was added at 10% v/v, to the media supplemented with required concentration of HF, followed by incubation at appropriate temperature for each organism. Abiotic control (containing the extract and growth medium, but no inoculum) was also included in the experiment. Catechin (50 μg/mL; Sigma-Aldrich, USA) was used as a positive control.

2.4. Measurement of Bacterial Growth and Pigment Production. At the end of the incubation, bacterial growth was quantified at 764 nm [17]. This was followed by pigment extraction and quantification, as per the method described below for each of the pigment. Purity of each of the extracted pigment was confirmed by running a UV-Vis scan (Agilent Cary 60 UV-visible spectrophotometer). Appearance of a single major peak (at λ_{max} reported in the literature) was taken as indication of purity.

2.4.1. Violacein Extraction. One millilitre of the *C. violaceum* culture broth was centrifuged (Eppendorf 5417R) at 15,300 g for 10 min at room temperature, and the resulting supernatant was discarded [18]. The remaining cell pellet was resuspended into 1 mL of DMSO and vortexed, followed by centrifugation at 15,300 g for 10 min. The purple-coloured violacein was extracted from the supernatant;

OD was measured at 585 nm. Violacein unit was calculated as OD_{585}/OD_{764}. This parameter was calculated to nullify the effect of any change in cell density on pigment production.

2.4.2. Prodigiosin Extraction. One millilitre of the *S. marcescens* culture broth was centrifuged at 10,600 g for 10 min [19]. Centrifugation was carried out at 4°C, as prodigiosin is a temperature-sensitive compound. The resulting supernatant was discarded. The remaining cell pellet was resuspended into 1 mL of acidified methanol (4 mL of HCl into 96 mL of methanol; Merck), followed by incubation in dark at room temperature for 30 min. This was followed by centrifugation at 10,600 g for 10 min at 4°C. Prodigiosin was obtained from the resulting supernatant; OD was taken at 535 nm. Prodigiosin unit was calculated as OD_{535}/OD_{764}.

2.4.3. Pyoverdine and Pyocyanin Extraction. One millilitre of the culture broth was mixed with chloroform (Merck, Mumbai) in 2 : 1 proportion followed by centrifugation at 12,000 rpm (15,300 g; REMI CPR-24 Plus) for 10 min [20, 21]. This resulted in formation of two immiscible layers. OD of the upper water-soluble phase containing yellow-green fluorescent pigment pyoverdine was measured at 405 nm. Pyoverdine unit was calculated as OD_{405}/OD_{764}.

The lower chloroform layer containing pyocyanin was mixed with 0.1 N HCl (Merck; at the rate of 20% v/v), resulting in a colour change from blue to pink. Absorbance of this pyocyanin in the acidic form was measured at 520 nm. Pyocyanin unit was calculated as OD_{520}/OD_{764}.

2.5. AHL Augmentation Assay. This assay was done to investigate whether the test formulation exerts its QS-inhibitory effect by inhibiting production of the signal AHL, or by interfering with the signal-response mechanism. Extraction of the *N*-acyl-homoserine lactone (AHL) was performed as described in [22]. OD of the overnight grown bacterial culture was standardized to 1.00 at 764 nm. It was centrifuged at 5000 g for 5 min. Cell-free supernatant was filter-sterilized using a 0.45 μm filter (Axiva, Haryana) and was mixed with equal volume of acidified ethyl acetate (0.1% formic acid (Merck) in ethyl acetate (Merck)). The ethyl acetate layer was collected and evaporated at 55°C, followed by reconstitution of the dried crystals in 100 μL phosphate buffer saline (pH 6.8). Identity of thus-extracted AHL was confirmed by thin-layer chromatography (TLC) [23]. The R_f value of purified AHL from the *C. violaceum* culture while performing TLC (methanol (60) : water (40); TLC Silica gel 60 F_{254} plates; Merck) was found to be 0.70, near to that (0.68) reported for *N*-hexonylhomoserine lactone (C6-HSL) [24]. The R_f value of purified AHL from *S. marcescens*, using the same TLC conditions as mentioned above, was found to be 0.83, near to that (0.80) reported for C12-HSL [25]. TLC of AHL extracted from *P. aeruginosa* resulted in three spots corresponding to R_f values of 0.43, 0.68, and 0.92 near to those (0.41, 0.68, and 0.84) reported for C8-HSL, C6-HSL, and C4-HSL respectively [24].

The bacterial culture growing in presence of test formulation was supplemented with 2% v/v AHL after 6 hours of incubation, and at the end of a total 24-hour incubation, pigments were extracted from AHL-supplemented experimental tubes, as well as AHL-nonsupplemented control tubes. If the QS-regulated pigment production is found inhibited in both these tubes in comparison to bacteria growing in absence of the extract as well as AHL, then the effect of the test extract was interpreted as a signal-response inhibitor because if the test formulation would have acted as a signal-supply inhibitor, then exogenous supply of AHL should restore pigment formation by the bacteria.

2.6. Hemolysis Assay. OD_{764} of the overnight grown (in presence or absence of HF) culture was standardized to 1.00. Cell-free supernatant was prepared by centrifugation at 15,300 g for 10 min [26]. 10 μL of human blood (collected in a heparinized vial) was incubated with this cell-free supernatant for 2 h at 37°C, followed by centrifugation at 800 g for 15 min. OD of the supernatant was read at 540 nm, to quantify the amount of hemoglobin released. 1% Triton X-100 (CDH, New Delhi) was used as a positive control. Phosphate buffer saline was used as a negative control.

2.7. Assay of Bacterial Susceptibility to Lysis in Presence of Human Serum. Serum was separated by centrifuging blood at 1,500 rpm (800 g) for 10 min [27]. The bacterial culture grown in media with and without HF was centrifuged, and the cell pellet was reconstituted in PBS so that the resulting suspension attains OD_{764} = 1. 200 μl of this bacterial suspension from control or experimental tubes was mixed with 740 μl of PBS and 60 μl of serum. After incubation for 24 h at 37°C, absorbance was read at 764 nm. The culture not exposed to HF incubated with human serum served as a control, against which OD (post-serum exposure) of the HF-treated cells was compared. Tubes containing bacterial cells (exposed neither to HF nor to serum) suspended in PBS were also included in the experimental setup, to nullify any interference from autolysis.

2.8. Catalase Assay. OD_{764} of the culture was adjusted to 1.00. 400 μL of phosphate buffer was added into a 2 mL vial followed by 400 μL of H_2O_2. To this, 200 μL of the bacterial culture was added, and the mixture was incubated for 10 min. Then, 10 μM of sodium azide (20 μL) was added to stop the reaction [28], followed by centrifugation at 12,000 rpm for 10 min. OD of the supernatant was measured at 240 nm to quantify the remaining H_2O_2 [29], with phosphate buffer as the blank.

2.9. Assay for Biofilm Formation, Eradication, and Viability. In this assay, both control and experimental groups contained nine test tubes. In each group, three subgroups were made. The first subgroup of three test tubes in the experimental group contained Pseudomonas broth supplemented with HF, whereas the remaining six tubes contained

Pseudomonas broth with no HF on the first day of the experiment. All these tubes were inoculated with inoculum (10% v/v) standardized to 0.5 McFarland turbidity standard (making total volume in a 1 mL tube), followed by incubation at 37°C for 24 h under static condition, which resulted in formation of biofilm as a ring on walls of the glass tubes. This biofilm was quantified by the crystal violet assay [30], preceded by quantification of the bacterial cell density and pigment. Contents from the remaining six test tubes from rest of the two subgroups were discarded following cell density and pigment estimation, and then the biofilms remaining on the inner surface of these tubes were washed with phosphate buffer saline (PBS; pH 7) to remove loosely attached cells. Now, 2 mL of minimal media (sucrose 15 g/L, K_2HPO_4 5.0 g/L, NH_4Cl 2 g/L, NaCl 1 g/L, $MgSO_4$ 0.1 g/L, and yeast extract 0.1 g/L; pH 7.4 ± 0.2) containing HF was added into each of these tubes so as to cover the biofilm completely, and tubes were incubated for 24 h at 37°C. At the end of incubation, one subgroup of 3 tubes was subjected to the crystal violet assay to know whether any eradication of the preformed biofilm has occurred under the influence of HF, and the last subgroup of 3 tubes was subjected to viability assessment through the MTT assay. For the crystal violet assay, the biofilm-containing tubes (after discarding the inside liquid) were washed with PBS in order to remove all nonadherent (planktonic) bacteria and air-dried for 15 min. Then, each of the washed tubes was stained with 1.5 mL of 0.4% aqueous crystal violet solution for 30 min. Afterwards, each tube was washed twice with 2 mL of sterile distilled water and immediately destained with 1500 μL of 95% ethanol. After 45 min of destaining, 1 mL of destaining solution was transferred into separate tubes and read at 580 nm. For the MTT assay [31], the biofilm-containing tubes (after discarding the inside liquid) were washed with PBS in order to remove all nonadherent (planktonic) bacteria and air-dried for 15 min. Then, 900 μL of minimal media was added into each tube, followed by addition of 100 μL of 0.3% MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HiMedia). After 2 h incubation at 37°C, resulting purple formazan derivatives were dissolved in DMSO and measured at 560 nm.

2.10. Cell Surface Hydrophobicity (CSH) Assay. Bacterial surface hydrophobicity was measured using the bacterial adhesion to hydrocarbon (BATH) assay as described by Hui and Dykes [32]. The *P. aeruginosa* culture was collected at the stationary phase and pelleted by centrifugation (NF800R; NUVE, Belgium) at 7,000 rpm for 10 min. This pellet was washed twice with phosphate buffer saline (PBS; pH 7.4) and then resuspended in PBS with HF (0.5% v/v) to $OD_{764} = 1.00$. The same procedure was repeated with the *P. aeruginosa* culture without HF, as a control. Each bacterial suspension was then incubated for 1 h at room temperature. 2 mL sample of each suspension was collected, and absorbance (A) at 764 nm was measured, using PBS as the blank. 1 mL of xylene (HiMedia, Mumbai) was added to the 2 mL cell suspension, and this mixture was vortexed for 2 min. The phases were then allowed to separate for 1 h. The absorbance of the aqueous phase (A_0) was again determined. The results were expressed as follows:

$$\% \text{ attachment to xylene} = \left(1 - \left(\frac{A}{A_0}\right)\right) \times 100. \quad (1)$$

2.11. Determination of the Effect of HF on Antibiotic Susceptibility of the Test Organisms. After *in vitro* assessment of the QS-inhibitory property of the test formulation, the effect of this HF on antibiotic susceptibility of the test pathogen was investigated. The bacterial cells pretreated with HF were subsequently challenged with sub-MIC concentrations of different antibiotics. All the antibiotics were procured from HiMedia, Mumbai.

2.12. In Vivo Assay. *In vivo* efficacy of the HF was evaluated using the nematode worm *Caenorhabditis elegans* as the model host, using the method described by Eng and Nathan [33] with some modification. This worm was maintained on the nematode growing medium (NGM; 3 g/L NaCl, 2.5 g/L peptone, 1 M $CaCl_2$, 1 M $MgSO_4$, 5 mg/mL cholesterol, 1 M phosphate buffer with pH 6, and 17 g/L agar-agar) with *E. coli* OP50 (procured from LabTIE B.V., JR Rosmalen, Netherlands) as the feed. Worm population to be used for the *in vivo* assay was kept on NGM plates not seeded with *E. coli* OP50 for three days, before being challenged with the test pathogen.

Test bacterium was incubated with the HF for 22–24 h. Following incubation, OD_{764} of the culture suspension was equalized to that of the control (not exposed to HF). 100 μL of this bacterial suspension was mixed with 900 μL of the M9 buffer containing 10 worms (L3-L4 stage). This experiment was performed in 24-well (sterile, nontreated) polystyrene plates (TPG24; HiMedia), and incubation was carried out at 22°C. The number of live vs. lead worms was counted everyday till five days by placing the plate (with lid) under light microscope (4x). Standard antibiotics- and catechin-treated bacterial suspension were used as a positive control. Straight worms were considered to be dead. On the last day of the experiment, when plates could be opened, their death was also confirmed by touching them with a straight wire, wherein no movement was taken as confirmation of death.

2.13. Statistical Analysis. All the experiments were performed in triplicate, and measurements were reported as mean ± standard deviation (SD) of 3 independent experiments. Statistical significance of the data was evaluated by applying the t-test using Microsoft Excel®. P values ≤ 0.05 were considered to be statistically significant.

3. Results

3.1. C. violaceum. *C. violaceum* was challenged with 0.025–1% v/v concentration of the HF. All the test concentrations were able to exert the inhibitory effect on *C. violaceum* growth as well as production of QS-regulated

pigment violacein, with latter getting affected more than the former (Figure 1(a)). Interestingly, a 40-fold increase in HF concentration (from 0.025 to 1% v/v) could cause only 2.91-fold more inhibition of violacein and only a 1.75-fold higher inhibition of growth. Exogenous addition of the QS signal (AHL) to the quorum-inhibited culture of *C. violaceum* was not found to reverse the inhibitory effect of HF on violacein production (Figure 1(b)), which suggests HF to act as a *signal-response inhibitor* of the QS machinery of this bacterium.

Pretreatment of *C. violaceum* before being challenged with sub-MIC concentrations of four different antibiotics enhanced its susceptibility to all of them, particularly to chloramphenicol and cephalexin (Figure 1(c)). Pretreatment of bacteria with higher (0.25% v/v) HF concentration was not found to modulate the antibiotic susceptibility to any notably greater extent (except in case of streptomycin), than pretreatment with lower (0.025% v/v) concentration. At both these test concentrations, HF was found to compromise the haemolytic potential of *C. violaceum* to a statistically identical extent; however, catalase activity was found to be modulated only at 0.25% v/v (Figure 1(d)). The *in vivo* assay reveled the ability of HF to confer protection on *C. elegans* in face of the *C. violaceum* challenge, wherein the magnitude of survival benefit was observed to increase with the dose of HF (Figure 1(e)). Worm population in the well corresponding to 0.25% v/v HF treatment was also able to generate progeny.

3.2. S. marcescens. HF till 0.05% v/v exerted no effect on pigment production in *S. marcescens* and an inhibitory effect at higher concentrations, with no effect on growth at any of the concentrations tested (Figure 2(a)). HF's effect on prodigiosin production by *S. marcescens* can be said to follow the threshold model of the dose-response relationship [34]; however, a 5-fold increase in concentration (from 0.1 to 0.5% v/v) resulted in only 1.48-fold higher inhibition of pigment production. This inhibition was not found to reverse upon augmentation of the quorum-inhibited *S. marcescens* culture with AHL, indicating HF to act as a *signal-response inhibitor* against this bacterium too (Figure 2(b)).

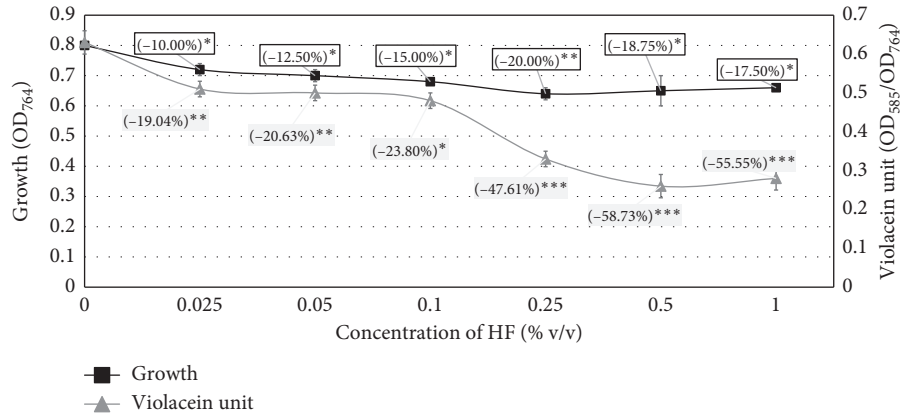
Pretreatment of *S. marcescens* with HF reduced its susceptibility to all four antibiotics tested. In fact, HF pretreatment made *S. marcescens* facing the antibiotic challenge grow either at par or even better than the control (not exposed to any antibiotic) *S. marcescens* culture (Figure 2(c)). Catalase activity of *S. marcescens* experienced a promotion, whereas haemolytic activity was heavily compromised under the influence of HF (Figure 2(d)). HF-treated *S. marcescens* could kill lesser number of *C. elegans*, as compared to HF-unexposed control *S. marcescens* (Figure 2(e)).

3.3. P. aeruginosa. HF at any of the concentrations employed did not exert any growth inhibitory effect on *P. aeruginosa*, while negatively affecting production of both the QS-regulated pigments negatively. The magnitude of the pigment inhibitory effect at majority of the HF concentrations was almost similar (Figure 3(a)), and this inhibition was not reversed following AHL augmentation, suggesting HF to act as a *signal-response inhibitor* (Figure 3(b)). Susceptibility of *P. aeruginosa* to gentamicin and cephalexin was raised owing to HF pretreatment, whereas that against ofloxacin and tetracycline remained unaffected (Figure 3(c)). Under the influence of HF, biofilm-forming ability of *P. aeruginosa* was suppressed up to ~40%; however, this formulation had no effect on preformed biofilm (Figure 3(d)). HF (0.5% v/v) could reduce CSH of *P. aeruginosa* by 9.32% ($p = 0.01$), wherein percent hydrophobicity of bacterial cells exposed to HF and control was found to be 21.22% and 30.55%, respectively.

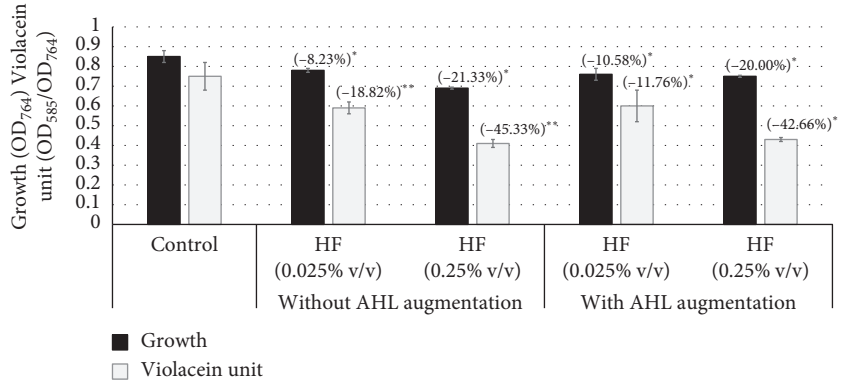
Exposure to HF induced catalase activity and restricted the haemolytic activity of *P. aeruginosa.* HF treatment could also make *P. aeruginosa* more prone to lysis in presence of human serum (Figure 4(a)). HF-exposed bacteria were able to kill lesser *C. elegans* worms as compared to the HF-unexposed *P. aeruginosa* (Figure 4(b)). The onset of death in worm population was also delayed in the wells corresponding to 0.025% v/v HF concentration, whereas generation of progeny worms was observed in the wells corresponding to 0.1% v/v HF. Paradoxically, pretreatment of bacteria with lower HF concentration attenuated their virulence towards *C. elegans* more than the higher concentration.

After confirming efficacy of HF against *P. aeruginosa in vitro* as well as *in vivo*, we subcultured the HF-treated *P. aeruginosa* in HF-free media to know whether there is any post-exposure residual effect of this formulation on next generations of bacteria. The *P. aeruginosa* culture obtained after one such subculturing on HF-free media was still altered with respect to pyocyanin and pyoverdine production, and the magnitude of this alteration was almost identical to that of *P. aeruginosa* receiving first exposure to HF. This pigment-modulatory effect was not observed in the *P. aeruginosa* culture obtained after second subculturing on HF-free media (Figure 5(a)). Such a long-lasting effect of any antimicrobial observed following a transient exposure of the parent culture to some extracts can be referred as the post-extract effect (PEE) [35–37]. Although this PEE seemed to disappear following second subculturing *in vitro*, the *in vivo* assay revealed that it did not disappear completely. *P. aeruginosa* obtained after second subculturing on HF-free media was still able to kill lesser worms that the HF-unexposed bacterial culture (Figure 5(b)).

In general, repeated exposure of a particular bacterial population to any given antimicrobial agent is expected to exert a strong selection pressure on the bacteria to develop resistant phenotypes. Whether this happens with *P. aeruginosa*, in face of continuous exposure to HF, was also investigated by us (Figure 5(c)). *P. aeruginosa* subcultured ten times on HF-containing media was still not able to kill as many worms as the HF-unexposed bacteria (Figure 4(b)), leading us to conclude that even after repeated exposure to this polyherbal formulation, *P. aeruginosa* could not become resistant to it.



(a)



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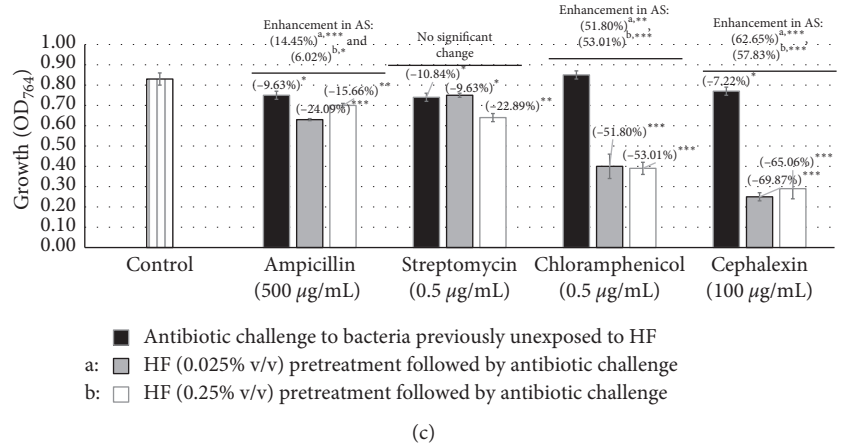

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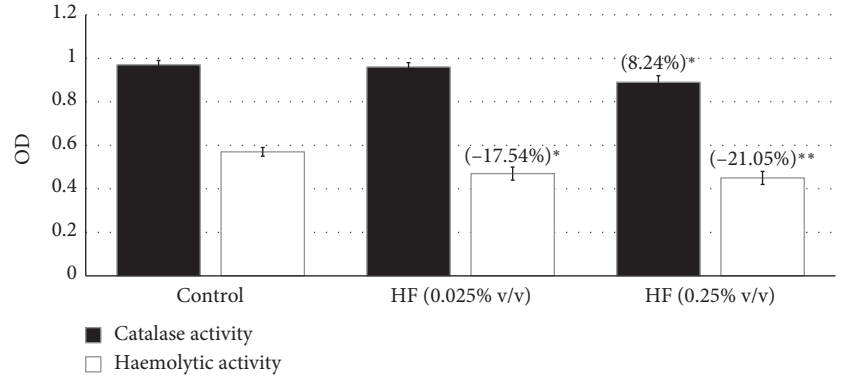

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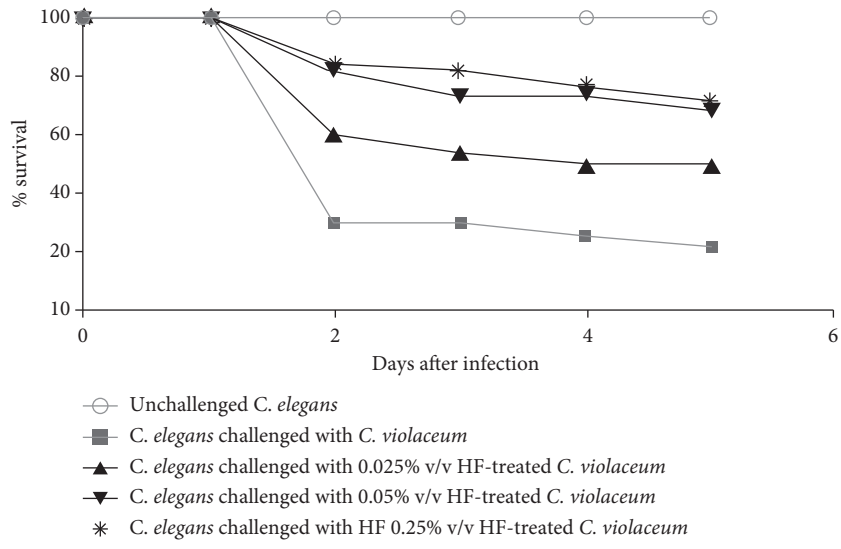
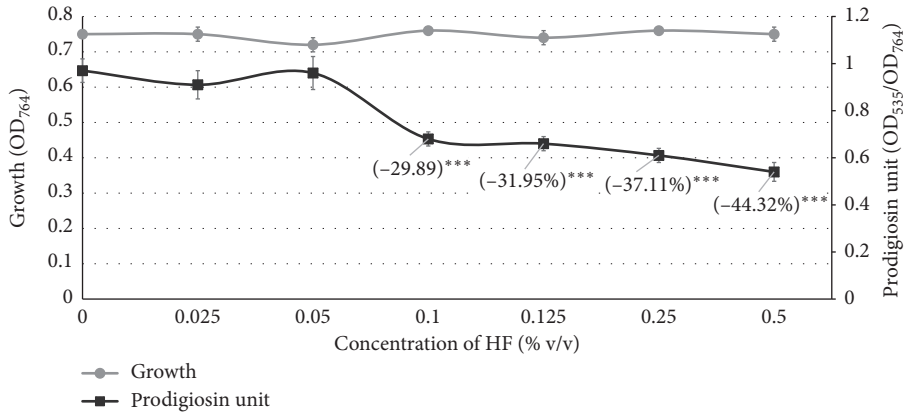


(e)

Figure 1: Effect of HF on *C. violaceum*. (a) Effect of HF on growth and QS-regulated violacein production in *C. violaceum*: bacterial growth was measured as OD_{764}, OD of violacein was measured at 585 nm, and violacein unit was calculated as the ratio OD_{585}/OD_{764} (an indication of violacein production per unit of growth). Catechin (50 μg/mL) did not exert any effect on growth of *C. violaceum* and inhibited violacein production by 47.69%*** ± 0.03. (b) HF acts as a *signal-response inhibitor* against *C. violaceum*. (c) HF pretreatment enhances susceptibility of *C. violaceum* to different antibiotics. (d) HF enhances catalase activity and inhibits haemolytic activity of *C. violaceum*: the catalase assay was done by monitoring disappearance of H_2O_2 at 240 nm. Chloramphenicol (0.5 μg/mL) enhanced catalase activity of this bacterium by 11.23%*± 0.01. Hemoglobin concentration was measured at OD_{540}. (e) HF treatment attenuates virulence of *C. violaceum* towards *C. elegans:* catechin (50 μg/mL) and ampicillin (500 μg/mL) employed as positive controls conferred 100% protection, and HF at 0.025% v/v, 0.05% v/v, and 0.25% v/v conferred 28%**, 46%***, and 50%*** survival benefit, respectively. Survival benefit refers to the difference between the number of worms surviving in experimental and control wells. HF at tested concentrations showed no toxicity towards the worm. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; AS: antibiotic susceptibility; QS: quorum sensing; HF: Herboheal formulation.

4. Discussion

Herboheal formulation was able to exert a quorum-inhibitory effect on all three gram-negative bacteria employed in this study, and it was found to exert its inhibitory effect by interfering with the signal-response part of the QS phenomenon, in all three cases. Since QS machinery of different gram-negative bacteria is believed to have a significant overlap [38], it can be expected from a quorum inhibitor effective against one gram-negative bacteria to be also effective against many other gram-negative bacteria. HF was indicated to act as a signal-response inhibitor against all three gram-negative bacteria used in this study. Since the use of AHLs as QS signals and role of LuxR analogues in mounting AHL-mediated response are common among gram-negative bacteria [38], HF can be expected to act as a broad-spectrum QS inhibitor against multiple gram-negative bacterial pathogens. HF could inhibit *in vitro* production of QS-regulated pigments in *C. violaceum* and *P. aeruginosa* at a concentration as low as 0.025% v/v and at 0.1% v/v in *S. marcescens*. It did not inhibit growth of *S. marcescens* and *P. aeruginosa* at any of the concentrations tested. In fact, it is expected from an ideal antivirulence agent not to have any notable inhibitory effect on growth of susceptible bacteria, which may make them exert lesser selection pressure on the target bacterial populations. Bactericidal antimicrobial agents are believed to apply a heavy selection pressure on the target bacteria by keeping them in a "mutate or die" situation. The newer concept of antipathogenic compounds focuses largely on disarming the pathogens without exerting a sharp killing effect on them. In contrast to the conventional microbicidal antibiotics, antivirulence agents curb the pathogenicity/virulence without necessarily displaying any notable growth-inhibitory effect. Such "pathoblockers" are likely to emerge as a new category of "tailored spectrum" superior therapeutics [39–41]. Based on this logic and early enthusiasm surrounding QS inhibitors, latter were viewed as "evolution-proof drugs" [42]. Although this enthusiasm may not be supported fully by experimental evidence, as reports on resistance to QS inhibitors [43–45] have also appeared in the literature, development of resistance against QS modulators can still be expected to emerge at a relatively slower pace. QS-targeting chemotherapeutic agents are believed to be less likely to generate resistance among pathogenic microbial populations as they target the adaptation and not the survival mechanisms of the pathogen [46, 47]. Treatment of bacteria used in this study by HF at 0.025–0.1% v/v was able to attenuate bacterial virulence enough so that at least 50% of the worms could not be killed in face of the bacterial challenge, as against 77.5–85% killing of worms by HF-unexposed bacteria. Besides challenging the nematode worm *C. elegans* with HF-treated bacteria, we also tested HF as a post-infection



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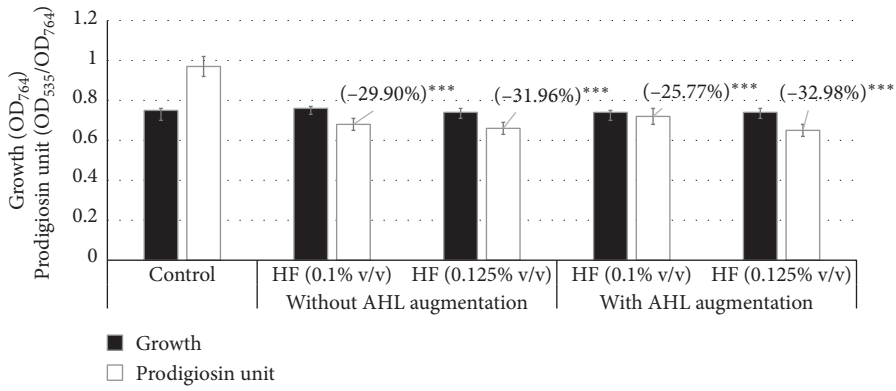

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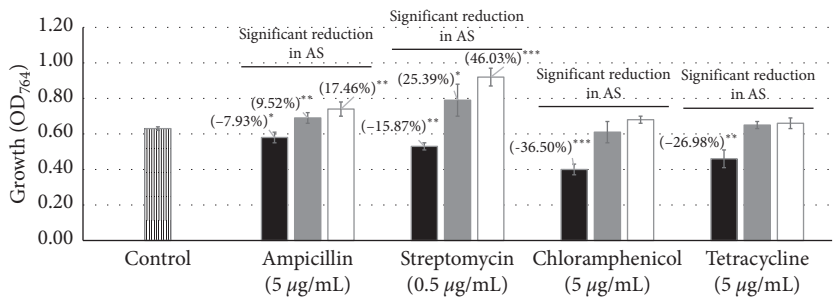

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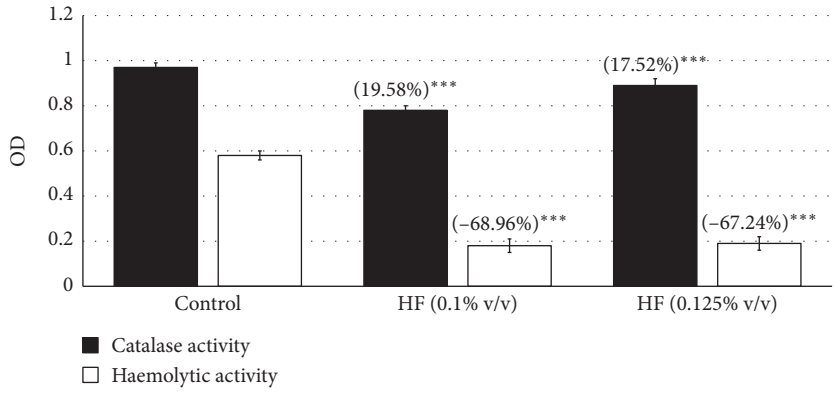

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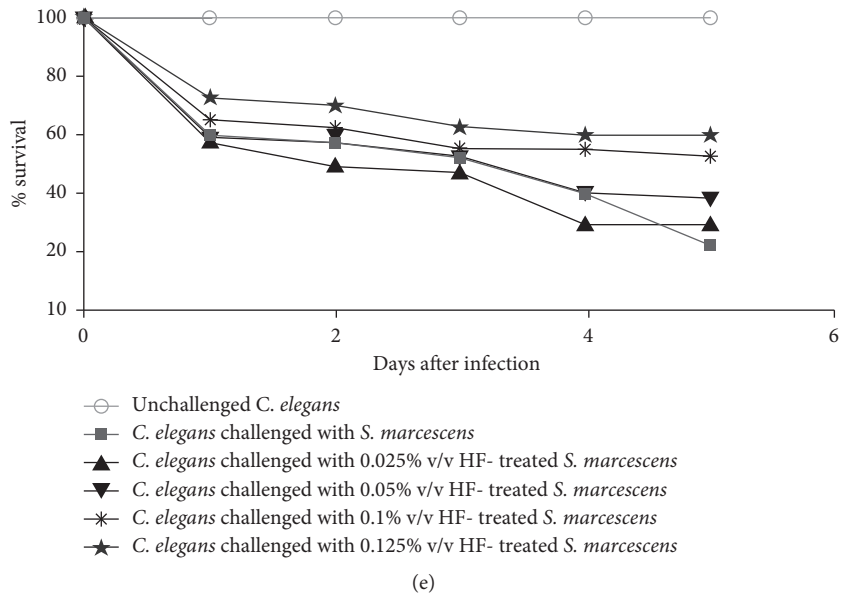


(e)

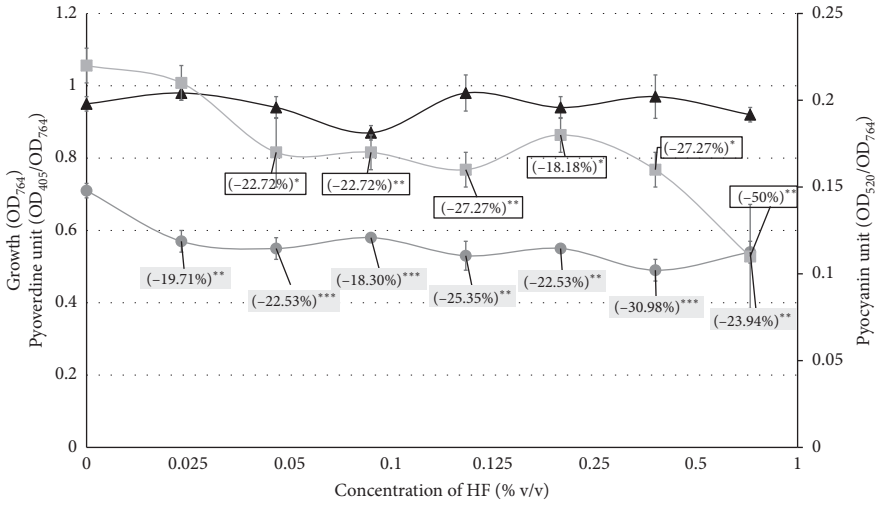
Figure 2: Effect of HF on *S. marcescens*. (a) Effect of HF on growth and QS-regulated prodigiosin production in *S. marcescens*: bacterial growth was measured as OD_{764}, OD of prodigiosin was measured at 535 nm, and prodigiosin unit was calculated as the ratio OD_{535}/OD_{764} (an indication of prodigiosin production per unit of growth). Catechin (50 μg/mL) inhibited prodigiosin production by 10%* ± 0.05 without affecting bacterial growth. (b) HF acts as a *signal-supply inhibitor* against *S. marcescens*. (c) HF pretreatment reduces susceptibility of *S. marcescens* to different antibiotics. (d) HF enhances catalase activity and inhibits haemolytic activity of *S marcescens*: the catalase assay was done by monitoring disappearance of H_2O_2 at 240 nm. Chloramphenicol (5 μg/mL) enhanced catalase activity of this bacterium by 13.40%* ± 0.02. Hemoglobin concentration was measured at OD_{540}. (e) HF treatment reduces virulence of *S. marcescens* towards *C. elegans*: catechin (50 μg/mL) and ofloxacin (0.1 μg/mL) employed as positive controls conferred 100% and 80% protection, respectively, on worm population. Pretreatment of bacteria with HF at 0.025%, 0.05%, 0.1%, and 0.125% conferred 7.5%***, 15%***, 30%***, and 37.5%*** survival benefit, respectively. Survival benefit refers to the difference between the number of worms surviving in experimental and control wells. HF at tested concentrations showed no toxicity towards *C. elegans*. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; AS: antibiotic susceptibility; QS: quorum sensing; HF: Herboheal formulation.

therapeutic by offering it to the already-infected *C. elegans*, wherein we allowed *P. aeruginosa* to establish infection for 6 hours or 24 hours before HF was applied (Table S1). In this modified *in vivo* assay too, HF was found to exert its antipathogenic effect notably.

While investigating antibiotic susceptibility of HF-exposed bacteria, in 10 out of 11 such experiments (not considering experiments at two different concentrations of HF as separate), we found antibiotic susceptibility was modulated following HF treatment. *C. violaceum* and *P. aeruginosa* were found to become more susceptible to cephalexin owing to HF pretreatment. Formulations like HF which can extend utility of the cephalexin type of broad-spectrum antibiotics assume importance in face of the fact that cephalexin is reserved as a third-line treatment for certain conditions, e.g., urinary tract infection in pregnant women (https://bpac.org.nz/BPJ/2011/december/cephalosporins.aspx). Since in these experiments only a fraction of the bacterial cells from HF-containing tubes was transferred to the tubes containing antibiotic, the observed modulation of susceptibility can better be explained in the context of PEE. This is based on the understanding that when OD was taken as an end-point measurement in antibiotic susceptibility assays, majority of the cells present in those tubes were daughter generations of the HF-exposed original inoculum but themselves were never directly exposed to HF.

Susceptibility of *S. marcescens* to all four test antibiotics was decreased owing to HF treatment. This suggests that if a patient is simultaneously taking allopathic and traditional medicinal agents, then wrong combinations of antibiotics and plant extracts can be harmful. In our study, we did not investigate the possibility of synergistic action of HF and antibiotics, as the test bacteria were not challenged with the HF-antibiotic combinations. However, it is not uncommon in real-world situations for patients to take conventional antibiotics as prescribed by an allopathic doctor, with simultaneous self-motivated intake of some herbal products (not usually informed to the doctor). Here, there is a probability of the pharmacological effects of the allopathic drug and the herbal product being antagonistic, which may result in either delayed patient recovery or even undesired side effects (https://www.pharmatutor.org/articles/herbs-interaction-allopathic-drugs-review).

HF was found to enhance catalase activity by 8–20% in all the gram-negative bacteria. It may be speculated that HF exposure might induce oxidative stress in these bacteria, and to counteract this elevated oxidative stress, the bacteria are forced to overwork their oxidative stress response machinery, of which catalase is an important component. Oxidative stress stems from the reactive oxygen species (ROS), and these ROS have been indicated as modulators of bacterial virulence [48]. Induction of redox-associated physiological alterations has



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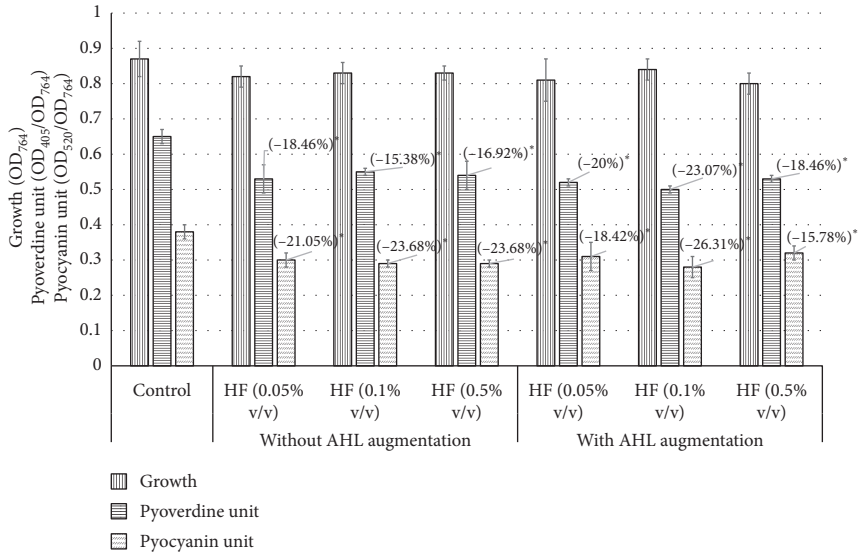

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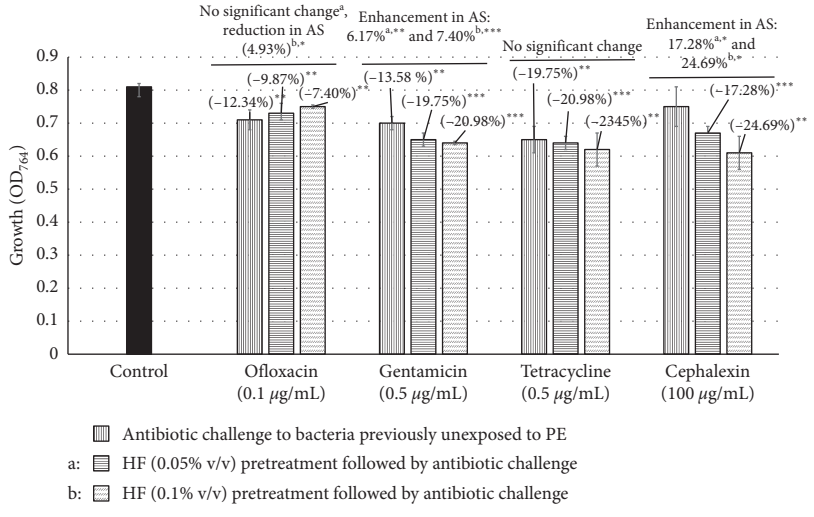

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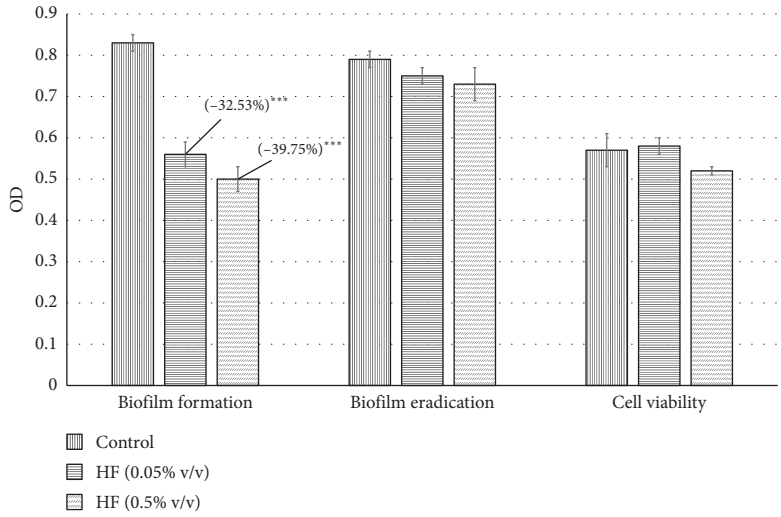

(d)

Figure 3: Effect of HF on *P. aeruginosa* growth, pigment production, antibiotic susceptibility, and biofilm. (a) Effect of HF on growth and QS-regulated pigment production in *P. aeruginosa*: bacterial growth was measured as OD_{764}, OD of pyoverdine was measured at 405 nm, and OD of pyocyanin was measured at 520 nm. Pyoverdine unit was calculated as the ratio OD_{405}/OD_{764} (an indication of pyoverdine production per unit of growth). Pyocyanin unit was calculated as the ratio OD_{520}/OD_{764} (an indication of pyocyanin production per unit of growth). Catechin (50 μg/mL) inhibited pyoverdine 17.13%** ± 0.06 and pyocyanin 23.65%* ± 0.04 production without affecting the bacterial growth. (b) HF acts as a *signal-response inhibitor* against *P. aeruginosa.* (c) HF treatment made *P. aeruginosa* more susceptible to gentamicin and cephalexin. (d) HF reduced *P. aeruginosa* biofilm formation but did not eradicate preformed biofilm nor had any effect on biofilm viability: the crystal violet assay was performed to measure biofilm formation and biofilm eradication, followed by the measurement of OD at 580 nm. Cell viability in biofilm was estimated through the MTT assay, wherein OD was measured at 560 nm. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; AS: antibiotic susceptibility; QS: quorum sensing; HF: Herboheal formulation.

been reported to be a part of the overall antibiotic effect exerted by conventional antibiotics [49]. Among the antibiotics employed by us as controls in this study, sub-MIC concentrations of tetracycline also induced catalase activity of *P. aeruginosa* and *S. marcescens* by 23.68% and 41.66%, respectively (data not shown). Generation of ROS has been suggested as a unifying mechanism of diverse antibiotics, and increasing importance is being attached to redox mechanisms in the context of antibiotic activation and resistance [50]. Infectious pathogens such as *Escherichia coli*, *Staphylococcus aureus*, and *Mycobacterium tuberculosis* are considered to be sensitive to changes in the intracellular oxidative environment. Thus, formulations that disturb the cellular oxidative environment can serve as novel therapeutics [51].

Haemolytic activity of all three bacteria was compromised by ~18–68% upon HF exposure. Hemolysis is considered an important virulence trait of many pathogens, as it allows the pathogenic bacteria access to the otherwise bound iron [52]. Iron requirement of pathogens inside the host far exceeds the amount of free iron present in human serum, and hence, therapeutic agents capable of compromising the haemolytic potential of bacteria can make their survival and replication inside the host difficult. HF-exposed *P. aeruginosa* was observed to produce lesser pyoverdine, which is an iron scavenger. This observation is important in light of the fact that *P. aeruginosa* wound infection is reported to involve activation of its iron acquisition system in response to fascial contact, and appropriate iron availability is also necessary for the bacteria to maintain a high population density in the wound [10].

HF at 0.5% v/v was found to increase the susceptibility of *P. aeruginosa* to human serum by 25%. This property of antimicrobial formulations like HF can aid the host immune system in rapid clearance of bacteria from the body, as lysis by serum will leave lesser bacteria to be dealt with by the body's defense mechanisms. Influences of the serum on expression of *P. aeruginosa* QS- and virulence-associated genes and QS-controlled virulence genes were investigated in [53]. They showed that, at early stages of growth, serum can repress the expression of many *P. aeruginosa* genes, and it can enhance them in the late phase. HF's ability to enhance *P. aeruginosa* susceptibility to human serum becomes even more important in light of the fact that serum-resistant phenotypes of *P. aeruginosa* are more frequently isolated from wounds and blood than infections at other sites [54]. Wound infections are believed to occur when microbial burden exceeds the innate clearance capacity of the host immune system [10]. Haemolysin and serum-resistance profile of clinical isolates are considered important aspects to be investigated [55].

HF at 0.5% v/v inhibited biofilm formation of *P. aeruginosa* by nearly 40%. This corroborates well with 9.32% reduction in CSH of this bacterium upon HF exposure. Surface hydrophobicity is an important determinant of bacteria's ability to form biofilm [32], and hydrophobic cells are believed to form stronger biofilms on medical implants constructed from hydrophobic materials [56]. Tribedi and Sil [57] indicated a direct correlation between CSH of *Pseudomonas* sp. and its ability to degrade nonpolar

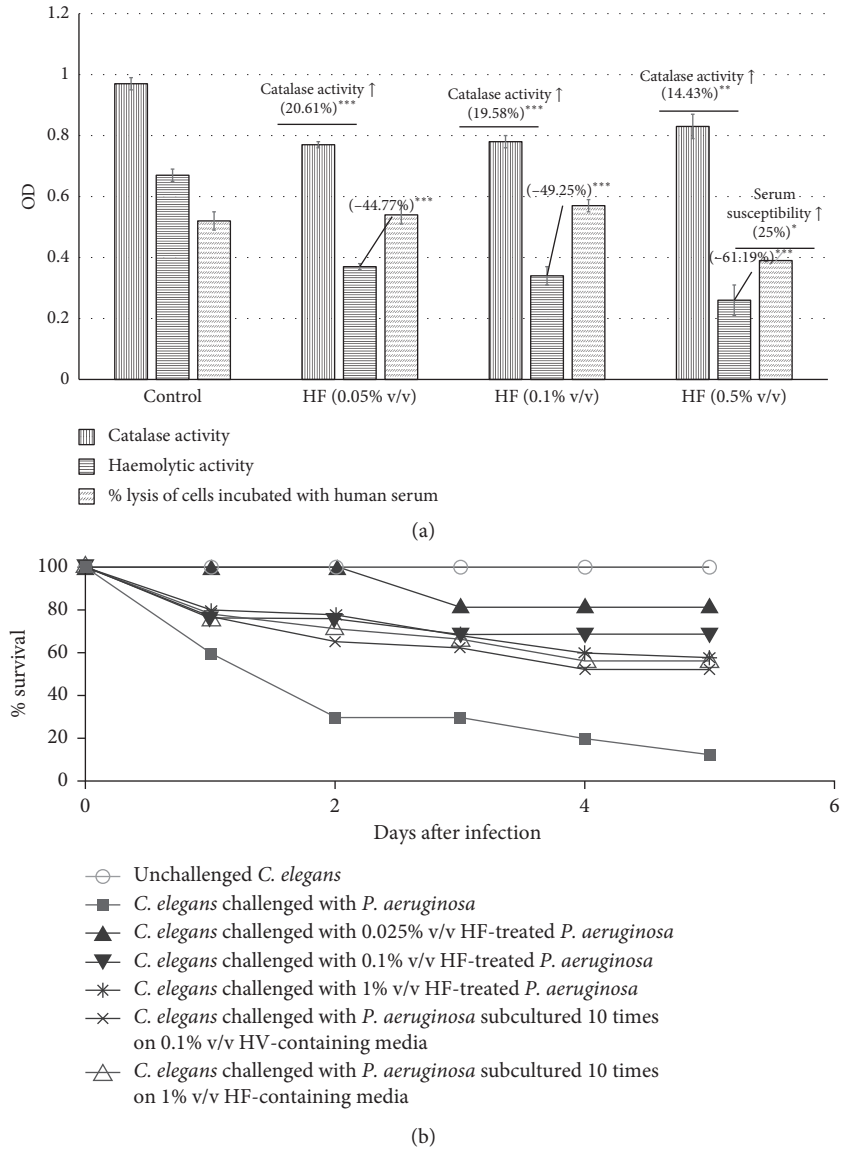

Figure 4: (a) HF enhances catalase activity, inhibits haemolytic potential, and increases susceptibility of *P. aeruginosa* to lysis by human serum: the catalase assay was done by monitoring disappearance of H_2O_2 at 240 nm. Tetracycline (0.5 μg/mL) inhibited catalase activity of this bacterium by 21.51%* ± 0.02. Hemoglobin concentration was measured at OD_{540}. "Control" in the serum-dependent lysis assay was HF-unexposed cells of *P. aeruginosa* incubated with human serum. (b) HF treatment reduces the virulence of *P. aeruginosa* towards *C. elegans*: catechin (50 μg/mL) and gentamicin (0.1 μg/mL) employed as positive controls conferred 100% and 80% protection, respectively. Pretreatment of bacteria with HF at 0.025% v/v, 0.1% v/v, and 1% v/v conferred 70%***, 55%***, and 45%*** survival benefit, respectively. Survival benefit refers to the difference between the number of worms surviving in experimental and control wells. HF at tested concentrations showed no toxicity towards *C. elegans*. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; HF: Herboheal formulation.

polymers. Gram-negative bacteria increase their CSH by releasing membrane vesicles, when subjected to stressful conditions [58]. CSH modification is considered to be a part of bacterial adaptive modifications in face of environmental changes. Reduced hydrophobicity may interfere with clumping of the bacterial cells by promoting the intercellular repulsion [59] and thus reduce biofilm formation.

We have demonstrated Herboheal to be effective against three different gram-negative bacteria. In general, it is difficult to find "hits" against gram-negative bacteria owing to presence of the outer membrane in their cell surface, which poses an additional entry barrier for majority of antimicrobials [60]. Gram-negative infections are believed to predominate in burn surgery, and *P. aeruginosa* is among one of the most common such burn wound pathogens [61]. In recent years, use of probiotic strains has also been indicated as a promising strategy (referred to as bacteriotherapy, or replacement therapy, or bacterial interference) for management of chronic wounds, wherein probiotic bacteria like lactobacilli are directly applied onto infected wound [62]; we investigated the effect of HF on two probiotic strains, namely, *L. plantarum* and *B. bifidum*. HF (at 0.025–1% v/v) could enhance growth of these bacteria by 6–27% (Figure 6). *L. plantarum* has earlier been reported for

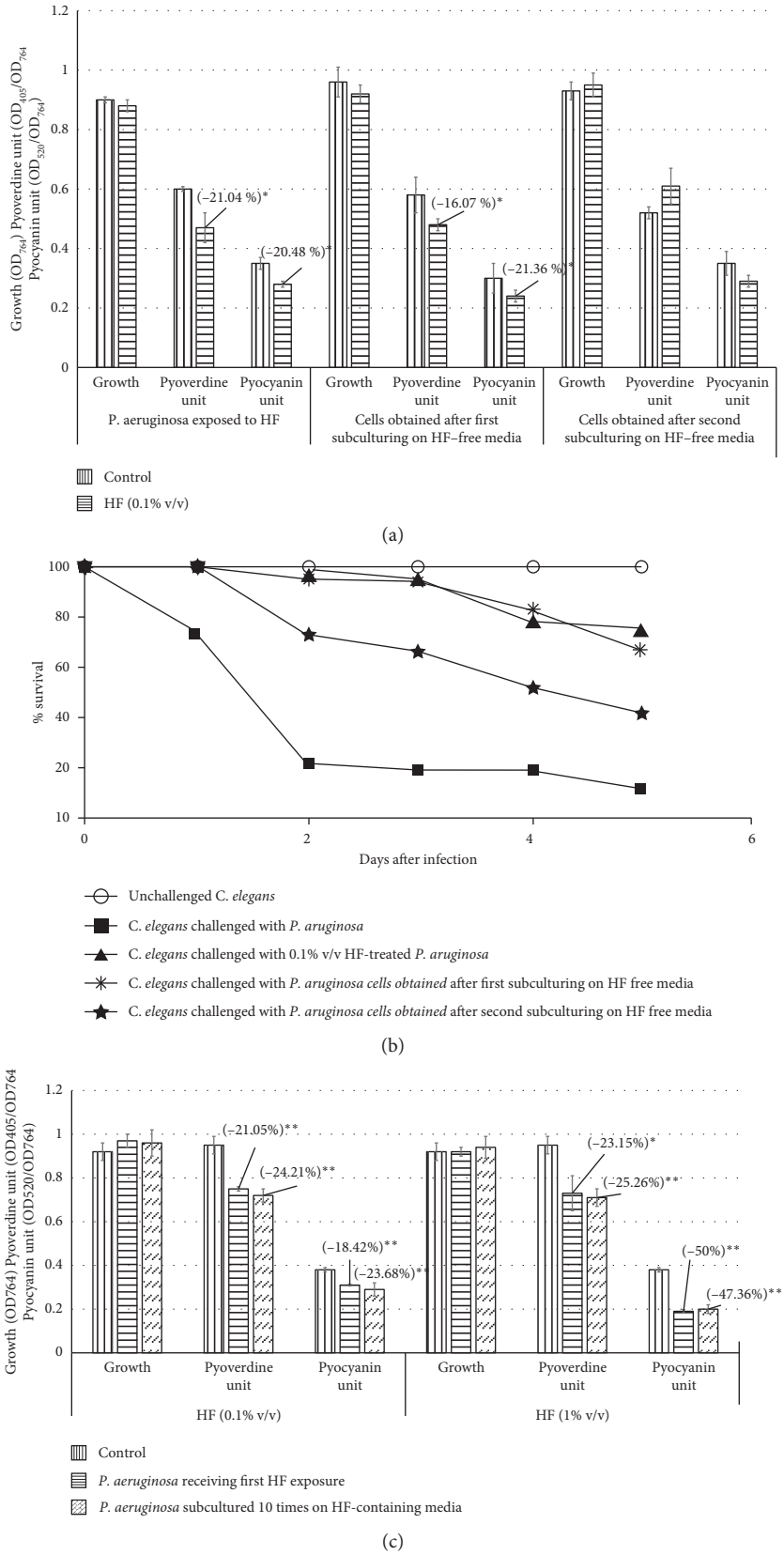

Figure 5: Demonstration of the PEE of HF on *P. aeruginosa in vitro* (a) and *in vivo* (b). (c) Effect of HF on *P. aeruginosa* growth, and pigment production remained unaltered even after repeated exposure to HF: *in vivo* data corresponding to (c) are part of Figure 4(b). $^{*}p < 0.05$; $^{**}p < 0.01$; HF: Herboheal formulation; PEE: post-extract effect.

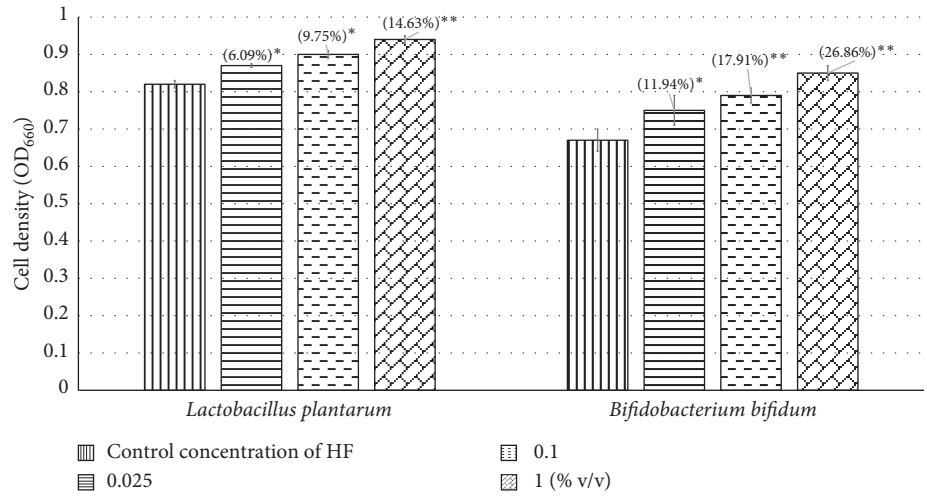

Figure 6: Growth-promoting effect of HF on probiotic strains. $^{*}p < 0.05$; $^{**}p < 0.01$. Bacterial growth was measured as OD_{660}.

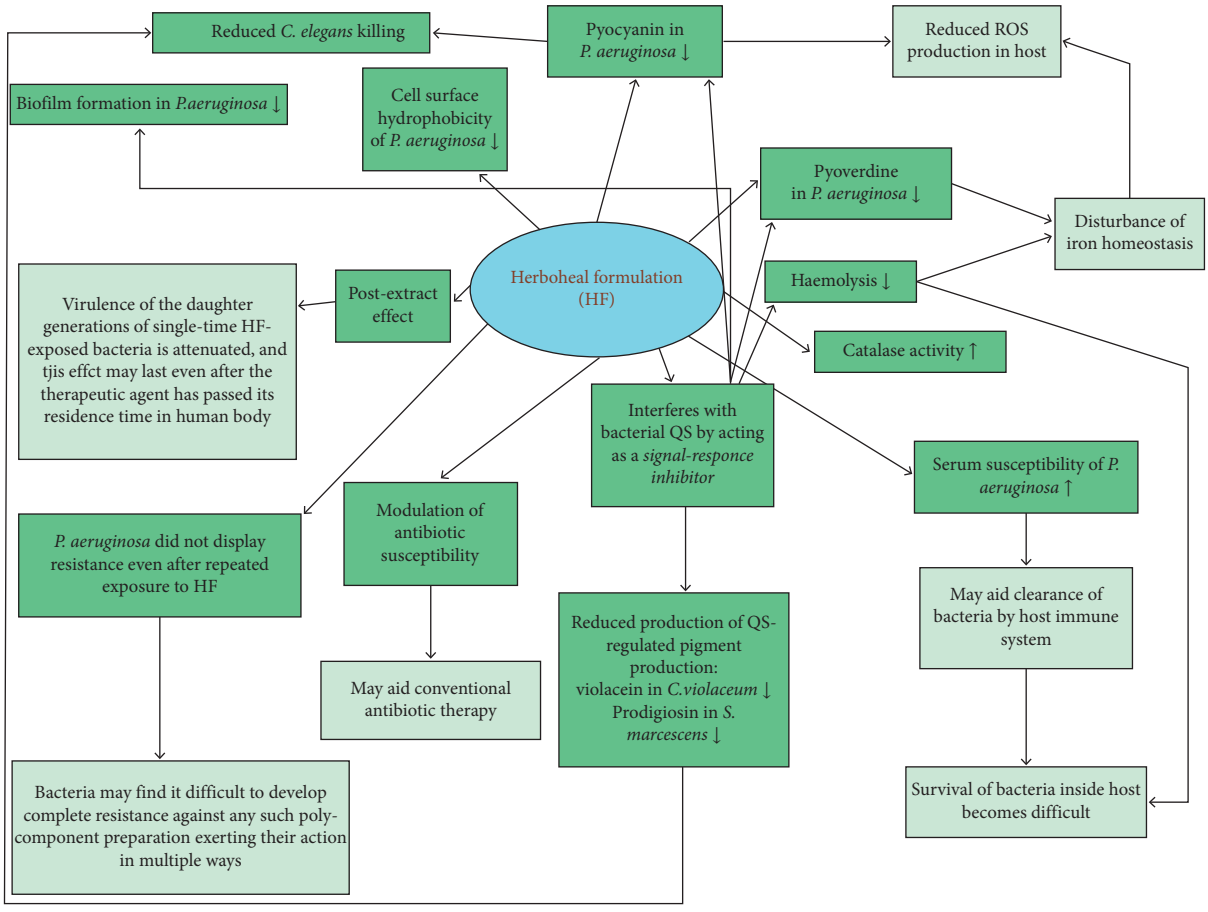

Figure 7: A summarized picture of the antipathogenic effect of Herboheal formulation against three different gram-negative bacterial pathogens.

its antipathogenic properties against *P. aeruginosa* by Ramos et al. [63], pointing to the potential use of its supernatants for treating infected chronic wounds. Similarly, Gan et al. [64] reported successful use of *L. fermentum* RC-14 and its secreted products in inhibiting surgical implant infections involving *S. aureus*. With lactobacilli being present in abundance in the vaginal tract, applying wound-care formulations with prebiotic (i.e., promoting growth of probiotic strains) properties on vaginal rashes/cuts and perineal wounds may help achieve faster healing. Such formulations may be effective in dealing with vaginal dysbiosis associated with bacterial vaginitis, at which current treatment options have limited success [65].

5. Conclusions

A summarized picture of the antipathogenic effect of the polyherbal Herboheal formulation investigated in this study against three different gram-negative bacterial pathogens is presented in Figure 7. The test formulation used in this study is a polyherbal formulation, and traditional medicine whether Indian, Arabian, or Chinese has always heavily relied on polyherbal and herbomineral formulations, which do not solely rely on activity of any single constituent. Their inherent polycomponent nature makes them less prone to development of resistance by the susceptible microorganisms, as for any organism developing simultaneous resistance against multiple components will always be more difficult than that against a single-molecule-based agent. We recently have reported antipathogenic potential of another polyherbal formulation described as *Panchvalkal* in *Ayurved* [16]. This concept of polyherbalism/multicomponent formulations is gaining wider acceptance today than ever before [66], and accordingly, there is an increasing interest among scientific community to validate traditional therapeutic practices. Generation of scientific evidence to verify the claims made regarding traditional medicine will make it more acceptable in the modern world. The current study is an example of such validation exercises, wherein we have shown the efficacy of Herboheal against three such bacteria which are known to be involved in wound infections. Further investigation regarding how the transcriptional and translational profiles of these bacteria change under the influence of Herboheal can explain the molecular mechanisms of its antipathogenic efficacy.

Abbreviations

QS: Quorum sensing
HF: Herboheal formulation
PEE: Post-extract effect
CSH: Cell surface hydrophobicity
AS: Antibiotic susceptibility.

Data Availability

All data pertaining to this study have been included within the manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Supplementary Materials

Appendix A: information on development and composition of the test formulation "Herboheal." Table S1: a comparison of efficacy of HF (0.1% v/v) when it is used against *P. aeruginosa* before infecting *C. elegans* versus when it is used on *C. elegans* already infected by *P. aeruginosa* having no previous exposure to the HF. (*Supplementary Materials*)

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Review Article

Nutraceuticals Inspiring the Current Therapy for Lifestyle Diseases

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Nutraceuticals are the pharmaceutically blended products that possess both nutritional as well as the medicinal value. Such a product is designed to improve the physical health, fight against day-to-day challenges such as stress, increase longevity, etc. Nowadays, emphasis is given to those herbs which are used as food and medicine due to its greater acceptance. Due to dynamic action, the popularity of nutraceuticals among people as well as healthcare providers has been increased over medicines and health supplements. This review documents herbs with a wide variety of therapeutic values such as immunity booster, antidiabetic, anticancer, antimicrobial, and gastroprotective. These herbs could be better options to formulate as nutraceuticals. Several nutraceuticals are described based on their availability as food, chemical nature, and mechanism of action.

1. Introduction

Hippocrates (460–377 BC), the father of modern medicine, almost 2500 years back established the relation of food and its importance for the treatment of various ailments in a very classical way optimizing various benefits [1]. Nutraceutical is composed of two words: nutrient and pharmaceutical. It is a food supplement that has a vital role in maintaining the healthy body and provides necessary supplements required for various metabolic processes to regulate body functions and thus prevents the body from diseases [1]. There is a vast cornucopia of herbs and foods which stimulate support and nourish our body system. Some have been used by different traditional systems of several countries and are now being evaluated by modern research. Use of pharmaceutical antibiotic would build up tolerances which make it ineffective in the long run. It is a better way to choose such herbs in our daily life, which would be not only capable of normalizing our body functions (even in disease condition) but also preventive and nutritive, and they also boost our immune system. An herb may not act as precisely as an antibiotic but can act as antibacterial (even antiviral) by boosting our body's own defense mechanism. To feel as a healthy well-being, one of the prominent approaches is to stay away from stress and other lifestyle diseases. The following are some examples of herbs used as food as well as medicine during infection, to boost the immune system or even in several other illnesses.

Astragalus membranaceus (Fabaceae) is a traditional Chinese herb. It is an extremely versatile and powerful immune enhancer antioxidant and also has hepatoprotective activity [2]. It also showed antidiabetic [3] and anticancer activity [4].

Triphala is one of the most revered tonics in Ayurveda. It is a combination of three important herbs, namely, *Terminalia bellerica* (Combretaceae), *Terminalia chebula* (Combretaceae), and *Emblica officinalis* (Phyllanthaceae). All these herbs act as a nutritive tonic. Triphala benefits almost all organs/systems of our body, particularly skin, liver, eyes, and digestive and respiratory system. The most well-known therapeutic uses are immunomodulating, antibacterial, antimutagenic, and adaptogenic, etc., which are well established [5, 6].

The northeast region of India is very rich in flora and fauna. The tribal people of the northeast region follow the

principle of Hippocrates. They use their food as medicine. *Paederia foetida* (Rubiaceae) is one of the tribal plants. A research study established its gastroprotective activity and antioxidant activity [7].

The yellow powder (turmeric) from South Asia, a curry ingredient, is well known for its preventive action. It is very active against various types of bacteria, fungus, virus, and also parasite. It is a potent inhibitor of HIV [8, 9]. Asian ginseng, probably the most westernized herb, is used as a tonic. It has been popular to promote immunity [10]. The most well-known ginseng is *Panax* ginseng, and it has protective effects in neurological disorders [11].

According to Ayurveda, garlic, onion, and ginger are the basis of all healing food recipes. Garlic is one of the most widely used natural health products. These are considered as food, spice, and medicine [12].

It has been the subject of intensive study for its possible effects against heart disease and cancer [13–15]. It increases the general immune system activity. Studies have also shown to be effective in treating AIDS and antimicrobial [16–18].

2. Classification of Nutraceuticals

2.1. Nutraceuticals Based on Food Availability

2.1.1. Traditional Nutraceuticals. These classes are generally sourced directly from nature, without any changes in the natural form. Various constituents such as lycopene in tomatoes, omega-3 fatty acids in salmon, or saponins in soy are available and consumed for different health benefits. Further, various types of traditional nutraceuticals are as follows:

(i) Chemical constituents

(a) Nutrients
(b) Herbals
(c) Phytochemicals

(ii) Probiotic microorganisms

(iii) Nutraceutical enzymes

(1) Chemical Constituents

(a) Nutrients

Primary metabolites such as amino acids, various vitamins, and fatty acids had well-defined functions in various metabolic pathways. Plant and animal products along with vitamin have many health benefits and are helpful in curing diseases related to heart, kidney, lungs, etc.

Natural products obtained from plants are beneficial in treating various disorders such as brittle bones and low hemoglobin count, and they provide strength to bones and muscles, help in neuron transmission, and maintain rhythm of heart muscles. Fatty acids, omega-3 PUFAs present in salmon, had influenced the overall inflammatory response and brain function and reduced cholesterol in the arteries.

(b) Herbals

Nutraceuticals along with herbs had an excellent impact on prevention of various chronic diseases to make life better. Salicin present in the willow bark (*Salix nigra*) had been proved for anti-inflammatory, analgesic, antipyretic, astringent, and antiarthritic response clinically. Flavonoids such as psoralen present in parsley (*Petroselinum crispum*) is useful in diuretic, carminative, and antipyretic.

Peppermint (*Mentha piperita*) contains various terpenoids especially menthol, a bioactive constituent, and cures cold and flu. Tannin contents of lavender (*Lavandula angustifolia*) help releasing stress and blood pressure and are useful for lung disorders such as asthma [19].

(c) Phytochemicals

They are mainly classified on the basis of phytochemicals. Carotenoids (isoprenoids) are present in vegetables, enhancing immune system, mainly killer cells accounting for an anticancer response. Legumes (chickpeas and soybeans), grains, and palm oil contain noncarotenoids, which remove cholesterol and are anticarcinogenic.

Flavonoids, a class of secondary metabolites, which are present in most of the plants, having more than 4000 varieties had been proven clinically for preventing various diseases such as cancer, diabetes, heart diseases, and kidney problem through its antioxidant properties and their bioactive components [20].

Phenolic acids are the largest class of secondary metabolites, mainly found in citrus fruits and red wine, and have the antioxidant activity of scavenging the free radicals produced as a result of various metabolic pathways such as protein, carbohydrate, and fat. They also have anticancer and antitumour activity.

One of the classical examples is curcumin (turmeric), used as phytochemicals in most of the kitchen.

(2) Probiotic Microorganisms. Metchnikoff coined the term "probiotic." Its application is well boosted in modern medicine due to its ability of making the intestine more friendly for processes such as absorption and metabolism. Probiotics are very important to make life smoother by removing the toxic flora of the intestine and maintaining a friendly environment, for example, useful consumption of *Bacillus bulgaricus* [21]. Currently various probiotic products are available in the market with adequate nutrients to counter various pathogens so that a number of ailments related to human body can be treated.

The antimicrobial property usually had an altering impact on the microflora, making the epithelial tissues more grounded and making a situation for the supplements for better retention, which is required by the body. Moreover, probiotics are very useful in lactose intolerance by the production of related enzymes (ß-galactosidase) and hydrolyzing lactose into its sugar components [22].

(3) Nutraceutical Enzymes. Enzymes are proteinous in structure, are produced by the cell, and act as a biocatalyst. It eases the metabolic rate and fastens the life process. The medical problem mainly related to the GIT whether GERD (gastroesophageal reflux disease) or constipation or diarrhoea or ulcerative colitis could be treated with enzyme supplements. The enzyme could be a better option for diabetic patients. Nowadays, enzyme therapies are used for

Table 1: Natural nutraceuticals along with mechanism.

Nutraceuticals	Mechanism/activity
Proanthocyanidin (chestnut fruits)	Inhibit IL-8 secretion by impairing NF-kappa-B signaling [24]
Fish-based diet	Severe osteoarthritis and hip and elbow dysplasia [25]
Curcuma extract	Decrease the level of PSA for prostate cancer [26]
Supplementation of live yeast fostered	Regulate inflammation and epithelial barrier in the rumen and express DFEB1 coding for an antimicrobial peptide [27]
Inulin-type friction dietary fiber	Immune responses against hepatitis-B [28]
Bovine milk-derived oligosaccharide and *B. lactis*	Modulate gut microbiota and immune system [29]
Lipid-based nutrient supplements	Prevent growth faltering in infants [30]
Partially hydrolyzed cow's milk proteins	Cow's milk allergy in children [31]
Lactic acid bacteria (LAB) probiotic	Endometrial inflammation and infection [32]
Lipid-based nutrient supplement (LNS)	Moderate acute malnutrition (MAM) [33]
Vitamin D supplementation	Extraskeletal benefits [34]
Neutral amino acid supplements	Optimize neurocognitive function [35]
Myo-inositol	Gestational diabetes [36]
Lactobacillus fermentum CRL1446	Enhances metabolism and oxidative parameters [37]
Dehydrozingerone and its dimer	Counteract the inflammation and oxidative stress [38]
25-Hydroxy vitamin D	Cognitive status in older adults [39]
Malic acid, a precursor of citrate	Antioxidant activity [40]
Combined omega-3 fatty acids	Prevents atrophy in AD-related brain [41]
Lactobacillus rhamnosus SP1	Insulin signaling and improves adult acne [42]
Omega-3 fatty acid ethyl esters	Breast cancer [43]
CoQ10 supplementation	Propofol inhibition on complex [44]
Omega-3 fatty acids and high-dose cholecalciferol	Type 1 diabetes [45]
Large neutral amino acid supplementation	Phenylketonuria (PKU) [46]
Low-fat yoghurt supplemented with a rooster comb extract	Muscle and joint function [47]
Lipid-based nutrient supplements	Home fortification in poor settings [48]
Cholecalciferol supplementation (HYPODD)	Arterial hypertension [49]
Omega-3 polyunsaturated fatty acid supplementation	Postmenopausal vascular disease [50]
Omega-3 fatty acids	Breast cancer prevention [51]
Myo-inositol supplementation	Gestational diabetes in obese pregnant women [52]

several rare diseases such as Gaucher disease, Hunter syndrome, Fabry disease, and Pompe disease. Although enzymes are produced by their own cells, microbial sources are preferred more over plant and animal sources as they are more economical.

2.1.2. Nontraditional Nutraceuticals. They are foods enriched with supplements or biotechnologically designed crops to boost the nutrients; for example, rice and broccoli are rich in β-carotene and vitamins, respectively. Food samples contain bioactive components which are engineered to produce products for human wellness. They are arranged as follows:

(1) Fortified Nutraceuticals. These types of nutraceuticals include breeding at the agriculture level or addition of compatible nutrients to the main ingredients such as minerals added to cereals, flour fortified with calcium, iron, and folic acid, and milk fortified with cholecalciferol commonly used for vitamin D deficiency [23].

(2) Recombinant Nutraceuticals. Biotechnology tools have been well applied through a fermentation process in various food materials such as cheese and bread to extract the enzyme useful for providing necessary nutrients at an optimum level.

2.2. Classification Based on Mechanism of Action. Nutraceuticals has been further classified in regard to specific therapeutic properties accounting for antimicrobial, anti-inflammatory, and antioxidant properties.

2.3. Classification Based on Chemical Nature. These types are classified depending upon their primary and secondary metabolite sources such as isoprenoid derivatives, phenolic substances, fatty acids, carbohydrates, and amino acid-based substances.

Different types of nutraceutical constituents of natural origin are described in Table 1. All the nutraceuticals are the resources of nature.

3. Conclusions

Natural products have been known for their therapeutic values for centuries. In the modern era, these substances have been used as an immunity booster; antidiabetic, anticancer, antimicrobial, and gastroprotective agents; and so

on. Therefore, these herbs could be better options to be formulated as nutraceuticals.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

In Vitro Antimicrobial Activity Screening of Ethanol Extract of *Lavandula stoechas* and Investigation of Its Biochemical Composition

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The aim of this study was to test antimicrobial activity of ethanol extract of *Lavandula stoechas* against 22 bacteria and 1 yeast. Also, biochemical composition of the extract was investigated. A wide range of Gram-positive, Gram-negative microorganisms, and multidrug resistant bacteria were selected to test the antimicrobial activity. As a result, the extract is observed to contain fenchone *(bicyclo[2.2.1]heptan-2-one, 1,3,3-trimethyl-, (1R)-)* and camphor *(+)-2-bornanone)* as major components and showed antimicrobial activity against all studied microorganisms except *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae*. The results of the study present that *L. stoechas* is active against MDR strains too.

1. Introduction

The World Health Organization (WHO) has predicted increasing antimicrobial resistance as a major threat for the public health for the twenty-first century. In order to prevent spreading of antibiotic resistance infections, scientists have been conducting intensive researches to determine new antimicrobial agents. One way to prevent antibiotic resistance of microorganisms is by using new compounds that are not based on existing antimicrobial agents.

The *Lavandula* genus is an important member of family Lamiaceae. It consists of 47 species of small evergreen shrubs having aromatic foliage and flowers [1]. *Lavandula* species are cultivated in France, Spain, and Italy. In Turkey, mainly two species, *Lavandula stoechas* and *Lavandula angustifolia*, and their subspecies and hybrid forms grow wildly or are cultivated [2]. The medicinal importance of the plant is well documented [3, 4], and the drugs prepared from this plant are registered in many Pharmacopeia [5]. *L. stoechas* L. is used in perfumery and cosmetics [6, 7]. Anticonvulsant, sedative, and antispasmodic activities were reported [8]. The essential oil (EO) of *L. stoechas* possesses weak antibacterial activity [9]. It is used in folk medicine as an antispasmodic, a sedative, and a diuretic and for rheumatic diseases [7]. The main purpose of this study was to investigate the antimicrobial activity of *L. stoechas* and reveal the major components of its ethanol extracts.

2. Materials and Methods

2.1. Endemic Plant Samples. Dried flowers of *L. stoechas* L. were purchased from the local market in Canakkale, Turkey, and identified by Dr. Mustafa Eray Bozyel.

2.2. Disk Diffusion Test. Plant samples were dried after collection and ground into small pieces with a grinder.

Ground *L. stoechas* samples were shaken in ethanol (Sigma-Aldrich) at 125 rpm for 2 days at room temperature. After that, all the mixture was filtrated through Whatman no. 1 filter paper into evaporation flasks. Filtrates were evaporated by a rotary evaporator (Buchi R3) at 45°C [10, 11]. Finally, the remnants were collected and weighed. 5.83, 23.4, and 35.1 mg samples were prepared. The activity of the extract was tested against 22 bacteria and 1 yeast, where most of the strains were standard; nostandard strains were isolated from food and the MDR strains were clinical isolates. Non-standard strains were identified in Ankara University, Department of Biology and Duzce University, Department of medical. All bacterial strains were incubated at 37°C for 24 hours; however, *Candida albicans* was incubated at 27°C for 48 hours [11]. Each bacteria and yeast were inoculated into 0.9% sterile saline solution and adjusted to 0.5 McFarland standard, in order to standardize inocula to contain about 10^8 cfu·mL^{-1} for bacteria and 10^7 cfu·mL^{-1} for *C. albicans* [11]. The antimicrobial activity of ethanol extract of *L. stoechas* was tested by the disk diffusion test, as mentioned before [12]. Firstly, Mueller–Hinton agar (BD Difco, USA) was poured into 90 mm sterile Petri dish in order to reach a meant depth of 4.0 mm ± 0.5 mm. The extracts were loaded on 6 mm Oxoid Antimicrobial Susceptibility Test Disks. Disks were left to dry overnight at 30°C in sterile conditions in order to prevent any remaining of solvent, which may interfere with the results. After that, prepared microorganisms, which were inoculated into saline solution, were streaked on the surface of Petri dishes. These plates were left to dry for 5 minutes at room temperature in aseptic conditions [12]. Next, disks were tightly applied to the surface of plates. Finally, these plates were incubated, and inhibition zone diameters were recorded [12].

Preculturing conditions for all microorganisms are as mentioned previously[13].

2.3. Broth Dilution Test. Broth dilution method for minimum inhibitory concentration (MIC) determination as described previously was employed [14]. Serial 2-fold dilutions were made to obtain a concentration range of 0.07–35.9 μg/mL. The MIC was defined as the lowest concentration of extract inhibiting any visible bacterial growth. All tests were conducted in triplicates.

2.4. GC-MS Analysis. For the identification of chemical components, each sample was analyzed by Agilent GC 6890N-Agilent MS 5973 equipped with HP5-MS capillary column (30m ∗ 0.25 mm; coating thickness 0.25 μm). Analytical conditions were an injector temperature of 350°C; carrier gas helium at 1 mL/min; injection mode: split, split ratio 10 : 1; volume injected: 1 μL of sample in ethanol extract; oven temperature programmed from 40°C to 350°C at 4°C/min; pressure: 48.2 kPa; and split flow: 9.9 mL/min. The MS scan conditions were a transfer line temperature of 280°C, an interface temperature of 280°C, and an ion source temperature of 230°C. Identification of the components was conducted by matching the retention times against National Institute of Standards and Technology (NIST Mass Spectrometry DATA CENTER) data library, and crosscheck was applied with previously published data [15, 16] The chemical components found to be higher than 1% were accepted as the major components, and the list of these components and information regarding them are given in Table 1.

2.5. Controls. Empty sterile disks and extraction solvent (ethanol) were used as negative controls. Ciprofloxacin and gentamicin used as pozitif controls (Table 2).

2.6. Statistics. The statistical analysis was executed using a parametric method, the one-way analysis of variance (ANOVA), with a significance level of 0.05. In order to put forward any correlation between concentration and antimicrobial activity, Pearson correlation coefficient was calculated. All statistical analysis were conducted by using R Studio, version 3.3.2 [17].

3. Results and Discussion

The diameters of inhibition zones, which were measured in millimeters, are given in Table 3 as the mean values of three parallels with standard errors. No activities were observed for the negative controls. Furthermore, statistical analysis proved that there are no significant differences between the activities of three parallels of each extract volumes ($p > 0.05$). On the other hand, a weak positive correlation is observed between the activities of extracts and the volumes tested, with a Pearson correlation coefficient of 0.3239.

In addition, the results of broth dilution test (MIC values) are given in Table 4.

According to Table 3, *L. stoechas* has antimicrobial activity against all studied microorganisms except *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae*. Three of them have high susceptibility (15–25 mm); seven of them have moderate susceptibility (14–10 mm); and eleven of them have low susceptibility (9–7 mm). *L. stoechas* shows antimicrobial activity against all tested MDR bacteria. These results are important since antimicrobial activity of this species were determined against large range of Gram-negative and Gram-positive bacteria.

According to GC-MS results, fenchone *(bicyclo[2.2.1] heptan-2-one, 1,3,3-trimethyl (1R)-)*, and camphor *(+)-2-bornanone)* are mainly found in the composition of *L. stoechas* ethanol extract. Similar results were obtained when compared with previous researches [18–20] (Figure 1).

The antimicrobial effect of the plant is known from previous investigations, but there is no broad-spectrum study like this [18, 20]. It has also been reported for the first time that the plant's ethanol extract is effective against multidrug-resistant microorganisms, which is one of the most important health hazards in the world [21].

Acinetobacter species, particularly *Acinetobacter baumannii*, have become significant pathogens especially in the nosocomial setting. *A. baumannii* has progressively been implicated in serious nosocomial infections, including bloodstream infection (BSI), nosocomial and ventilator-related

Table 1: The major chemical components of *L. stoechas* according to the GC-MS analysis.

No.	Retention time	Compound name	Formula	Molecular weight (g/mol)	Area (%)
1	14.529	Eucalyptol	$C_{10}H_{18}O$	154.249	6.22
2	16.940	Bicyclo[2.2.1]heptan-2-one, 1,3,3-trimethyl-, (1R)-	$C_{10}H_{16}O$	152.233	18.81
3	19.163	(+)-2-Bornanone	$C_{10}H_{16}O$	152.233	8.64
4	22.138	Fenchyl acetate	$C_{12}H_{20}O_2$	196.286	1.10
5	24.593	Bornyl acetate	$C_{12}H_{20}O_2$	196.286	3.40
6	26.015	Myrtenyl acetate	$C_{12}H_{18}O_2$	194.270	5.18
7	30.067	Benzaldehyde, 2-hydroxy-4-methyl-	$C_8H_8O_2$	136.148	1.64
8	31.687	Cubedol	$C_{15}H_{26}O$	222.366	1.10
9	32.350	Cubedol	$C_{15}H_{26}O$	222.366	1.08
10	32.854	Bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-trimethyl-	$C_{10}H_{14}O$	150.218	1.40
11	33.844	Acetic acid, 4a-methyl-1,2,3,4,4a,5,6,7-octahydronaphthalen-2-yl ester			1.42
12	34.749	Veridiflorol	$C_{15}H_{26}O$	222.366	4.50
13	35.121	Viridiflorol	$C_{15}H_{26}O$	222.366	2.99
14	37.869	Unknown	—	—	1.02
15	39.683	Andrographolide	$C_{20}H_{30}O_5$	350.449	1.15
16	48.039	Palmitic acid	$C_{16}H_{32}O_2$	256.424	2.48
17	52.456	Behenic alcohol	$C_{22}H_{46}O$	326.600	1.06
18	75.553	Tetrapentacontane	$C_{54}H_{110}$	759.451	1.48
19	77.908	Tetrapentacontane	$C_{54}H_{110}$	759.451	1.37
20	82.903	Tetrapentacontane	$C_{54}H_{110}$	759.451	4.84
21	86.068	Tetrapentacontane	$C_{54}H_{110}$	759.451	4.83
22	91.031	Tetrapentacontane	$C_{54}H_{110}$	759.451	2.53
23	91.398	Stigmast-5-en-3-ol, (3.beta.)-	$C_{29}H_{50}O$	414.707	3.25

Table 2: Pozitif controls (inhibition zones in mm).

	Ciprofloxacin	Gentamicin
B. subtilis DSMZ 1971	36	30
C. albicans DSMZ 1386	—	—
E. aerogenes ATCC 13048	30	23
E. durans	24	14
E. faecalis ATCC 29212	19	13
E. faecium	28	28
E. coli ATCC 25922	—	20
K. pneumoniae	30	22
L. innocula	18	13
L. monocytogenes ATCC 7644	20	28
P. aeruginosa DSMZ 50071	28	15
P. fluorescens P1	19	12
S. enteritidis ATCC 13075	36	24
S. infantis	24	24
S. Kentucky	34	13
S. typhimurium SL 1344	35	23
S. aureus ATCC 25923	22	24
S. epidermidis DSMZ 20044	34	25

—: no activity observed.

Table 3: Disk diffusion test result for *L. stoechas* (inhibition zones in mm).

	5.83 mg loaded disk	23.4 mg loaded disk	35.1 mg loaded disk
Bacillus subtilis DSMZ 1971	11.00 ± 0.00	13.00 ± 0.00	14.00 ± 0.58
Candida albicans DSMZ 1386	7.00 ± 0.00	9.00 ± 0.00	11.00 ± 0.00
Enterobacter aerogenes ATCC 13048	—	—	8.00 ± 0.00
Escherichia coli	—	—	8.00 ± 0.00
Escherichia coli ATCC 25922	—	—	—
Enterococcus durans	—	10.00 ± 0.00	11.00 ± 0.00
Enterecoccus faecalis ATCC 29212	7.00 ± 0.00	9.00 ± 0.00	11.00 ± 0.00
Enterecoccus faecium	11.00 ± 0.58	16.00 ± 0.58	15.00 ± 1.15
Klebsiella pneumoniae	—	—	—

Table 3: Continued.

	5.83 mg loaded disk	23.4 mg loaded disk	35.1 mg loaded disk
Listeria innocua	—	8.00 ± 0.00	10.00 ± 0.00
Listeria monocytogenes ATCC 7644	9.00 ± 0.00	14.00 ± 1.15	13.00 ± 0.58
Pseudomonas aeruginosa DSMZ 50071	—	8.00 ± 0.00	10.00 ± 0.00
Pseudomonas fluorescens P1	7.00 ± 0.00	9.00 ± 0.00	13.00 ± 0.00
Staphylococcus aureus	10.00 ± 0.00	10.00 ± 0.00	14.00 ± 0.58
Staphylococcus aureus ATCC 25923	12.00 ± 0.58	13.00 ± 0.00	18.00 ± 0.00
Salmonella enteritidis ATCC 13076	7.00 ± 0.00	9.00 ± 0.00	10.00 ± 0.00
Staphylococcus epidermidis DSMZ 20044	15.00 ± 0.00	19.00 ± 0.58	20.00 ± 0.00
Salmonella infantis	—	—	10.00 ± 0.00
Salmonella kentucky	—	7.00 ± 0.00	10.00 ± 0.00
Salmonella typhimurium SL1344	8.00 ± 0.58	—	10.00 ± 1.15
Acinetobacter baumannii MDR	—	10.00 ± 0.00	11.00 ± 0.00
Proteus vulgaris MDR	10.00 ± 1.15	14.00 ± 1.15	11.00 ± 1.15
Streptococcus pneumoniae MDR	10.00 ± 0.00	14.00 ± 1.15	13.00 ± 0.58

—: no inhibition.

Table 4: MIC values for *L. stoechas* (MIC values in μg/mL).

	MIC
Bacillus subtilis DSMZ 1971	35.9
Candida albicans DSMZ 1386	35.9
Enterobacter aerogenes ATCC 13048	—
Escherichia coli	—
Escherichia coli ATCC 25922	—
Enterococcus durans	35.9
Enterecoccus faecalis ATCC 29212	35.9
Enterecoccus faecium	17.95
Klebsiella pneumoniae	—
Listeria innocua	35.9
Listeria monocytogenes ATCC 7644	35.9
Pseudomonas aeruginosa DSMZ 50071	35.9
Pseudomonas fluorescens P1	35.9
Staphylococcus aureus	17.95
Staphylococcus aureus ATCC 25923	17.95
Salmonella enteritidis ATCC 13076	35.9
Staphylococcus epidermidis DSMZ 20044	8.98
Salmonella infantis	—
Salmonella kentucky	35.9
Salmonella typhimurium SL1344	35.9
Acinetobacter baumannii MDR	35.9
Proteus vulgaris MDR	35.9
Streptococcus pneumoniae MDR	35.9

—: no inhibition.

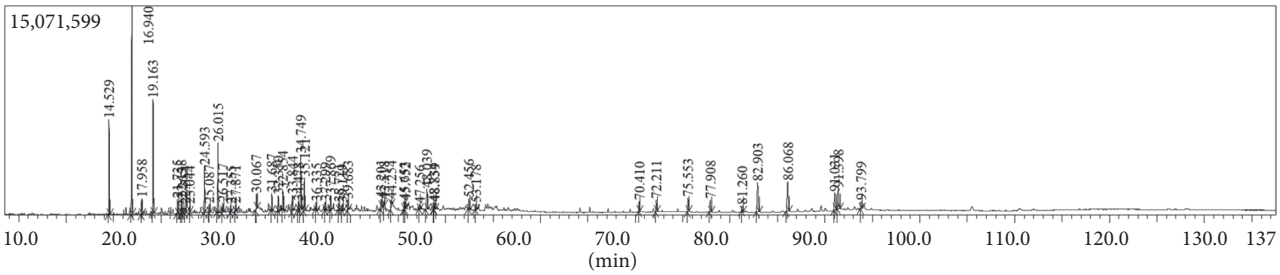

Figure 1: GC-MS chromatography of *L. stoechas*.

pneumonia, and meningitis. These infections are particularly common in critically ill patients, with mortalities as high as 40–64% for pneumonia and 17–46% for BSI [22]. The extensive use of broad-spectrum antibiotic agents within hospitals has led to the rapid emergence of multidrug-resistant (MDR) *A. baumannii* strains. Only a few antimicrobial agents are active against MDR *A. baumannii* infections [22]. In our study, we observed 11 mm of inhibition zone against *A. baumannii* (MDR) strain. Moreover, we have found 14 mm of inhibition zones against *Proteus vulgaris* (MDR) and *Streptococcus pneumoniae* (MDR) strains. Our results present that *L. stoechas* is active against MDR strains too.

Previous studies tested the antimicrobial activity of *L. stoechas* and found MIC values between 0 and 6500 μg/mL [23]. When the result of this study were compared with the results of the previous study, it may be observed that the MIC values obtained in our study were much lower, which is better. The reason of this difference should be related with the strains used in two studies. Although *B. subtilis*, *S. aureus*, *S. typhimurium*, *E. coli*, and *C. albicans* were used in both studies, the strains were different. Only *L. monocytogenes* ATCC 7644 were common in these two studies but the MIC values were different. This difference could be related with the composition of extracts which were directly affected by the environment; those plant samples were collected.

Another previous study was tested the antimicrobial activity of two subspecies of *L. stoechas*. The only common microorganism used in our study and this previous study was *C. albicans*. They observed a MIC value > 100 μg/mL for both subspecies, which are quite higher than our results [24]. But this difference is logical since the plant samples, *C. albicans* strain, and collection area were different.

Ez Zoubi et al. [25] tested the antimicrobial activity of the essential oil of *L. stoechas* against *E. coli*, *K. pneumoniae*, *Proteus mirabilis*, and *P. aeruginosa*. As a result, they observed MIC values between 2.5 and 10 μg/mL. Although the strains of these microorganisms were not defined, the difference in the MIC values between this study and our study was mainly due to using different types of extracts, namely, essential oil and ethanol extracts.

4. Conclusion

Our study clearly presents that *L. stoechas* should have a possible medicinal uses, especially against MDR bacteria. However, further researches are needed in order to analyse the active substances and their activity mechanisms in details.

Abbreviations

GC-MS: Gas chromatography-mass spectrophotometry method.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

An earlier version of this study was presented as an abstract in the Second Japan-Turkey International Symposium on Pharmaceutical and Biomedical Sciences, 2017.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Oleander Stem and Root Standardized Extracts Mitigate Acute Hyperglycaemia by Limiting Systemic Oxidative Stress Response in Diabetic Mice

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The extracts of different parts of *Nerium oleander* L. are used as antidiabetic remedy in the traditional medicinal systems of different parts of the world. Despite these uses in ethnomedicinal system, the antihyperglycemic potentials of oleander stem (NOSE) and root (NORE) extracts have not been pharmacologically evaluated. Therefore, we aimed at evaluating the antidiabetic ethnomedicinal claims of NOSE and NORE, primarily focusing on glucose homeostasis and associated metabolic implications. Alloxan-treated mice with hyperglycaemia (blood glucose >200 mg/dL) were treated with oleander 70% hydromethanolic extracts (200 mg/kg) for 20 consecutive days, and the results were compared with positive control glibenclamide. Blood glucose level was 52–65% lowered ($P < 0.001$) in oleander treated groups, which was otherwise 4.62 times higher in diabetic mice, compared to control. Insulin resistance was lowered 51–36% irrespective of any significant ($P > 0.05$) changes in insulin sensitivity throughout the treatments. Improved serum insulin remained associated with lowered glucose level ($r_P = -0.847$ and -0.772; $P < 0.01$). Markers of hyperglycaemia-related hepatic glycogen, glycated haemoglobin (HbA1c), hyperlipidaemia, hepatic injury, and diabetic nephropathy were normalized as well. Improvement of systemic intrinsic antioxidant enzymes (catalase and peroxidase) were correlated ($r_P = -0.952$ to -0.773; $P < 0.01$) with lower lipid peroxidation by-product malondialdehyde (MDA) in the circulation. Principal component analysis coupled with hierarchical cluster analysis represented shift in metabolic homeostasis in diabetic mice, which was further normalized by oleander and glibenclamide treatment. Additionally, molecular docking studies of the phenolic acids measured by HPLC with intracellular cytoprotective transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) revealed strong molecular interactions. The results collectively support the ethnomedicine antidiabetic claims of oleander stem and root and suggest that the oleander mediated elevation of systemic antioxidant status is likely responsible for the improved glycaemic control.

1. Introduction

Diabetes mellitus (DM) is a systemic disorder, primarily characterized by loss of glucose homeostasis, impaired insulin signalling, carbohydrate, and lipid metabolism, ultimately resulting in progressive systemic complications such as hyperlipidemia, hyperglycaemia, nephropathy, hepatic injury, vascular dysfunction, etc. The global prevalence of diabetes has quadrupled in the last 30 years [1]. It is expected to deteriorate based on estimate that 642 million

people will be diabetic, and an additional 481 million will suffer from impaired glucose tolerance by the end of 2040 [2].

Nerium oleander L. (syn. *Nerium indicum* Mill and *Nerium odorum* Aiton) is an ethnomedicinal shrub which is used for the treatment of diabetic complications in India, Pakistan, Algeria, and Morocco, and its medicinal properties are recognized in Ayurveda as well [3]. Prior pharmacognostic studies have demonstrated that oleander at 200 mg/kg/day lowers hyperinsulinemia and hyperglycaemia in association with attenuating streptozotocin-induced diabetic hepatotoxicity in rats [4]. Methanolic exact of oleander leaf also ameliorates alloxan-induced diabetes by improving antioxidant enzymes and limiting lipid peroxidation in mice [5]. These benefits over glycaemic control were irrespective of type of extracts as prior reports suggest that several fractions of oleander leaf limits hyperglycaemia in rats [6].

However, despite its tremendous traditional therapeutic use, pharmacognostic studies are focused primarily only on the bioactivities of its leaves. Interestingly, recent prospective studies have demonstrated potent free-radical scavenging properties and comparable bioactivities of oleander stem and root extracts to modulate macrophage bioactivities and limit haloalkane-induced hepatotoxicity in murine models [7–9]. These reports were additionally associated with detailed phytochemical fingerprinting using chromatographic and spectroscopic techniques that revealed several bioactive constituents in different parts of oleander.

Therefore, based on the ethnomedicinal use against diabetic complications and pharmacognostic reports of hepatoprotective activities, we hypothesized that oleander stem and root hold the potentiality to lower hyperglycaemia and improve glucose homeostasis and insulin resistance by improving systemic oxidative stress against a free-radical induced murine model of type 1 diabetes (T1D). The extracts were further analysed by HPLC, and *in silico* docking studies were performed against the intrinsic cytoprotective transcription factor to validate the hypothesis.

2. Materials and Methods

2.1. Chemicals and Solvents. All chemicals were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), unless otherwise indicated. Spectrophotometric kits to measure serum chemistries were procured from Crest Biosystem; Goa, India. HPLC standards were purchased from Sigma-Aldrich (St. Louis, MO, USA) and ChromaDex (Irvine, CA, USA). MilliQ water (Ω 18.2) from departmental central facility was used for all the experiments.

2.2. Animals. Inbred Swiss albino mice (7-8 wk; male) were maintained (25 ± 2°C; 12 h photoperiod) in the departmental animal house, with food and water *ad libitu*m. Female mice were excluded from the study based on the report that female mice are resistant to experimental obesity and insulin resistance [10]. All *in vivo* experiments were reviewed and approved by the university ethical committee (840/ac/04/CPCSEA).

2.3. Plant Sample and Extract Preparation. Stem and root of white flowered variety of oleander were collected from departmental garden (26.71°N, 88.35°S) during August 2015. Plant samples were authenticated, and voucher specimen was stored at the university herbarium with an accession no. 09618. Hydromethanolic (70%) extracts of stem (NOSE) and root (NORE) were prepared as described previously [8]. In brief, shade-dried and grinded 100 g of oleander stem and root were separately mixed with 1000 mL of 70% methanol and twice extracted for 12 h at 37°C. Extraction was performed under reduced pressure and at physiological temperature to preserve the heat labile phytochemicals. The resultant was filtered and concentrated under reduced pressure and lyophilized. The final yield of NOSE and NORE were 11.82% and 15.22% of dry weight.

2.4. Experimental Design. Freshly prepared alloxan monohydrate (in 154 mM NaCl) was intraperitoneally injected (200 μL; 150 mg/kg) to each mouse. Mouse with blood glucose level >200 mg/dL post 3 d of injection was considered diabetic and randomized (body weight (BW); $P > 0.05$) into following cohorts ($n = 6$): *T1D* group receive normal saline; *Glib* group received glibenclamide (5 mg/kg/d) as reference drug; *NOSE* and *NORE* groups were gavaged 200 mg/kg/d NOSE and NORE, respectively. Another cohort of 6 nondiabetic mice (not alloxanized; glucose <60 mg/dL) were considered as control (Ctrl). Doses of NOSE and NORE were selected based on prior reports of acute toxicity study which demonstrated that up to 2 g/kg BW oral gavaging of the extracts display no clinical and toxicological symptoms in mice, in addition to ameliorate haloalkane-induced hepatotoxicity at 200 mg/BW dose [9]. Based on these evidences, 200 mg/BW dose was selected for the present study which was $1/10^{th}$ of the highest dose (2 g/kg) selected in acute toxicity study.

Blood glucose level was measured from the tail vein using one-touch glucometer (Bayer, contour TS meter), and BW was monitored periodically. After 20 d, mice were fasted 12 h and whole blood was collected from the heart under mild ether anaesthesia, allowed to colt for 30 min and centrifuged (2000 × g; 15 min) to obtain serum. Mice were sacrificed by cervical dislocation, and liver, both kidneys, and thigh skeletal muscle were collected, washed in phosphate buffer saline, snap-frozen, and stored in −80°C freeze until further use.

2.5. Measurement of Hyperglycaemic Metabolic Parameters. Serum insulin was estimated by the ELISA method using Accu-Bind Universal ELISA kit (Monobind Inc., USA) according to manufacturer's instructions, and glycated haemoglobin (HbA1c) level was measured by ion-exchange HPLC using D-10™ Dual HbA1c program (Bio-Rad #220-0201). Insulin resistance was calculated using the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR): glucose (mg/dL) × insulin (mU/L). Insulin sensitivity was calculated using the Quantitative Insulin Sensitivity Check Index (QUICKI): 1/log (fasting insulin (mU/mL) + log (fasting glucose (mg/dL)). Hepatic glycogen content was

measured according to a standardized Anthrone reagent method [11] against a glucose standard curve prepared in parallel to the samples.

2.6. Measurement of Serum Chemistries. Serum biochemical parameters (acid phosphatase (ACP); alanine aminotransferase (ALP); alanine transaminase (ALT); aspartate transaminase (AST); blood urea nitrogen (BUN); cholesterol, triglyceride, creatinine, and uric acid) were spectrophotometrically measured as primary markers of diabetic hepatic injury, hyperlipidemia, and diabetic nephropathy, using commercially available kits (Crest Biosystem; Goa, India).

2.7. Measurement of Antioxidant Enzymes and Lipid Peroxidation. Catalase (CAT) activity was studied by measuring breakdown of H_2O_2 at 240 nm [12], and peroxidase (PX) activity was measuring the oxidation of guiacol at 436 nm according to standardized methods [13]. The extent of lipid peroxidation was measured in terms of serum malondialdehyde (MDA) content by colorimetric thiobarbituric acid reactive substances (TBARS) assay kit (Cayman Chemical, USA) as per the manufacturer's instructions.

2.8. Oral Glucose Tolerance Test (OGTT). In brief, a separate cohort of alloxanized (glucose >200 mg/dL) and nondiabetic mice were subjected to separate groups ($n = 4$) and treated as previous. After 20 d, all mice were subjected to 12 h fasting and a single-dose (2.5 g/kg) of glucose was orally gavaged. Blood glucose was measured from tail vein at 0, 30, 60, 120, and 180 min after glucose administration.

2.9. HPLC Analysis. In order to be analysed in reverse-phase HPLC and identify relatively polar phenolic compounds, NOSE and NORE were delipified following the method of Bligh and Dyer [14]. In brief, the methanolic extracts were mixed with 4 volumes of chilled acetone prior to incubating at −20°C for 1 h. After incubation, the mixtures were spun at 16,000 × g (4°C for 15 min). The supernatant methanolic fractions were carefully collected and were subjected to thin layer chromatography (TLC) on silica gel plate. Acetic acid (10%) in chloroform was used as solvent, and corresponding bands of secondary metabolites were eluted by acetonitrile after detection with 20% (w/v) Na_2CO_3 and diluted Folin–Ciocaltaeu reagent (1 : 3; v/v). Samples were analyzed (20 μL injection; 0.4 mL/mL flow) in a HPLC (Agilent, USA) coupled with Zorbax SB-C18 column (4.6 × 150 mm, 3.5 μm) and equipped with a diode array detector. Gradient concentrations of mobile phase A—methanol (M) and B—water (W) with 0.02% H_3PO_4 were 25% A + 75% B for 5 min, 30% A + 70% for 10 min, 45% A + 55% for 30 min, and 80% A + 20% B for 45 min. Peaks were identified by relative retention time (RRT) against respective standards (Sigma, USA; ChromaDex, USA) and spectral patterns. Quantifications were done by postcalibration with response factor of standards.

2.10. Molecular Docking Studies. Molecular docking was performed as we describe previously [15]. In brief, X-ray diffraction structures of Nrf2 (nuclear factor erythroid 2-related factor 2; PDB ID: 5FNQ) was obtained from the Research Collaboratory for Structural Bioinformatics (RSCB; http://rcsb.org) Protein Data Bank (PDB) database. DNA counterpart was removed from 2UZK using Autodock Vina V1.5.6. Protein sequence was prepared for docking after removal of water molecules, followed by addition of polar hydrogen atoms. Grid measurement was performed after Gasteiger charge calculation and subsequently the .pdb files were converted into .pdbqt files via SMILES server (https://cactus.nci.nih.gov/translate/). Three-dimensional structures of the compounds were retrieved from the NCBI-PUBCHEM database (https://pubchem.ncbi.nlm.nih.gov), prepared for docking analysis using Autodock Vina. The docked conformations were visualized using PyMol V1.7.4.

2.11. Statistical Analysis. Statistical analysis was performed using KyPlot version 5.0 (KyensLab Inc.). Group differences were measured using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. Data of OGTT were analyzed using one-way ANOVA. All data are reported as the mean ± SD of six measurements. Linear bivariate correlation (one-tailed) was performed to explore the pairwise association between individual variables by Pearson's method (r_P). $P < 0.05$ was considered significant. Principal component analysis (PCA) was performed to represent and the divergence of systemic and metabolic status of individual mouse from each group ($n = 6$) using a correlation-based matrix and represented as a PCA loading plot. Further, the divergence of systemic and metabolic status of individual animals were measured using Pearson's distance with complete linkage method and represented by a dendrogram. Multivariate analysis was performed by Minitab® 18.

3. Results and Discussion

The present finding demonstrates that oleander stem and root extract significantly lowers hyperglycaemia and insulin resistance without affecting insulin sensitivity. T1D associated systemic oxidative stress was lowered by improvement of intrinsic antioxidant defence associated with limiting lipid peroxidation. Attenuation of diabetes associated hyperlipidemia, hepatic injury, and nephrotoxicity remained linked with improved antioxidant status. Further, strong molecular interaction of HPLC-identified phytochemicals with Nrf2 indicates oleander mediated antidiabetic protection through antioxidant activities.

After 20 days of intervention, significant ($P < 0.01$; 19%) BW increase was seen only in case of T1D group (Figure 1(a)). The lowest BW increase was noted (3.15%) for NOSE-treated mice. However, the lowest rate of BW gain was seen in the control (70 mg/d) group. Uncontrolled hyperglycaemia is the hallmark of T1D, and oleander extracts showed profound effects on glucose metabolism in the alloxanized mice. A gradual decrease of blood glucose load

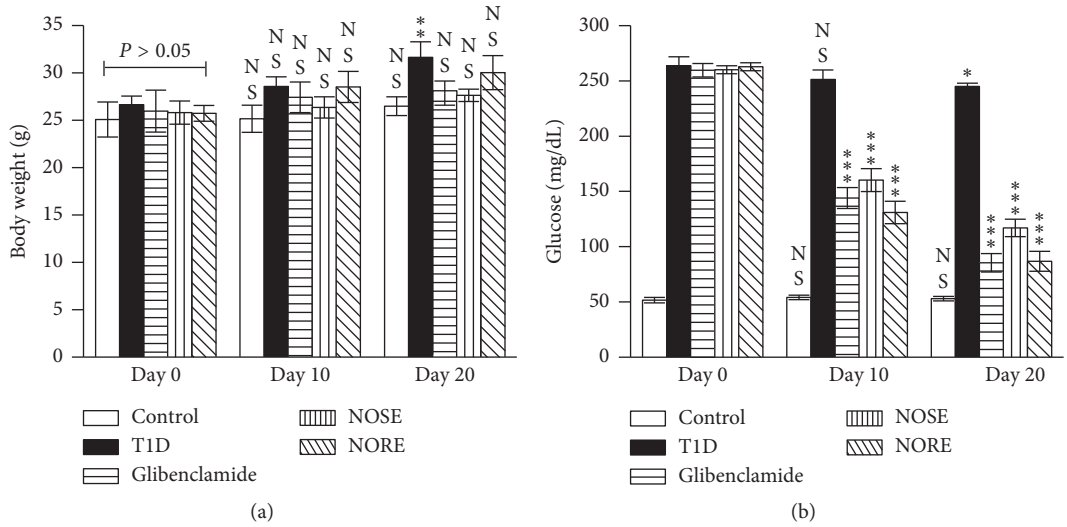


Figure 1: Effects of oleander extracts on body weight (BW) and glucose metabolism. Alloxanized T1D mice (glucose >200 mg/dL) were treated with 200 mg/kg oleander stem (NOSE) and root (NORE) extracts for 20 consecutive days, and glibenclamide (Glib) was used as standard. After the intervention, (a) BW increase of NOSE- and NORE-treated mice were lower compared to day 0, which was otherwise significantly higher in T1D group at day 20. (b) In agreement to the hypothesized antidiabetic activity, blood glucose level was decreased in the alloxanized mice due to NOSE and NORE treatment. Data were represented as mean ± SD of six measurements. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, and $^{NS}P > 0.05$ compared to control.

was observed in the NOSE and NORE groups (Figure 1(b)). After the intervention, significant ($P < 0.001$) reduction (NOSE: 55% and NORE: 67%) of blood glucose was observed which was otherwise reduced merely 7% in T1D group. Autoimmune pancreatic β-cell toxicity lowers circulatory insulin level which leads to persistent hyperglycaemia and abnormal energy metabolism in T1D. However, the etiology of alloxan toxicity leading to insulin-dependent diabetes T1D is governed by β-cell oxidative stress and redox imbalance [16]. This was reflected by significantly lower ($P < 0.001$; 64%) serum insulin level in alloxanized mice compared to control (Figure 2(a)). NOSE and NORE considerably increased ($P < 0.01$) the serum insulin level 35% and 32%. Indeed, lowered blood glucose level remained correlated with the increased insulin level ($r_P = -0.847$, $P < 0.001$; $r_P = -0772$, $P < 0.01$), indicating better glucose utilization by the liver due to improved insulin level. NOSE and NORE further improved insulin resistance which would otherwise remain impaired in the alloxanized mice. In support, HOMA-IR model showed 66% elevated ($P < 0.001$) insulin resistance in T1D mice, indicating alloxan mediated double-diabetes [17], which was 51% and 36% lowered by NOSE and NORE, respectively (Figure 2(b)). In fact, linear association of lowered HOMA-IR with lowered glucose ($r_P = 0.878$ to 0.860, $P < 0.001$), and increased insulin ($r_P = -0.585$ and -0.412; $P < 0.05$) level highlights improvement of impaired glucose metabolism. This indicates better peripheral uptake of elevated systemic glucose by insulin-target organ and tissue (i.e. skeletal muscle, liver, and adipose) through lowered insulin resistance. However, insulin sensitivity as measured by QUICKI model was marginally impaired (4%; $P > 0.05$) in the alloxanized mice, and compared to T1D group, both the oleander extracts (4–6%) resulted no significant ($P > 0.05$) increase in insulin sensitivity (Figure 2(c)). This suggests that oleander stem and root extracts did not directly attenuate alloxan-induced pancreatic β-cell toxicity. Thus, the improved insulin level mediated glucose uptake was likely not due to cytotoxic protection of oleander but due to improved pancreatic insulin signalling and/or improved hepatic glucose metabolism. However, our result contradicts a prior report which concludes hypoglycaemia associated improved insulin sensitivity by oleander shoot distillate in streptozotocin-induced diabetic rats [18]. Thus, future interventions are required to study the effect of oleander on insulin signalling.

Circulatory glucose in excess is converted and stored primarily in the liver as glycogen. Elevated serum glucose level in the alloxanized mice and subsequent increased insulin resistance significantly ($P < 0.001$) decreased the hepatic glycogen level up to 57.14%, which was further improved 26% ($P > 0.05$) and 40% ($P < 0.01$) by NOSE and NORE treatment (Figure 2(d)). Even though, 20 days of intervention failed to restore hepatic glycogen level to control, the improved glycogen level remained associated with improved insulin resistance ($r_P = -0.581$ and -0.49; $P < 0.05$) and serum insulin level ($r_P = 0.945$ and 0.931; $P < 0.01$), indicating elevated systemic insulin affecting hepatic glycogenesis. Indeed, this was further supported by significant correlation ($r_P = -0.767$ and -0.745; $P < 0.01$) between lowered blood glucose and hepatic glycogen level, indicating improved glucose to glycogen turnover in the liver.

The potentials of oleander extracts to improve body's ability to utilize excess glucose was further evaluated by OGTT. Thirty-minute postglucose (2.5 g/kg) gavage, blood glucose was significantly ($P < 0.001$) spiked up in all mice (Figure 3(a)). At postprandial 180 min, the blood glucose

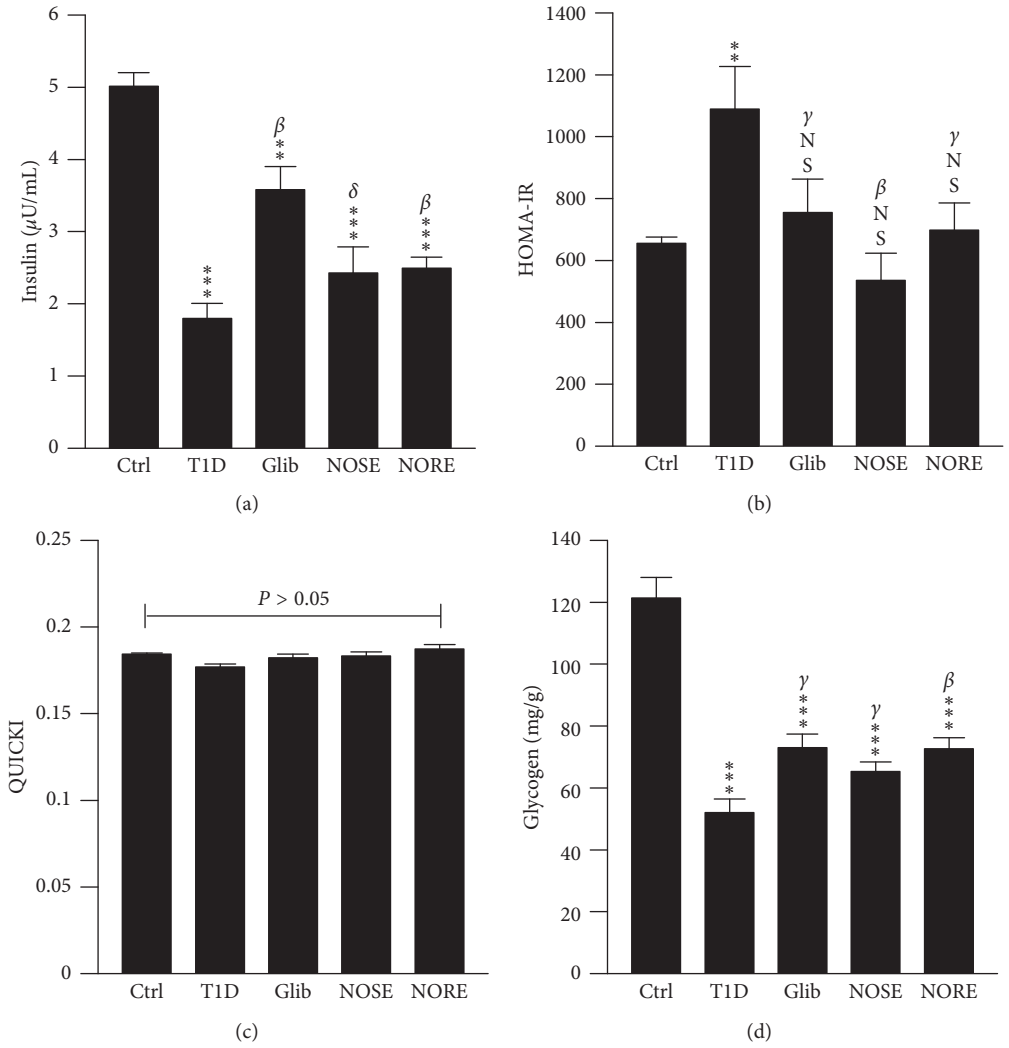


Figure 2: Effects of oleander extracts on the systemic glucose metabolism. The 20 d treatment with NOSE and NORE resulted in the improvement in glucose homeostasis which was otherwise impaired in the alloxanized animals. (a) NOSE and NORE normalized serum insulin level which was associated with systemic glucose load. (b) The elevated insulin resistance, measured by Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) model, was mitigated; however, (c) insulin sensitivity remained unchanged ($P > 0.05$) throughout the treatments as measured by the Quantitative Insulin Sensitivity Check Index (QUICKI) model. (d) Hepatic glycogen level was improved in comparison to T1D group, however not up to the level of control. Data were represented as mean ± SD of six measurements; $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{NS}P > 0.05$ compared to control. $^{\beta}P < 0.01$, $^{\gamma}P < 0.05$, and $^{\delta}P > 0.05$ compared to T1D.

level was 25%, 40%, and 34% lower in T1D, NOSE, and NORE groups, respectively, indicating more efficient removal of excess glucose from the circulation and improvement of peripheral glucose uptake due to oleander extracts. In fact, NOSE-treated ($r_P = -0.9942$; $P < 0.01$) and NORE-treated ($r_P = -0.9997$; $P < 0.001$) groups not only demonstrated comparatively more time-dependent activity than T1D ($r_P = -0.9901$; $P < 0.01$), but the rate of initial blood glucose spike was lower in NOSE ($\Delta_{glu/min}$: 5.27 mg/dL/min; 231%) and NORE ($\Delta_{glu/min}$: 5.43 mg/dL/min; 242%) compared to both T1D ($\Delta_{glu/min}$: 6.52 mg/dL/min) and Glib ($\Delta_{glu/min}$: 5.64 mg/dL/min). Following postintervention glucose levels, improvement of systemic glucose level was additionally reflected by HbA1c levels (Figure 3(b)). NOSE and NORE significantly ($P < 0.01$) normalized HbA1c level 14–15%, respectively, which was otherwise 53% higher in T1D group compared to control ($P < 0.001$). Lowered HbA1c was in association with lowered blood glucose level ($r_P = 0.940$ and 0.866; $P < 0.01$). Moreover, lowered HbA1c remained negatively correlated with serum insulin ($r_P = -0.900$ and -0.939; $P < 0.01$) and hepatic glycogen ($r_P = -0.859$ and -0.860; $P < 0.01$), highlighting oleander stimulated excess glucose uptake and improvement of glucose metabolism in the alloxanized mice.

The Liver plays a critical role in energy homeostasis by regulating glycolysis, gluconeogenesis, and glycogenesis [19]. Strong evidence indicates interplay between hepatic complications and diabetes [20], which was further reflected by 178%, 100%, 107%, and 40% increase in ACP, ALP, ALT, and AST in the T1D mice (Table 1). All the hepatic injury marker enzymes were lowered due to NOSE and NORE treatment, reflecting improvement of liver injury in the

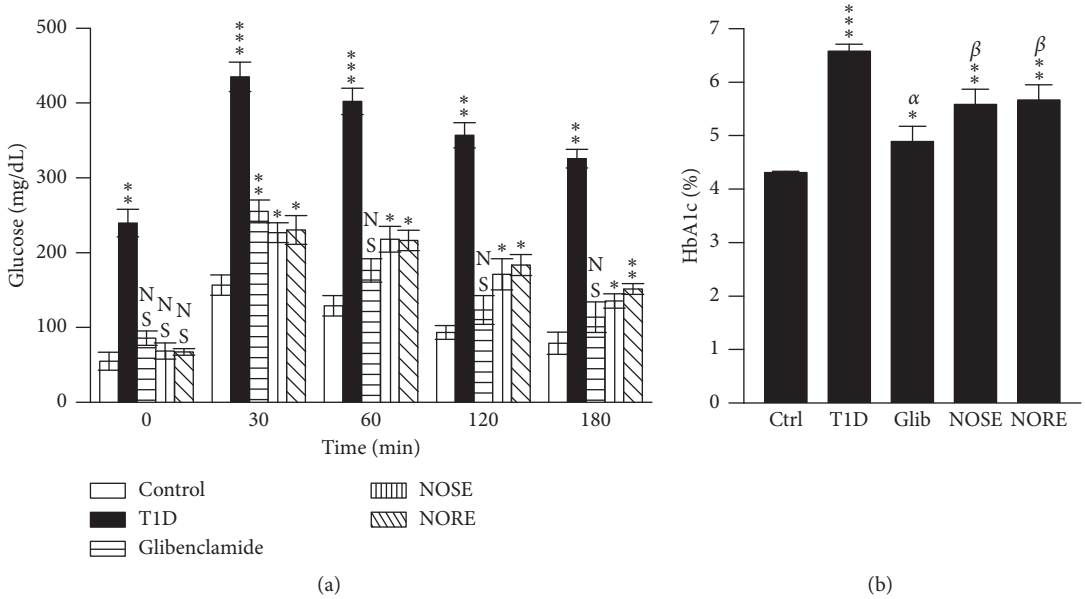


Figure 3: Effects of oleander on oral glucose tolerance (OGTT) and glycated haemoglobin (HbA1c) levels. (a) The treatment effects on acute glucose administration was measured in a separate cohort of diabetic (glucose >200 mg/dL) and nondiabetic control mice, which demonstrated a time-dependent improved peripheral glucose uptake after 20 d of treatment. (b) Improved glycated haemoglobin level further indicated increased glucose metabolism due to 20 d of oleander treatment. Data represented as mean ± SD of 4 measurements for OGTT and 6 measurements for HbA1c. $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{NS}P > 0.05$ compared to control. $^{\alpha}P < 0.001$ and $^{\beta}P < 0.01$compared to T1D.

Table 1: Effect of NOSE and NORE on serum enzymatic and biochemical parameters.

	Control	Alloxan			
		T1D	Glibenclamide	NOSE	NORE
ACP	3.39 ± 0.09	$8.06 \pm 0.76^{**}$	$5.79 \pm 0.25^{**\delta}$	$6.15 \pm 0.38^{**\gamma}$	$5.06 \pm 0.24^{**\gamma}$
ALP	10.90 ± 1.07	$21.85 \pm 1.52^{*}$	$11.61 \pm 1.25^{NS\alpha}$	$14.0 \pm 0.37^{*\gamma}$	$12.44 \pm 0.36^{NS\beta}$
ALT	43.12 ± 1.57	$89.13 \pm 7.05^{**}$	$54.72 \pm 2.30^{**\gamma}$	$65.27 \pm 2.38^{***\gamma}$	$54.88 \pm 5.97^{NS\gamma}$
AST	68.32 ± 8.08	95.87 ± 4.86^{NS}	$74.90 \pm 1.91^{NS\gamma}$	$85.72 \pm 3.89^{NS\beta}$	$73.95 \pm 2.24^{NS\gamma}$
Cholesterol	78.31 ± 6.49	$121.29 \pm 3.04^{*}$	$75.67 \pm 3.07^{NS\beta}$	$104.92 \pm 2.49^{NS\gamma}$	$85.50 \pm 4.44^{NS\alpha}$
Triglyceride	92.1 ± 4.15	$139.19 \pm 5.77^{*}$	$95.91 \pm 4.24^{NS\beta}$	$121.95 \pm 5.96^{*\gamma}$	$109.97 \pm 4.25^{*\gamma}$
Creatinine	0.18 ± 0.01	$0.33 \pm 0.01^{*}$	$0.19 \pm 0.01^{NS\gamma}$	$0.27 \pm 0.00^{**\gamma}$	$0.25 \pm 0.01^{*\beta}$
BUN	8.55 ± 1.08	$24.90 \pm 3.00^{*}$	$12.19 \pm 3.08^{NS\delta}$	$17.20 \pm 0.50^{*\gamma}$	$14.08 \pm 1.02^{*\gamma}$
Uric acid	1.77 ± 0.15	2.50 ± 0.20^{NS}	$1.90 \pm 0.12^{NS\alpha}$	$2.09 \pm 0.03^{NS\delta}$	$1.86 \pm 0.09^{NS\gamma}$

^{NS}P = nonsignificant ($P > 0.05$), $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs control. $^{\alpha}P < 0.001$, $^{\beta}P < 0.01$, $^{\gamma}P < 0.05$, and $^{\delta}P > 0.05$ compared to T1D. ACP: acid phosphatase; ALP: alanine aminotransferase; ALT: alanine transaminase; AST: aspartate transaminase; BUN: blood urea nitrogen; NOSE: *Nerium oleander* stem extract; NORE: *Nerium oleander* root extract; T1D: type 1 diabetes.

alloxanized T1D mice. This corroborates previous *in vivo* and *in vitro* report showing oleander stem and leaf hydromethanolic extract attenuates haloalkane-induced hepatotoxicity [9]. In support, the extent of lowered hepatic marker enzymes remained in accordance with improved hepatic glycogen ($r_P = -0.938$ to -0.692; $P < 0.01$ to 0.001), serum insulin ($r_P = -0.932$ to -0.751; $P < 0.01$), and glucose ($r_P = 0.976$ to 0.878; $P < 0.01$), demonstrating association of oleander mediated lower hyperglycaemic complications with lowered hepatic injury.

Preclinical and clinical evidence suggest direct association of diabetic hyperlipidaemia with progressive fatty-liver disease [21]. This was evident by significant ($P < 0.05$) 54.88% increase in total serum cholesterol and 51% in triglyceride levels (Table 1). Oleander extracts lowered serum total cholesterol (13% and $P < 0.05$; 29% and $P < 0.001$) and triglyceride (12% and 22%; $P < 0.05$) levels, indicating its hypolipidemic potentials. In support, previous reports have demonstrated that oleander floral extract limits high-fat diet induced dyslipidaemia associated with BW gain [22]. Insulin signalling is known to modulate adiposity and hepatic triglyceride deposition, leading to fatty-liver phenotype in diabetes and obesity [23]. Consequently, improvement of hyperlipidaemia by NOSE and NORE treatment likely resulted through improved insulin resistance. A positive association between lowered triglyceride and insulin resistance ($r_P = 0.654$, $P < 0.05$; $r_P = 0.66$, $P < 0.01$) further supports this view.

Diabetic nephropathy is the secondary effect of uncontrolled hyperlipidemia, impaired glycaemic homeostasis,

and metabolic dysfunction, and alloxan treatment results in interstitial nephritis and tubular atrophy [24]. Acute renal injury in the alloxanized mice was noted by elevation of creatinine (83%, $P < 0.05$), BUN (191%, $P < 0.05$), and uric acid (41%, $P > 0.05$), which serves as markers of nephrotoxicity (Table 1). After oleander treatment, the lowered nephrotoxicity markers remained correlated with lowered serum glucose and triglyceride levels, indicative of limiting hyperglycaemia and hyperlipidemia mediated lowering of diabetic nephropathy [25]. NOSE and NORE treatment lowered serum creatinine, BUN, and uric acid 18 to 24%, 31 to 43, and 16 to 26%, respectively.

Hyperglycaemia-related increased glucose-oxidation, cellular protein nonenzymatic glycation, and subsequent degradation results in free-radical generation and redox imbalance [26]. Consequently, progressive glycation of cellular cytoprotective enzymes leads to lowered antioxidative protection and consequently hypersusceptibility to the elevated oxidative stress [27]. Oleander extracts overall improved the systemic antioxidant status in the liver, kidneys, and skeletal muscle as reflected by elevated CAT and PX activities, which were otherwise 27–64% lowered ($P < 0.001$ to 0.01) in alloxanized mice (Figures 4(a) and 4(b)). Liver CAT and muscle PX activities were elevated 11.14% and 16.29% NOSE, irrespective of significant treatment effect compared to T1D group. Improvement of systemic antioxidant enzymatic activities were negatively associated with improved hypoglycaemia ($r_P = -0.927$ to -0.822; $P < 0.01$), insulin resistance ($r_P = -0.840$ to -0.544; $P < 0.01$ to 0.05), and hepatic ALT level ($r_P = -0.309$ to -0.810; $P < 0.01$), which indicates strong association of oleander-mediated improved antioxidative protection with mitigation of hypoglycaemia hepatic injury. This corroborates with previous report demonstrating significant neutralization of the major physiologically relevant intracellular O_2 and N_2 free-radicals by hydromethanolic extracts of oleander [7]. The improvement of systemic antioxidant status was further confirmed by measuring serum MDA levels. NOSE and NORE treatments lowered the MDA level 17% ($P < 0.05$) and 18% ($P < 0.01$), respectively, which was or else 42% ($P < 0.001$) higher in T1D compared to control (Figure 4(c)). High association of lowered MDA level with increase CAT and PX activities further supports oleander-mediated improved systemic antioxidant status.

Further, multivariate analysis was performed to reflect the systemic shift of metabolic and antioxidative status due to T1D and its mitigation by oleander and standard glibenclamide. In agreement to the systemic shift in the metabolic and antioxidant status, T1D mice were clustered away from the control mice (Figure 5(a)). However, treatment with Glib and oleander extracts in T1D mice resulted in a shift away from T1D and closer to control. This reflected mitigation of T1D complications by the oleander extracts. NOSE and NORE clustered in close proximity; however, NORE-treated mice remained comparatively closer to Glib group and control groups, indicating superior bioactivities of NORE compared to NOSE. Moreover, the nearness of treatment effects on individual animals were measured using Pearson distance based on similarity approach and represented using a dendrogram (Figure 5(b)). This as well confirmed the clustering patterns from PCA loading plot, demonstrating distance divergence of T1D animals (7–12) from the remaining clusters at the root. The control animals (1–6) diverge at Pearson distance 41.02, generating separate branching from the treatment groups. Further at Pearson distance 59.98, NOSE group (19–24) diverge from NOSE and Glib. This reflects the clustering patterns of PCA plot, which indicates nearness of NORE to control and glib compared to NORE. A detailed account of amalgamation steps and final partitions are provided in the Supplementary Materials.

In the present study (Figures 6(a) and 6(b)), HPLC-UV (275 nm) analysis identified 4-hydroxybenzoic acid (0.321 μg/mg dw), vanillic acid (0.307 μg/mg dw), syringic acid (0.556 μg/mg dw), ferulic acid (2.72 μg/mg dw), and myricetin (0.012 μg/mg dw) in NOSE. Similarly, vanillic acid (0.014 μg/mg dw), syringic acid (0.023 μg/mg dw), ferulic acid (0.0003 μg/mg dw), and myricetin (0.0006 μg/mg dw) were the phenolic compounds identified in NORE. Prior reports suggest that 4-hydroxybenzoic acid exerts antihyperglycaemia by improving peripheral glucose uptake without affecting insulin secretion and hepatic glycogen content [28]. Vanillic acid lowers insulin resistance and dyslipidaemia and glucose uptake in high-fat diet-fed rats [29]. Syringic acid is known to lower hyperglycaemic status in alloxanized mice by elevating plasma insulin and C-peptide levels in systemic circulation [30]. Potent antioxidant and anti-inflammatory activities of ferulic acid have been demonstrated to contributing towards lowering diabetic nephropathy [31]. Moreover, several studies have reported beneficial role of myricetin in mitigating diabetes-related complications such as hyperglycaemia, insulin resistance, hyperlipidemia, obesity, etc. [32]. Apart from the phenolic acids identified in the present study, recent studies have identified a vast array of bioactive constituents in the stem and root of oleander, several of which are well established for their potent antidiabetic activities [8, 9].

Following the trail of oleander mediated improvement of systemic antioxidant status, synergistic effects of the phytochemicals with the redox sensitive transcription factor Nrf2 which regulate intrinsic antioxidant enzymes were studied. A comprehensive molecular docking study of all the phytochemicals being out of scope of the present study, the HPLC-identified phenolic acids were only focused for direct interaction with Nrf2, which transcriptionally regulates the expression of intracellular antioxidant enzymes and its activation is associated with limiting diabetes [33]. Out of the 5 compounds, only ferulic acid, 4-hydroxybenzoic acid, and syringic acid interacted with Nrf2 with high binding energies of −5.2, −5.3, and −5.1 kcal/mol, predicting potential modulation of Nrf2 activation status (Figure 7). Indeed, pharmacological studies also demonstrated that bioactive fractions of oleander induce Nrf2 activation [34].

4. Conclusion

The phenotypic observations of the present study therefore provided a convincing evidence that support the use of oleander extracts as antidiabetic remedy in traditional medicine.

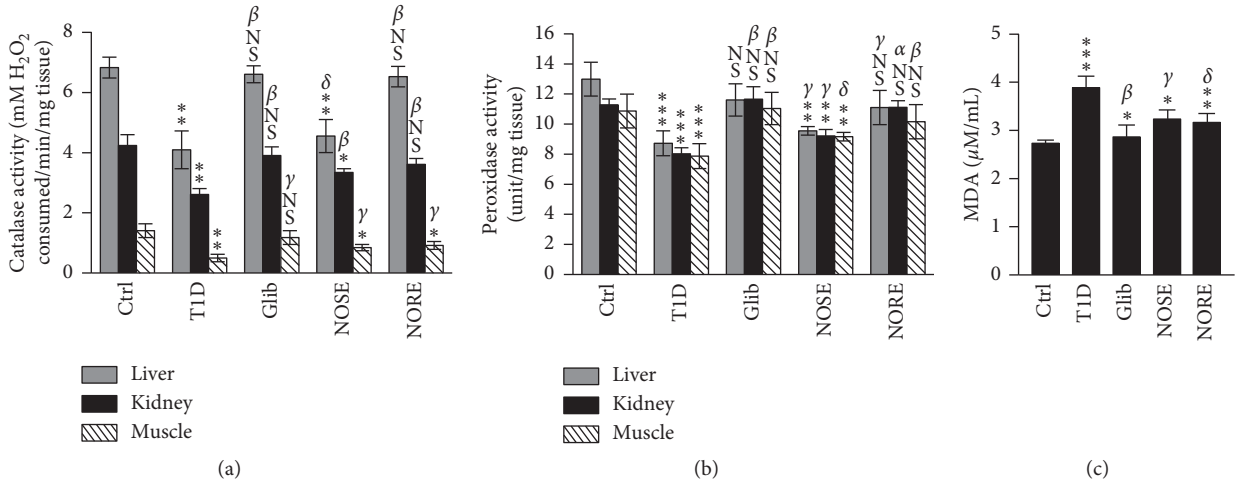


FIGURE 4: Effects of oleander extracts on intracellular antioxidant protection and systemic oxidative stress in T1D. Consecutive 20 days of treatment of alloxanized mice (glucose >200 mg/dL) with oleander stem (NOSE) and root (NORE) extracts increased the (a) catalase and (b) peroxidase activities in the primary hyperglycaemia-associated tissue of the liver, kidneys, and skeletal muscle. (c) The improved systemic antioxidant defences and lowered intracellular oxidative stress were highlighted by lowered lipid peroxidation (MDA). $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, and $^{NS}P > 0.05$ compared to control. $^{\alpha}P < 0.001$, $^{\beta}P < 0.01$, $^{\gamma}P < 0.05$, and $^{\delta}P > 0.05$ compared to T1D.

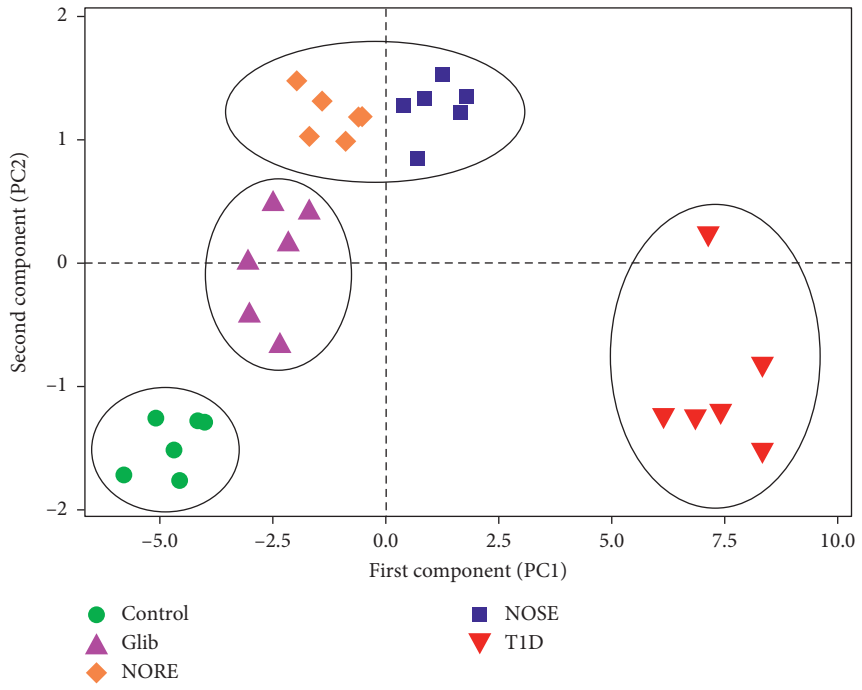
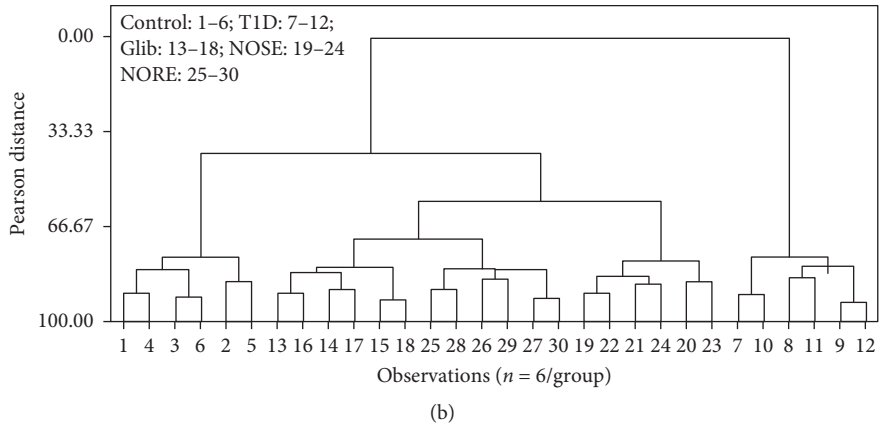
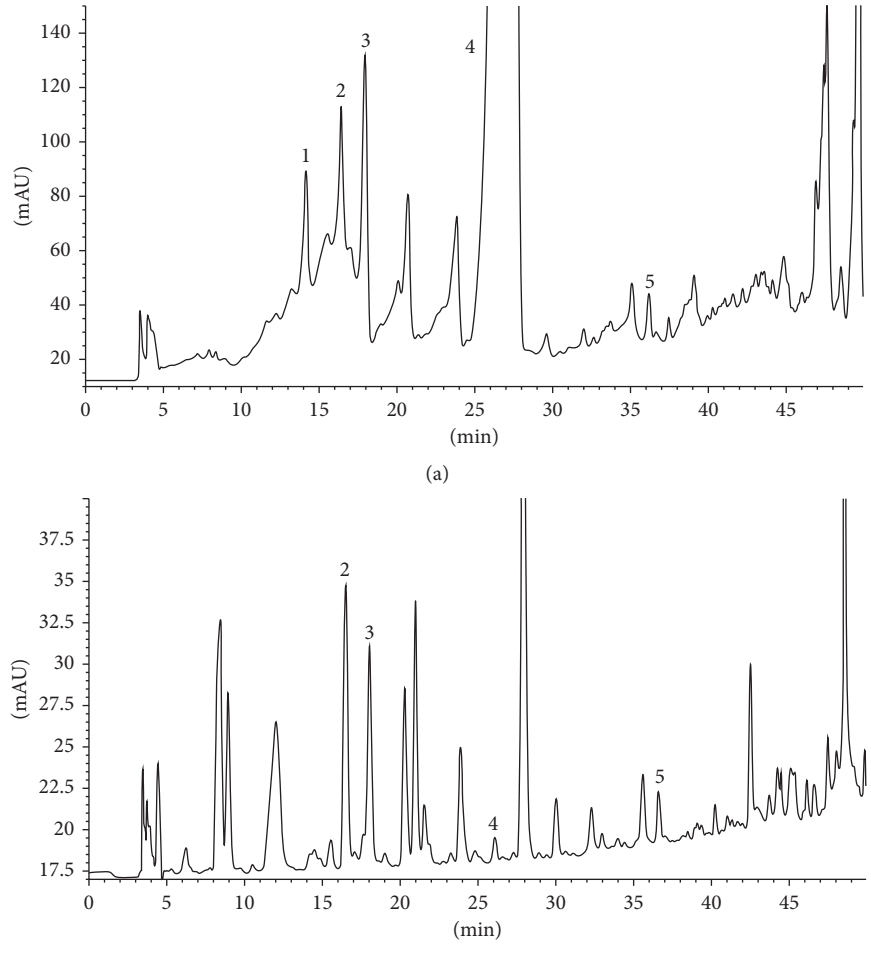


FIGURE 5: Continued.



(b)

Figure 5: Oleander treatment for 20 consecutive days improves systemic metabolic homeostasis and antioxidant status. (a) The loading plot of principal component analysis (PCA) demonstrated a drastic shift of the spatial arrangement of T1D group mice from the remaining clusters. This was brought back to normal by glibenclamide and oleander treatment, spatial arrangement of which was near to control and far from T1D group. PC1 and PC2 accounted for cumulatively 88.2% and 81.8% variance, respectively. (b) The divergence of 5 different clusters of the 5 experimental groups were measured (Supplementary Materials) and represented using a dendrogram.



(a)

(b)

Figure 6: HPLC chromatograms of NOSE and NORE. Measurements were taken at 275 nm. (a) Chromatogram of NOSE and (b) chromatogram of NORE. Peak represents (1) 4-hydroxybenzoic acid; (2) vanillic acid; (3) syringic acid; (4) ferulic acid; (5) myricetin. Chromatogram of reference standards is represented in Supplementary Figure 1.

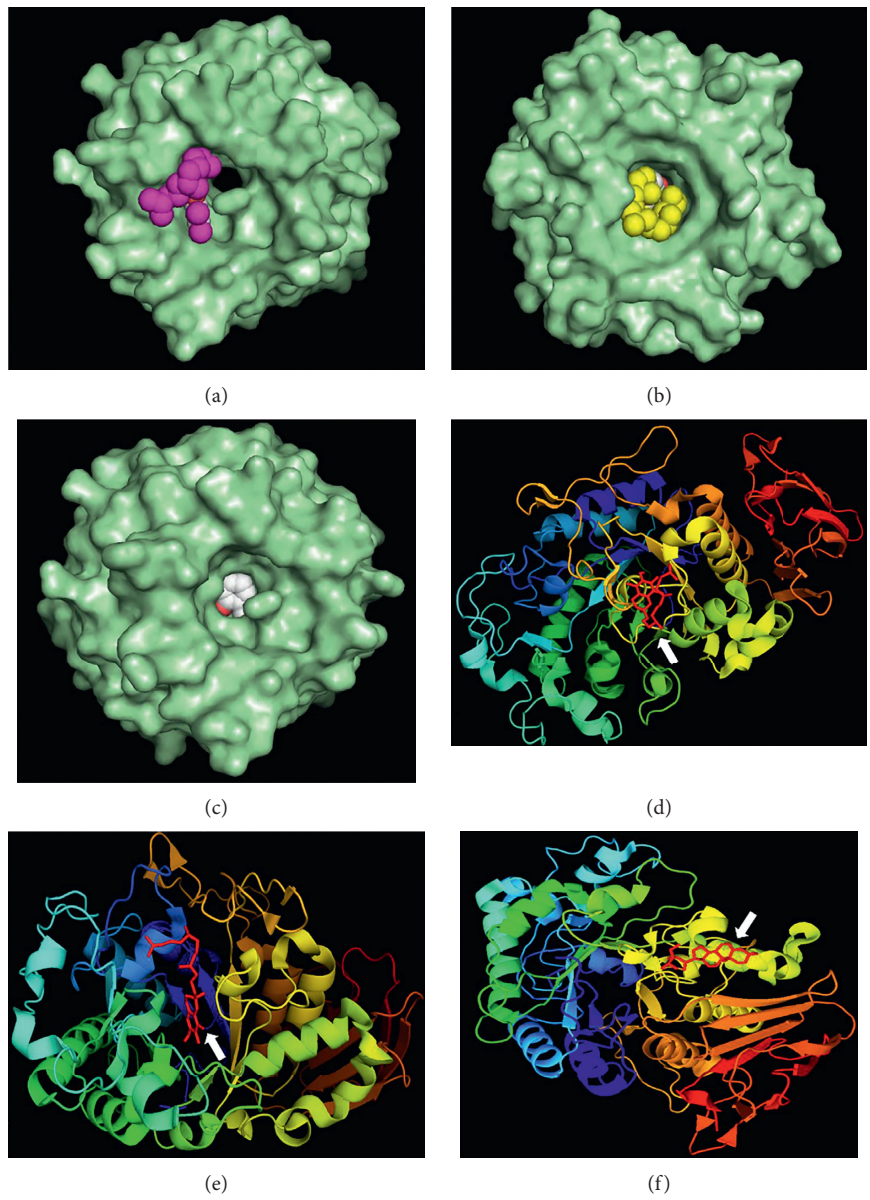


Figure 7: Molecular interaction modelling of HPLC-identified compounds with cytoprotective transcription factor Nrf2. (a, d) 4-hydroxy benzoic acid; (b, e) ferulic acid; (c, f) syringic acid.

NOSE and NORE not only limit acute hyperglycaemia and insulin resistance but also attenuates diabetes associated complications such as dyslipidaemia and hepatic and renal injury. Although the molecular mechanism contributing to these pharmacological effects is out of the scope of the present study, indeed data implicate that oleander-induced improvement of systemic antioxidant status is likely responsible for the potent antiiabetic activity. These data collectively indicate the need of further pharmacognostic investigations to identify the additive benefits of the bioactive phytochemicals in oleander.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval

The animal study protocol was verified and approved by the Ethical Committee of University of North Bengal.

Conflicts of Interest

None of the authors express any conflicts of interest.

Acknowledgments

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Chaudhuri affirms that Biswajit Halder contributed to the paper and vouches for Biswajit Halder's coauthorship status.

Supplementary Materials

Supplementary Figure 1: HPLC chromatogram of reference standards of phenolic acids and flavonoids. 1: 4-hydroxy benzoic acid (RT: 14.044), 2: vanillic acid (RT: 16.327), 3: syringic acid (RT: 17.873), 4: *p*-coumaric acid (RT: 23.767), 5: ferulic acid (RT: 25.705), 6: rutin (RT: 32.489), 7: myricetin (RT: 36.144), and 8: quercetin (42.217). (*Supplementary Materials*)

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Research Article

Total Phenolic Content and Antioxidant and Antibacterial Activities of *Pereskia bleo*

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Different solvent extracts of *Pereskia bleo* leaves were evaluated for total phenolic content (TPC) and antioxidant activities based on the Folin–Ciocalteu test and DPPH scavenging activities. The antibacterial activities against four bacteria, namely, Gram-positive bacteria: *Streptococcus pyogenes* ATCC 19615 (SP) and *Staphylococcus aureus* ATCC 29737 (SA) and Gram-negative bacteria: *Escherichia coli* ATCC 10536 (EC) and *Pseudomonas aeruginosa* ATCC 9027 (PA), were also performed based on the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays. The findings demonstrated that both the methanolic and chloroform extracts displayed strong activities against SA, SP, EC, and PA while the hexane extract demonstrated the weakest activities towards all the four bacteria. The methanolic extract also exhibited higher TPC and possessed higher antioxidant activity with the IC_{50} value 33.83 μg/mL compared to the chloroform and hexane extracts. As such, the methanolic extract has a higher ability to scavenge free radical compared to other extracts. Due to the interesting result, activities are shown by the methanolic and chloroform crude extracts of *P. bleo*; hence, the study has been extended to the isolation of bioactive compounds to uncover its great potential as a natural source for antibacterial and antioxidant agents.

1. Introduction

Medicinal plants have been used to promote human health, and it has been documented since ancient times. Even though accessibility to modern healthcare nowadays become faster and easier, still there are people who prefer to promote health by using fresh medicinal plants which are locally grown. For example, in the way to compliment the allopathic medicine, Malays prefer Jamu medicine and Chinese prefer Traditional Chinese medicine (TCM) while Indians prefer Ayurvedic medicine. Therefore, even though there is an abundance of modern medicine in the market, traditional medicine still maintained its popularity in the developing world.

P. bleo is well known as the medicinal plant of the Cactaceae family, which consists of 100 genera and about 2000 species [1]. The origin of the genus *Pereskia* is from South America, and they have been cultivated in tropical countries [2] such as Malaysia, Indonesia, Singapore, and India. Some *Pereskia* species are used as traditional medicines to treat hypertension, diabetes, cancer, high blood pressure, skin inflammation, and skin injuries. Previous reports revealed that the preparation methods of this plant varied subject to the uses. For example, a decoction from the leaves is prepared and then used as a warm bath to alleviate muscle ache while the leaves are boiled for making tea and then drinking it warm or cold for prevention of cancer and detoxification of the body.

Previous phytochemical studies of *Pereskia* genus have revealed the occurrence of a variety of compounds including carotenoids, alkaloids, flavonoid, lactone, sterols, terpenoids, fatty acids, phytosterol glycoside, and phenolic compounds [1]. Besides, studies of *Pereskia* species on its biological activities have been reported on antioxidant, anticancer, antinociceptive, and antibacterial. Moreover, previous phytochemical studies of *Pereskia* genus have revealed the occurrence of a variety of compounds. Although there are many reviews on the traditional uses for a variety of

prophylactic and therapeutic purposes, only few *Pereskia* species have been investigated on its phytochemical and biological activities. Therefore, *P. bleo* is selected to further study on its biological activities.

The intention of this study is to explore the biological activities of the *P. bleo* crude extract in exploring the potential of natural sources for the drug discovery which can be used for further innovation development of pharmaceutical, medicinal, health, and household products in order to increase its commercial values. Studies on medicinal plants not only discover new therapeutics but also assist in setting appropriate guidelines and policy in the usage of the traditional herbal medicine since there are scientific evidence and proper understanding of traditional usage of the plants available.

2. Materials and Methods

2.1. Plant Material. The studied plant sample which is the leaves of *P. bleo* was collected from Perlis, Malaysia. The sample was air-dried at a room with room temperature and good air ventilation. A dried sample was cut into smaller pieces to make it easier for it to be grounded using an electric cutting mill to produce fine powder sample.

2.2. Extraction. The air-dried leaves (926 g) of *P. bleo* were extracted at 24 hours interval by the cold extraction method, and the process was repeated twice using methanol as a solvent. Solvents removal under diminished pressure using a BUCHI model R-200 rotavapor produces 39.1371 g dark-green methanol crude extracts. Chlorophyll was removed from the methanol extract before being performed the fractionation. The fractionation process which was performed using liquid-liquid partition have yielded 0.334 g green hexane fraction, 5.8916 g dark-yellow chloroform fraction, and 25.3989 g dark-brown methanol fraction.

2.3. Total Phenolic Content. The Folin–Ciocalteu test was chosen to measure TPC of *P. bleo* extracts. This test was performed by referring to the method developed by Velioglu et al. [3] with some modifications. The crude sample was prepared by liquefying 10 mg of the extract in 10 mL of the solvent to yield a concentration of 1 mg/mL. About 100 μL of the extract (1 mg/mL) was combined and mixed with 0.75 mL of the Folin–Ciocalteu reagent (diluted 10-fold with deionized water previously) in the test tube. The liquid mixture was allowed to stand for 5 minutes at a room temperature. The mixture was then added about 0.75 mL of sodium carbonate (Na_2CO_3), and the test tube was shaken gently to mix them. After 90 minutes, the absorbance of the mixture was measured using the UV-Vis spectrophotometer at 725 nm.

A calibration curve of standard reference was established using gallic acid (range of concentration from 0.01 to 0.05 mg/mL) as standard references plotted. TPC was revealed as gallic acid equivalents in milligrams per 100g of the extract.

2.4. Antioxidant Activity. The DPPH was used to determine free radical scavenging activity as previously described by Shimada et al. [4]. About 3.94 mg of DPPH was first dissolved in 100 mL of ethanol to a concentration of 0.1 mL. About 1 mL of DPPH solution was added to 3 mL of the samples with different concentrations (250, 125, 62.5, 31.25, and 15.62 μg/mL).

For the control test, the same amount of ethanol was added. All the mixture was mixed well by shaking vigorously and left to stand for 30 minutes at a room temperature. After that, the UV-Vis spectrophotometer was used to measure the value of absorbance of each mixture at 517 nm. The calculation for the percentage of inhibition (I%) of the DPPH radical is as follows:

$$I\% = \left[\left(\frac{A_o - A_s}{A_o}\right)\right] \times 100\%, \quad (1)$$

where A_S represents the absorbance value of the sample while A_0 represents the absorbance value of the control reaction (contain all reagents except the sample). A graph of inhibition percentages (I%) against concentrations of the sample was plotted. From the graph, 50% inhibition (IC_{50} value) provides the value of concentrations for each sample. All experiments were carried out in triplicate to minimize the precision error. Mean ± SD of triplicates was reported as IC_{50} values.

2.5. Antimicrobial Activity. Antimicrobial activities were conducted using MIC and MBC assays. The MIC method as described by Gulluce et al. [5] involves the broth microdilution technique using 96-well microplates. In this study, four types of bacteria are used which are SP, SA, EC, and PA.

2.6. Minimum Inhibitory Concentration. The MIC represents the lowest concentration that had absence of microscopically noticeable growth. The interpretation of *in vitro* data is based on attainable sample concentrations. Nutrient broth (NB) was prepared as the medium. About 3.6 mg crude extract samples were weighted and dissolved in 2 mL DMSO (stock solution, 1800 μg/mL). Inoculations of the microbial strains were produced from 24 hours broth cultures and suspensions regulated to 0.5 McFarland standard turbidity. About 100 μg/mL sterile NB was added into 96-well plate in rows B to H. After that, about 100 μg/mL of the stock solution was added into rows A and B. Well-mixed mixture of sample and NB at row B were shifted to each well in order to achieve a two-fold serial dilution of samples (1800, 900, 450, 225, 112.5, 56.25, 28.13, and 14.07 μg/mL). Lastly, about 100 μg/mL incubated bacteria were added to all wells (from A to H). The 96-well plate was capped with a lid, airtight, and incubated for 24 hours at 37°C. The microbial growth was recognized by turbidity and the presence of pallet at the base of the wells.

2.7. Minimum Bactericidal Concentration. MBC was used to reconfirm the results of MIC by determining the number of the surviving organism through observing the growth of the bacteria. MBC represents the concentration at which 99% of the bacteria were killed. About 10 μg/mL of the solution in the 96-well plate (from MIC analysis) at the first clear stage

was transferred on the Petri dish containing nutrient agar (NA) using micropipette and spread using sterilized cotton bud. The Petri dish was incubated for 24 hours at 37°C. If the NA inside the Petri dish is clear during observation, meaning no bacteria growth, so the MBC result is the same as the MIC result. Meanwhile, if the NA turns cloudy or not clear, the MBC result showed that concentration is one step higher than MIC. The agar was prepared by dissolving NA powder (20 g/L) in distilled water and then mixed. The mixture was autoclave at 121°C for 15 minutes. When the NA solution is warm enough, the media is poured aseptically into Petri dishes.

3. Results and Discussion

3.1. Total Phenolic Content. TPC activity is the process to figure out the amount of phenolic content in the samples. Phenolic compounds that contained in the plants have redox properties, and the properties allow them acting as antioxidants [6, 7]. The results (Table 1) showed that the methanolic extract exhibited higher TPC as compared to the chloroform and hexane extracts which are approximately about 40.82 mg GAE/g for methanolic extract, 31.91 mg GAE/g for chloroform extract, and 25.2 mg GAE/g for hexane extract. Higher phenolic content in the methanolic extract is responsible for bioactivity; therefore, this extract is expected to exhibit good result in antioxidant and antibacterial activities.

3.2. Antioxidant Activity. Scavenging activity of DPPH is based on one-electron reduction which represents the free radical reducing activity of antioxidants. Ascorbic acid (AA) and quercetin (Qc) were used as positive control. All the samples were run in triplicates. The results (Figure 1) showed percentage inhibition of DPPH radical of the *Pereskia bleo* crude extract and standard at different concentrations. The lowest IC_{50} was detected in the methanolic extract (PbM) followed by hexane extract (PbH) and chloroform extract (PbC) with IC_{50} values of 33.83 μg/mL, 143.55 μg/mL, and 379.41 μg/mL, respectively. As the lower IC_{50} value possesses a higher antioxidant activity, the methanol extract has a higher ability to scavenge free radical compared to hexane and chloroform extracts. High antioxidant activity showed by the methanolic extract has a positive relationship with TPC activity, where high TPC gives a high antioxidant capacity due to the linear correlation between the two parameters. Previous studies have shown that the capacity of the antioxidant is highly associated with the total flavonoid content and total phenolic compounds of the plant leaves' crude extract [8–10]. Finding from this study was supported by the findings reported by Sharif et al. [11], where the leaves of the *P. bleo* methanol extract showed the lowest IC_{50} with a value of 68.75 μg/mL. The study also reported the presence of high amounts of phytol, fatty acid, and sterols in the methanolic extract.

3.3. Antimicrobial Activity. Antimicrobial activities of *P. bleo* extracts against all four tested bacteria ,namely, *Staphylococcus aureus* (SA), *Streptococcus pyogenes* (SP), *Pseudomonas aeruginosa* (PA), and *Escherichia coli* (EC) were qualitatively and quantitatively appraised by the presence or absence of bacteria based on minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays. Based on the MIC values, the methanolic extract displayed strong activities towards Gram-positive bacteria, SA and SP with the MIC value 225 μg/mL, as well as that of against Gram-negative bacteria, PA and EC with the MIC value 450 μg/mL. In addition, the chloroform extract also exhibited strong activities against SA, SP, and EC with the MIC value 225 μg/mL, as well as that of against PA with the MIC value 450 μg/mL. However, the hexane extract demonstrated weak activities towards all four bacteria with the MIC value 1800 μg/mL (Table 2).

Table 1: Total phenolic content of *P. bleo* hexane, chloroform, and methanolic extracts.

Sample extract	Total phenolic content (mg GAE/g extract)
Hexane	25.20 ± 0.01
Chloroform	31.91 ± 0.01
Methanolic	40.82 ± 0.01

The evaluation of antibacterial activity was extended to MBC. From the result, the MBC values were confirmed by the absence of bacterial growth on the nutrients agar streaked from the lowest clear MIC values. Both methanolic and chloroform extracts of *P. bleo* showed strong inhibition activity towards the tested bacteria SA, SP, PA, and EC. Most of the bacterial strains used in this study are related to the food spoilage. For example, SA considered as one of the most common sources of food-borne disease while EC and PA produce toxins that induce human gastroenteritis diseases. Moreover, antibacterial activities studied by Abbdewahab [12] against two bacteria which are *Salmonella choleraesuis* and *Pseudomonas aeruginosa* has reported that methanol and hexane extracts of *P. bleo* show positive result in inhibiting both bacteria. The dichloromethane extract of *P. bleo* also demonstrated a good antibacterial effect against *Staphylococcus aureus* [2, 12]. Besides antibacterial, methanol leaves' extract of *P. bleo* also show great antifungal activity against a plant pathogenic fungus, *Cladosporium cucumerinum* [13]. As the methanolic and chloroform extracts of *P. bleo* showed strong inhibition activity towards the tested bacteria, the present study suggested that this plant extract could have a great potential to be used as food poisoning control and natural preservatives in preserving food for replacing chemical preservative.

4. Conclusions

The methanolic and chloroform extracts of *P. bleo* showed strong inhibition activity towards the tested bacteria SA, SP, PA, and EC. The methanolic extract exhibited highest TPC (40.82 mg GAE/g) and possess highest antioxidant activity (IC_{50} value 33.83 μg/mL) compared to chloroform and hexane extracts. Linear correlation among two parameters of the high TPC value which will give great antioxidant capacity are proved in this study where high antioxidant

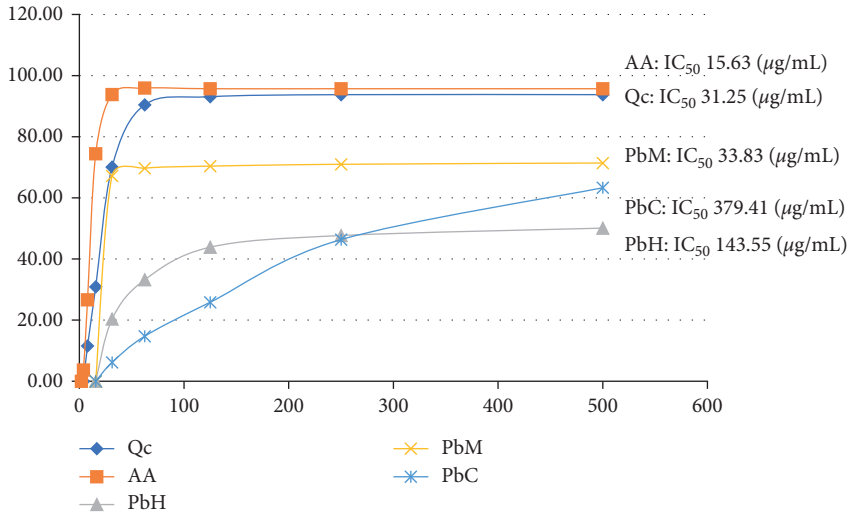

Figure 1: Graph for percent inhibition of DPPH radical of *P. bleo* crude extract and standard at different concentrations.

Table 2: Inhibitory concentration of MIC activity for crude extracts of *Pereskia bleo*.

Sample extract	SA	SP	PA	EC
Hexane	1800	1800	1800	1800
Chloroform	225	225	450	225
Methanolic	225	225	450	450

Note. SA, *Staphylococcus aureus*; SP, *Streptococcus pyogenes*; PA, *Pseudomonas aeruginosa*; EC, *Escherichia coli* (unit in μg/mL).

activity demonstrated by the methanolic extract is backed by the high TPC value. Due to the interesting results exhibited by the methanolic and chloroform extracts of *P. bleo*, the study has extended to the isolation of bioactive compounds to uncover its great potential natural source for antibacterial and antioxidant agents.

Data Availability

No data were used to support this study.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Research Article

Evaluation of Novel 3-Hydroxyflavone Analogues as HDAC Inhibitors against Colorectal Cancer

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Alteration of epigenetic enzymes is associated with the pathophysiology of colon cancer with an overexpression of histone deacetylase 8 (HDAC8) enzyme in this tissue. Numerous reports suggest that targeting HDAC8 is a viable strategy for developing new anticancer drugs. Flavonols provide a rich source of molecules that are effective against cancer; however, their clinical use is limited. The present study investigated the potential of quercetin and synthetic 3-hydroxyflavone analogues to inhibit HDAC8 enzyme and evaluated their anticancer property. Synthesis of the analogues was carried out, and cytotoxicity was determined using MTT assay. Nonspecific and specific HDAC enzyme inhibition assays were performed followed by the expression studies of target proteins. Induction of apoptosis was studied through annexin V and caspase 3/7 activation assay. Furthermore, the analogues were assessed against *in vivo* colorectal cancer. Among the synthesized analogues, QMJ-2 and QMJ-5 were cytotoxic against HCT116 cells with an IC_{50} value of 68 ± 2.3 and 27.4 ± 1.8 μM, respectively. They inhibited HDAC enzyme in HCT116 cells at an IC_{50} value of 181.7 ± 22.04 and 70.2 ± 4.3 μM, respectively, and inhibited human HDAC8 and 1 enzyme at an IC_{50} value of <50 μM with QMJ-5 having greater specificity towards HDAC8. A reduction in the expression of HDAC8 and an increase in acetyl H3K9 expression were observed with the synthesized analogues. Both QMJ-2 and QMJ-5 treatment induced apoptosis through the activation of caspase 3/7 evident from 55.70% and 83.55% apoptotic cells, respectively. *In vivo* studies revealed a significant decrease in colon weight to length ratio in QMJ-2 and QMJ-5 treatment groups compared to DMH control. Furthermore, a reduction in aberrant crypt foci formation was observed in the treatment groups. The present study demonstrated the potential of novel 3-hydroxyflavone analogues as HDAC8 inhibitors with anticancer property against colorectal cancer.

1. Introduction

Cancer is a multifactorial disease and the second largest cause of death globally [1]. Over the years, knowledge about the pathophysiology of cancer has increased radically owing to the emergence of drug resistance and through the identification of newer hallmarks of cancer. Genetic predisposition was thought be one of the prime concerns in understanding the etiological background of cancer; however, the treatment modalities were still not successful based on either biochemically mediated or targeting genetically predisposed factors. This has created an arousal of interest in the field of epigenetic research. Researchers are earnestly attempting to develop newer therapies for cancer to improve the quality of life of patients. To support the continuous efforts made in developing anticancer agents, epidemiological survey shows that the overall mortality rate caused due to cancer has decreased by 25% from 1991 to 2014 [2].

Cancer is categorized into various types among which colorectal cancer (CRC) is the third most lethal malignancy diagnosed worldwide. The incidence of CRC is anticipated to increase by 60% globally with a mortality of 1.1 million by

2030 [3]. CRC is classified into four different stages, and the survival rate is solely dependent on the stage of diagnosis [4]. Patients who are diagnosed in the early stages (Stages I and II) have a 5-year survival rate of 90%, whereas patients who are diagnosed in the late stages (Stages III and IV) of CRC have a survival rate of 13.1% [5]. There are numerous risk factors associated with CRC that includes familial history, inherited genetic mutations, and lifestyle factors [6]. It is also noteworthy to mention the role of epigenetics in CRC. Studies have shown that epigenetic alterations play a crucial role in the transformation of normal colon epithelial cells into adenomatous polyps [7]. The epigenetic enzyme histone deacetylase (HDAC) has emerged out as an important regulator in the maturation and transformation of colon cells with several HDACs being upregulated in colon tumors [8]. Among the HDAC enzymes, expression of HDAC8 is highly prevalent in colon cancer where its expression level is higher compared to healthy tissues [9]. Studies have shown that knockdown of HDAC8 inhibits the proliferation of various cancer cells including colon cancer cells [10]. Although HDAC8 belongs to class I HDACs, unlike other members, HDAC8 functions as a deacetylase without being a part of multiprotein repressive complex making it a druggable target. Since the currently available chemotherapeutic agents for CRC has their own limitations, there is a scope of developing new drug candidates which could act as an alternative from the existing therapy.

Natural products are one of the most efficient and productive sources of lead molecules for the development of novel drugs. Among them, chalcones and polyphenols are some of the most widely studied molecules as anticancer agents [11]. Flavonoids, a distinct class of polyphenolic compounds ubiquitously present in plants, serve as phytonutrients and protect us against various forms of cancer [12]. Among the flavonoids, quercetin, a naturally occurring polyhydroxylated flavonol, has been found to exhibit broad spectrum of biological activities [13]. The presence of C2=C3 double bond and the 3-OH group in quercetin is highly decisive for its antitumor properties [14]. Studies have reported the potential of quercetin in eliciting epigenetic changes through the alteration of DNA methylation and histone acetylation levels [15]. However, the ability of quercetin in inhibiting HDAC8 in colon cancer has not been reported. Since flavonols have less adverse effect than chemotherapeutic drugs, they could provide better therapeutic options. Thus, in the present study, the polyphenol quercetin and a series of synthetic 3-hydroxyflavone analogues were evaluated for their *in vitro* HDAC8 inhibition and anticancer potential along with *in vivo* antitumor effects against colorectal cancer.

2. Materials and Methods

2.1. Chemicals and Instruments. Starting materials utilized in the synthesis of 3-hydroxyflavone analogues were procured from Sigma-Aldrich Co. LLC, St. Louis, MO, USA; Spectrochem Pvt. Ltd., Mumbai, MH, India; Merck Specialities Pvt. Ltd, Mumbai, MH, India; and TCI Co. Ltd., Tokyo, Japan. Dulbecco's Modified Eagle's Medium (DMEM), phosphatase inhibitor cocktail, protease inhibitor cocktail, Nonidet-P 40 (NP-40), BOC-Ac-Lys-AMC substrate, SAHA, and cell lysis buffer were obtained from Sigma-Aldrich Co. LLC, St. Louis, MO, USA. HDAC1 and HDAC6 enzymes were procured from Enzo Life Sciences, Inc. USA. Fetal bovine serum (FBS) was procured from Invitrogen Bio-Services Pvt. Ltd., Bangalore, India. 5-Fluorouracil was procured from Biochem Pharmaceutical Industries Ltd., Mumbai, India. 1,2-Dimethylhydrazine (DMH) was procured from TCI Chemicals (India) Pvt. Ltd. Buffers and chemicals for western blotting were obtained from Bio-Rad Laboratories Inc., Hercules, CA, USA. Primary antibodies, HDAC8 was obtained from Santa Cruz Biotechnology Inc. USA; $p21^{Waf1/Cip1}$ was obtained from Cell Signaling Technology Inc., Danvers, USA; acetyl histone H3[Ac-Lys^{9}] and GAPDH were obtained from Sigma-Aldrich Co. LLC, USA. Determination of the melting point was achieved using the capillary melting point apparatus from Toshniwal Systems and Instruments Pvt. Ltd., Chennai, TN, India. Thin layer chromatography was performed on precoated silica gel plates (Merck #60F254) and was visualized using a UV light source (254 or 366 nm) and/or iodine vapor. IR spectrophotometer (FTIR-8300, Shimadzu Co., Kyoto, Japan) was used to record the IR spectra utilizing KBr pellets. LC-MS (ESI) (LCMS-210A, Shimadzu Co., Kyoto, Japan) was used to record the mass spectra. ^{1}H and ^{13}C NMR spectra were recorded at 400 MHz (Ascend 400, Bruker Biosciences Corporation, Billerica, MA, USA) utilizing DMSO (D6) as a solvent. δ values (ppm) were used to report the chemical shifts and the signal multiplicities were represented by s (singlet), d (doublet), t (triplet), and m (multiplet).

2.2. Synthesis

2.2.1. General Procedure for the Synthesis of (2E)-1-(2-Hydroxyphenyl)-3-(4-methylphenyl)prop-2-en-1-ones. Synthesis of chalcone intermediates was carried out using a previously described method with modifications [16, 17]. In brief, a solution of 2-hydroxyacetophenone (5 mM) in 10 ml methanol was mixed with 40% w/v aqueous potassium hydroxide (KOH) (10 ml), and the reaction was stirred for 20 min. To this reaction mixture, 4-methylbenzaldehyde (5 mM) was added and the reaction was stirred for 12 h or till the completion of a starting material. Furthermore, the mixture was poured on ice cold water and was neutralized using concentrated hydrochloric acid to obtain solid precipitate. The precipitate was then filtered, washed with ice cold water, and dried. The dried product was recrystallized from methanol to obtain the desired chalcone.

2.2.2. General Procedure for the Synthesis of 3-Hydroxy-2-(4-methylphenyl)-4H-1-benzopyran-4-ones. Cyclization of chalcones into 3-hydroxyflavones was carried out using a previously described method with modifications [16, 17]. In brief, the chalcone intermediate (1 mM) was added in 5 ml of methanol to which 10% w/v aqueous potassium hydroxide (5 ml) was added and stirred for 20 min under ice cold condition. Next, 15% v/v hydrogen peroxide (H_2O_2) (2 ml) was added carefully into the reaction mixture, and stirring was

continued for another 12 h. The reaction mixture was then poured on to ice cold water and neutralized using concentrated hydrochloric acid to obtain the final precipitate. It was then filtered, washed with ice cold water, and dried. The precipitate was recrystallized from methanol to obtain the final product. The synthetic scheme is shown in Figure 1.

2.3. Physical Parameters and Spectral Data for the Synthesized Analogues

2.3.1. QMJ-1: 2-[4(Dimethylamino)phenyl]-3-hydroxy-4H-1-benzopyran-4-one. Yield = 76%, m. p. 270 ± 1°C uncorrected; IR (KBr): 3305.99 (-OH, Str), 1606.7 (C=O, Str), 1118.71 (C-O-C, Str), and 1070.4 (N-C, Str) cm^{-1}; ^{1}H NMR (DMSO-d6); δ = 3.022 (s, 6H, N-C2H6), 6.842 (d, 2H, Ar), 7.425 (t, 1H, Ar), 7.716 (m, 2H, Ar), 8.081 (m, 3H, Ar), and 9.188 (s, 1H, -OH) ppm. ^{13}C NMR (DMSO-d6); δ = 111.88, 118.39, 118.61, 121.91, 124.77, 125.09, 129.43, 133.56, 137.73, 147.28, 151.50, 154.74, and 172.41 ppm. MS: *m/z* 281.

2.3.2. QMJ-2: 3-Hydroxy-2-(4-methylphenyl)-4H-1-benzopyran-4-one. Yield = 72%, m. p. 250 ± 1°C uncorrected; IR (KBr): 3284.77 (-OH, Str), 1610.56 (C=O, Str), 1116.78 (C-O-C, Str), and 2916.37 (C-H, Str) cm^{-1}; ^{1}H NMR (DMSO-d6); δ = 2.395 (s, 3H, -CH3), 7.374 (t, 2H, Ar), 7.473 (d, 1H, Ar), 7.751 (m, 2H, Ar), 8.114 (t, 3H, Ar), and 9.555 (s, 1H, -OH) ppm; ^{13}C NMR (DMSO-d6); δ = 21.52, 145.89, 139.24, 173.3, 124.99, 125.24, 134.11, 118.86, 154.99, 121.78, 128.98, 128.05, 129.61, and 140.27 ppm. MS: *m/z* 251.

2.3.3. QMJ-3: 2-[4-(Dimethylamino)phenyl]-3-hydroxy-6-methyl-4H-1-benzopyran-4-one. Yield = 63%, m. p. 206 ± 2°C uncorrected; IR (KBr); 3269.34 (-OH, Str), 1600.92 (C=O, Str), 1168.86 (C-O-C, Str), 1058.92 (N-C, Str), and 2900.94 (C-H, Str) cm^{-1}; ^{1}H NMR (DMSO-d6); δ = 2.438 (d, 3H, -CH3), 3.017 (s, 6H, N-C2H6), 6.836 (d, 2H, Ar), 7.582 (d, 2H, Ar), 7.868 (s, 1H, Ar), 8.105 (d, 2H, Ar), and 9.106 (s, 1H, -OH) ppm. ^{13}C NMR (DMSO-d6); δ = 111.87, 118.43, 121.60, 124.25, 129.37, 134.19, 134.78, 137.69, 147.10, 151.45, 153.09, and 172.33 ppm. MS (ESI): *m/z* (M−1) 294.00.

2.3.4. QMJ-4: 3-Hydroxy-6-methyl-2-(4-methylphenyl)-4H-1-benzopyran-4-one. Yield = 61%, m. p. 182 ± 1°C uncorrected; IR (KBr); 3255.84 (-OH, Str), 1608.63 (C=O, Str), 1174.65 (C-O-C, Str), and 2918.30 (C-H, Str) cm^{-1}; ^{1}H NMR (DMSO-d6); δ = 2.395 (s, 3H, -CH3), 2.450 (s, 3H, -CH3), 7.372 (d, 2H, Ar), 7.630 (d, 2H, Ar), 7.900 (s, 1H, Ar), 8.129 (d, 2H, Ar), and 9.480 (s, 1H, -OH) ppm. ^{13}C NMR (DMSO-d6); 20.91, 21.51, 118.68, 121.50, 124.31, 128.02, 129.06, 129.62, 134.50, 135.40, 139.18, 140.21, 145.75, 153.37, and 173.24 ppm. MS (ESI): *m/z* (M−1) 265.

2.3.5. QMJ-5: 3-Hydroxy-7-methyl-2-(4-methylphenyl)-4H-1-benzopyran-4-one. Yield = 81%, m. p. 145 ± 1°C uncorrected; IR (KBr); 3261.63 (-OH, Str), 1610.56 (C=O, Str), 1166.93 (C-O-C, Str), and 2918.30 (C-H, Str) cm^{-1}; ^{1}H NMR (DMSO-d6); δ = 2.394 (s, 3H, -CH3), 2.481 (s, 3H, -CH3), 7.286 (d, 1H, Ar), 7.373 (d, 2H, Ar), 7.576 (s, 1H, Ar), 7.986 (d, 1H, Ar), 8.110 (d, 2H, Ar), and 9.449 (s, 1H, -OH) ppm; ^{13}C NMR (DMSO-d6); δ = 21.51, 21.76, 145.46, 140.14, 173.17, 125.05, 126.55, 139.08, 118.33, 155.13, 119.58, 129.07, 127.95, 129.62, and 145.04 ppm. MS (ESI): *m/z* (M−1) 265.25.

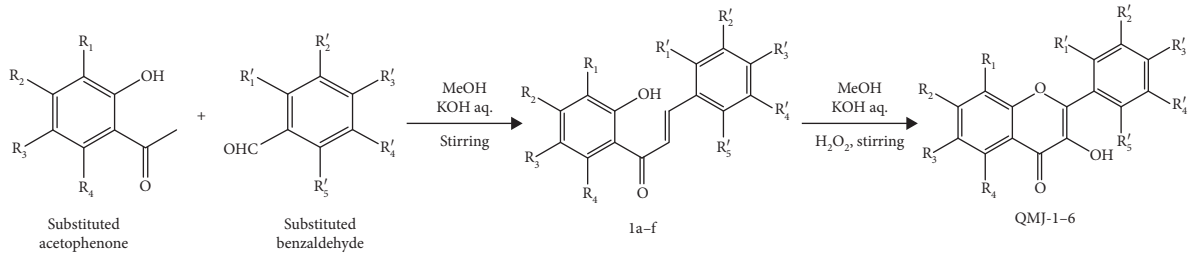
2.3.6. QMJ-6: 3-Hydroxy-7-methoxy-2-(4-methylphenyl)-4H-1-benzopyran-4-one. Yield = 72%, m. p. 195 ± 1°C uncorrected; IR (KBr); 3277.06 (-OH, Str), 1604.77 (C=O, Str), 1166.93 (C-O-C, Str), 2845.00 (-OCH_3, Str), and 2972.31 (C-H, Str) cm^{-1}; ^{1}H NMR (DMSO-d6); δ = 2.400 (s, 3H, -CH3), 3.926 (s, 3H, -OCH3), 7.043 (s, 1H, Ar), 7.063 (s, 1H, Ar), 7.374 (d, 2H, Ar), 8.002 (s, 1H, Ar), 8.150 (d, 2H, Ar), and 9.437 (s, 1H, -OH) ppm. ^{13}C NMR (DMSO-d6); δ = 21.49, 56.55, 100.74, 115.11, 115.63, 126.60, 127.79, 129.09, 129.58, 138.94, 139.97, 145.17, 156.93, 164.10, and 172.76 ppm. MS (ESI): *m/z* (M+1) 283.17.

2.4. In Vitro Studies

2.4.1. Cell Lines and Their Maintenance. Human colorectal carcinoma cell line (HCT116) and African green monkey kidney epithelial cells (VERO) were obtained from National Centre for Cell Science, Pune, MH, India. For their maintenance, the cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) which was supplemented with 10% fetal bovine serum (FBS) and 1 × penicillin/streptomycin at 37°C in CO_2 incubator (NU-5501E/G, NuAire Inc., Plymouth, MN, USA) in a humidified atmosphere of 5% CO_2 and 95% air.

2.4.2. Cytotoxicity Study. Cytotoxicity for the synthesized analogues was determined using MTT assay [18]. In brief, HCT116 and VERO cells were harvested from the tissue culture flask and seeded in sterile 96 well plates at a density of 5000 cells per well. After overnight adherence, different concentrations of test compounds were added and incubated for 48 h. Next, 50 μl of the MTT reagent (HiMedia Laboratories, Mumbai, India) (2 mg/ml in the sterile phosphate buffer saline (PBS)) was added into each well and incubated for 3 h. The formazan crystals formed were solubilized using 100% dimethyl sulfoxide (DMSO), and the optical density was measured at 540 nm using the microplate reader (ELx800, BioTek Instruments Inc., Winooski, VT, USA).

2.4.3. Whole Cell HDAC Inhibition Assay. Confluent HCT116 cells were harvested and seeded in 96 well sterile black well plates at a density of 2000 cells per well and incubated overnight. Furthermore, the cells were treated with different concentrations of test compounds for a period of 18 h followed by the addition of 15 mM BOC-Ac-Lys-AMC substrate and incubated for 1 h. The reaction was then terminated by adding 50 μl of stop solution (trypsin 2 mg/ml, 1% NP40, and 1 μl SAHA) in the HDAC assay buffer (25 mM Tris-HCl (pH 8.0), 137 mM NaCl, 2.7 mM KCl, and 1 mM $MgCl_2$). The reaction was then allowed to proceed for 15 min at 37°C. Finally, the fluorescence intensity was measured at



Substituted chalcones, 1a–f

Comp.	R_1	R_2	R_3	R_4	R'_1	R'_2	R'_3	R'_4	R'_5
1a	H	H	H	H	H	H	NC_2H_6	H	H
1b	H	H	H	H	H	H	CH_3	H	H
1c	H	H	CH_3	H	H	H	NC_2H_6	H	H
1d	H	H	CH_3	H	H	H	CH_3	H	H
1e	H	CH_3	H	H	H	H	CH_3	H	H
1f	H	OCH_3	H	H	H	H	CH_3	H	H

Quercetin analogues, QMJ-1–6

Comp.	R_1	R_2	R_3	R_4	R'_1	R'_2	R'_3	R'_4	R'_5
QMJ-1	H	H	H	H	H	H	NC_2H_6	H	H
QMJ-2	H	H	H	H	H	H	CH_3	H	H
QMJ-3	H	H	CH_3	H	H	H	NC_2H_6	H	H
QMJ-4	H	H	CH_3	H	H	H	CH_3	H	H
QMJ-5	H	CH_3	H	H	H	H	CH_3	H	H
QMJ-6	H	OCH_3	H	H	H	H	CH_3	H	H

Figure 1: Synthesis of 3-hydroxyflavone analogues was carried out by the reaction of various substituted acetophenones with substituted benzaldehyde using 40% KOH and methanol to form chalcones (1a–f). The chalcones were cyclized using 10% KOH, methanol, and 15% H_2O_2 to produce 3-hydroxyflavones (QMJ-1–6).

360 nm excitation and 460 nm emission using a fluorescence microplate reader (FLx800, BioTek Instruments Inc., Winooski, VT, USA) [19].

2.4.4. HDAC8 Enzyme Inhibition Assay. The enzymatic reaction was conducted as per Enzo Life Science, NY, USA (BML-AK518) kit protocol. In brief, 10 μl of different concentration of test compounds were added to a 40 μl reaction mixture containing the HDAC assay buffer, HDAC8 substrate, and recombinant human HDAC8 enzyme. The reactions were incubated for 20 min at 30°C. Then, 50 μl of the HDAC developer was added in each well, and the plate was incubated at room temperature for an additional 45 min. Fluorescence intensity was measured at an excitation of 360 nm and an emission of 460 nm using a fluorescence microplate reader (FLx800, BioTek Instruments Inc., Winooski, VT, USA).

2.4.5. HDAC1 and 6 Enzyme Inhibition Assays. The HDAC1 and 6 assays were carried out with few modifications from the previously described method [20]. In brief, 10 μl of different concentration of test compounds was added to 40 μl of reaction mixture containing HDAC assay buffer, HDAC substrate, and recombinant human HDAC1 and 6 enzymes. The reactions were incubated for 30 min at 37°C. Then, 50 μl of trypsin stop solution was added in each well, and the plate was incubated at room temperature for an additional 20 min. Fluorescence intensity was measured at an excitation of 360 nm and an emission of 460 nm using a fluorescence microplate reader (FLx800, BioTek Instruments Inc. Winooski, VT, USA).

2.4.6. Western Blotting. After various treatments for 24 h, cells were lysed, and protein quantification of the lysates was estimated using the BCA™ kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Proteins samples (30 μg) were resolved on a 10% SDS-PAGE and transferred onto a PVDF membrane and blocked with 5% nonfat milk in TBST (tris-buffered saline with Tween-20) buffer for 1 h followed by washing in TBST buffer. The membrane was then probed with primary antibodies (GAPDH, HDAC8, $p21^{Waf1/Cip1}$, and acetyl-histone H3[Ac-Lys^9]) and incubated overnight at 4°C. The membrane was washed with TBST buffer and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Signal was captured using SuperSignal™ West Pico Plus chemiluminescent substrate (Thermo Fischer Scientific Inc., Waltham, MA, USA) on gel documentation system (G : BOX Chemi XRQ, Syngene). The protein band densities were estimated using the software ImageJ (version 1.46r, NIH, Bethesda, MD, USA) [21].

2.4.7. Annexin V Staining. Mechanism of cell death induced by synthesized analogues was determined using annexin V staining. In brief, as per annexin V flow cytometry kit protocol (#MCH100105), 1×10^6 cells were seeded in a cell culture dish and after overnight adherence, were incubated with test compounds. Cells were then detached by trypsinization, centrifuged, and washed with PBS. Cell suspension (100 μl) was mixed with Muse annexin V reagent (100 μl) and incubated for 20 min at room temperature. The stained cells were quantitatively analyzed for live, early, and late apoptosis using Muse cell analyzer (#0500-3115 Merck Millipore).

2.4.8. Caspase 3/7 Activation Assay. The downstream signaling pathway of apoptosis was determined using the caspase 3/7 activation assay. In brief, as per the caspase 3/7 activation kit protocol (#MCH100108), 1×10^6 cells were seeded in the cell culture dish and after overnight adherence,

were incubated with the test compounds. The cells were detached by trypsinization, centrifuged, and washed with PBS. Cell suspension (50 μl) was mixed with the caspase 3/7 antibody reagent (5 μl) and incubated at 37°C temperature for 30 min. 7-AAD (7-amino-actinomycin D) working solution (150 μl) was added, and stained cells were then analyzed in Muse Cell Analyzer (#0500-3115 Merck Millipore).

2.4.9. Cell Cycle Analysis. Cell cycle analysis was performed to estimate the effect of synthesized analogues on the different phases of the cell cycle. In brief, 1×10^6 cells were seeded in the cell culture dish and allowed to attach overnight followed by incubation with test compounds. After 48 h, the cells were detached by trypsinization, centrifuged, and washed with PBS. The cell pellets were fixed in 70% v/v ice cold ethanol and stored at −20°C for 24 h. After fixing, the pellets were dislodged in PBS and stained with propidium iodide solution and analyzed using Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) [22].

2.5. In Vivo Studies

2.5.1. Animals. Animal care and handling were carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), after seeking the approval of the research proposal by institutional animal ethics committee (IAEC) (IAEC/KMC/23/2015). Male Wistar rats inbred at the central animal research facility (CARF), Manipal academy of higher education (MAHE), was used in the present study. The experimental room had a temperature of 23 ± 2°C and a humidity of 50 ± 5%. The animals were housed in sterile polypropylene cages containing sterile paddy husk and were provided with 12 h light and dark cycles.

2.5.2. Acute Toxicity Studies. The safe dose of the promising two analogues (QMJ-2 and QMJ-5) and quercetin were evaluated using Organization for Economic Cooperation and Development (OECD), 425 guidelines. Limit test was performed using 2000 mg/kg dose. Animals were observed for the first 4 h continuously and then monitored each day for a period of 14 days.

2.5.3. Preparation of the Test Compounds and Standard Drug. The two test compounds (QMJ-2 and QMJ-5) and quercetin were suspended in 0.25% w/v sodium carboxymethyl cellulose (CMC) and were administered orally (*p.o.*). 5-Fluorouracil (5-FU) which was used as a standard was procured in the form of injection and was administered intraperitonially (*i.p.*).

2.5.4. 1,2-Dimethylhydrazine-(DMH-) Induced Colon Cancer in Wistar Rats. Colon cancer in experimental animals was induced using the highly specific carcinogen DMH, with few modifications from the previously described procedure [23]. DMH was administered at a dose of 30 mg/kg *i.p.* once a week for 20 weeks. The incidence of aberrant crypt foci (ACF) and adenocarcinoma were confirmed by sacrificing few animals after 20 weeks of induction with DMH. Furthermore, the animals were randomized into five experimental groups, and various treatments were administered for a period of 21 days followed by the assessment of different parameters in the experimental animals.

(1) Experimental Groups

Group 1: animals received 0.25% CMC in water *p.o.*

Group 2: animals received 0.25% CMC in water *p.o.* (DMH control)

Group 3: animals received 5-FU at 10 mg/kg *i.p.*

Group 4: animals received quercetin at 100 mg/kg *p.o.*

Group 5: animals received QMJ-2 at 100 mg/kg *p.o.*

Group 6: animals received QMJ-5 at 100 mg/kg *p.o.*

(2) ACF Formation and Adenocarcinoma Incidence. The entire colon was excised from the experimental animals and was used to study the incidence of adenocarcinoma. The count and size were noted from each animal. For ACF formation, the distal part of the colon was excised, and the tissue was then cut open and fixed with 10% buffered formalin for 12 h. Following this, the tissue was stained with 0.1% methylene blue in PBS for 5 min. The tissues were then observed under microscope for ACF formation and were calculated as the number of counts/5 cm^2 in colon tissue.

(3) Colon Weight/Length Ratio and Organ Index. Length of the excised colon was measured in centimeters, and weight was measured in g. Colon weight/length ratio was then calculated. For the determination of organ index, major organs including spleen, liver, and kidney of the experimental animals were weighed in g and the respective index was calculated.

(4) Histopathology of Colon. Colon tissue was collected at the end of the study period from different groups and stored in 10% neutralized buffered formalin and processed for histopathological changes. The stained slides were subsequently analyzed under microscope for anatomical changes.

2.6. Statistical Analysis. Experimental data were analyzed using one-way ANOVA followed by Tukey's multiple comparison tests using Prism 5.03 (Graph Pad Software Inc., La Jolla, CA, USA). All values are expressed as mean ± SEM of 6 animals. Significance was considered at $p < 0.05$.

3. Results

3.1. In Vitro Studies

3.1.1. Cytotoxicity Study. Cytotoxic potential of the synthesized analogues was estimated using MTT assay. QMJ-1, QMJ-2, QMJ-3, QMJ-5, and QMJ-6 were found to be cytotoxic against colon cancer cells after 48 h of treatment. Among them, QMJ-2 and QMJ-5 were most cytotoxic with an IC_{50} value of 68 ± 2.3 and 27.4 ± 1.8 μM, respectively.

Table 1: MTT assay.

Sl. no.	Compound	IC_{50} (μM ± SEM)	
		HCT116	Vero
1	QMJ-1	102.5 ± 1.8	186.4 ± 4.9
2	QMJ-2	68 ± 2.3	140 ± 6.8
3	QMJ-3	70.3 ± 4.1	80.1 ± 3.7
4	QMJ-4	>400	>400
5	QMJ-5	27.4 ± 1.8	55.6 ± 3.8
6	QMJ-6	94 ± 2.1	153 ± 2.3
7	Quercetin	107.6 ± 1.2	201.5 ± 7.5
8	SAHA	3.1 ± 0.35	4.6 ± 1.6

All values are expressed as mean ± SEM of three experiments.

However, in Vero cells, the IC_{50} values of QMJ-2 and QMJ-5 was found to be 140 ± 6.8 and 55.6 ± 3.8 μM. Table 1 shows the IC_{50} value of the synthesized analogues on both cell lines.

3.1.2. Whole Cell HDAC Inhibition Assay. The ability of the synthesized analogues to inhibit the epigenetic enzyme HDAC was estimated using whole cell HDAC enzyme inhibition assay in HCT116 cells. Dose-dependent enzyme inhibition was observed in QMJ-2- and QMJ-5-treated cells with an IC_{50} value of 181.7 ± 22.04 and 70.2 ± 4.3 μM, respectively. However, the other analogues were less efficacious with higher IC_{50} values. The nonspecific HDAC inhibitor SAHA was found to have an IC_{50} value of 1.2 ± 0.1 μM. Table 2 shows the IC_{50} values obtained from this assay.

3.1.3. HDAC8, 1, and 6 Enzyme Inhibition Assays. The test compounds were evaluated for their isoform-specific HDAC enzyme inhibition. QMJ-2 and QMJ-5 were found to be specific toward class I HDAC enzyme with an IC_{50} value of 34.11 and 47.7 μM, respectively, against HDAC1 enzyme and 36.03 and 24.0 μM, respectively, against HDAC8 enzyme. The IC_{50} value of QMJ-2 and QMJ-5 was found to be 165.1 and 163.35 μM, respectively, against HDAC6 enzyme. Quercetin inhibited HDAC1 and HDAC8 with an IC_{50} value of 26.72 and 15.4 μM, respectively, and inhibited HDAC6 at an IC_{50} value of 43.39 μM. The nonspecific HDAC inhibitor SAHA had an IC_{50} value of 0.026 and 0.7 μM against HDAC1 and HDAC8 enzyme, respectively, whereas 0.098 μM against HDAC6 enzyme. Table 3 shows the results of HDAC8, 1, and 6 enzyme inhibition assays.

3.1.4. Western Blotting Analysis. Protein expression studies indicated a significant reduction in HDAC8 expression in SAHA-, QMJ-5-, and quercetin-treated groups compared to normal. Moreover, a significant increase in the expression of acetylated histone H3K9 was observed in SAHA- and QMJ-5-treated groups compared to normal control. The expression of the cell cycle regulatory protein $p21^{Waf1/Cip1}$ was also increased in SAHA-, QMJ-2-, and QMJ-5-treated groups which were significant compared to normal control. Figure 2 shows the expression of target proteins in various treatment groups along with their relative density.

Table 2: Whole cell HDAC enzyme inhibition assay.

Sl. no.	Compound	HDAC inhibition IC_{50} (μM ± SEM)
1	QMJ-1	1023.1 ± 5.2
2	QMJ-2	181.7 ± 22.04
3	QMJ-3	1124 ± 6.2
4	QMJ-4	1011.2 ± 3.4
5	QMJ-5	70.2 ± 4.3
6	QMJ-6	984.2 ± 2.5
7	Quercetin	167.8 ± 6.9
8	SAHA	1.2 ± 0.1

The values are expressed as mean ± SEM of three experiments.

Table 3: Isoform-specific HDAC enzyme inhibition assay.

Sl. no.	Compound	HDAC8	HDAC1	HDAC6
1	QMJ-2	36.03 ± 4.17	34.11 ± 3.83	165.1 ± 11.1
2	QMJ-5	24.00 ± 2.8	47.7 ± 6.3	163.35 ± 7.05
4	Quercetin	15.4 ± 1.53	26.72 ± 3.77	43.39 ± 2.17
3	SAHA	0.7 ± 0.025	0.026 ± 0.002	0.098 ± 0.012

The IC_{50} (μM) values are expressed as mean ± SEM of three experiments.

3.1.5. Annexin V Staining. Induction of apoptosis was determined by annexin V staining in HCT116 cells. It was observed that the percentage of live cells in the normal control group was 70.85%, whereas treatment with QMJ-2 and QMJ-5 decreased the percentage of live cells drastically to 57.25% and 51.35%, respectively. Furthermore, it was found that the percentage of cells undergoing late apoptosis was 35.20% and 38.65% in QMJ-2 and QMJ-5 groups, respectively, compared to that of 21.30% in the normal control group. Percentage of cells undergoing apoptosis both in SAHA- and in quercetin-treated groups were found to be 27.65% and 35.80%, respectively. Figure 3 shows the apoptotic profile of various treatment groups.

3.1.6. Caspase 3/7 Activation Assay. The activation of executioner caspase 3/7 was determined in HCT116 cells. It was found that QMJ-2- and QMJ-5-treated cells activated apoptosis through the induction of caspase 3/7. The percentage of live cells was found to be 37.40% and 8.05% in QMJ-2- and QMJ-5-treated groups when compared to 69.40% in normal control. An increase in the percentage of apoptotic cells was observed in QMJ-2- and QMJ-5-treated groups and was found to be 55.70% and 83.55%, respectively, when compared to that of 24.60% in the normal control group. Furthermore, percentage of cells undergoing apoptosis in both SAHA- and quercetin-treated group was found to be 56.30% and 59.65%, respectively. Figure 4 shows the results from the caspase 3/7 activation assay.

3.1.7. Cell Cycle Analysis. In normal control group, the percentage of cells in G_0/G_1, S, and G_2/M phase was found to be 57.8%, 14.4%, and 29.2%, respectively. Treatment with QMJ-2 and QMJ-5 caused an increase in the percentage of cells in G_0/G_1 phase. Treatment with SAHA arrested G_2/M phase (37.5%) of the cell cycle. Figure 5 shows the histogram plot of the cell cycle in various treatment groups.

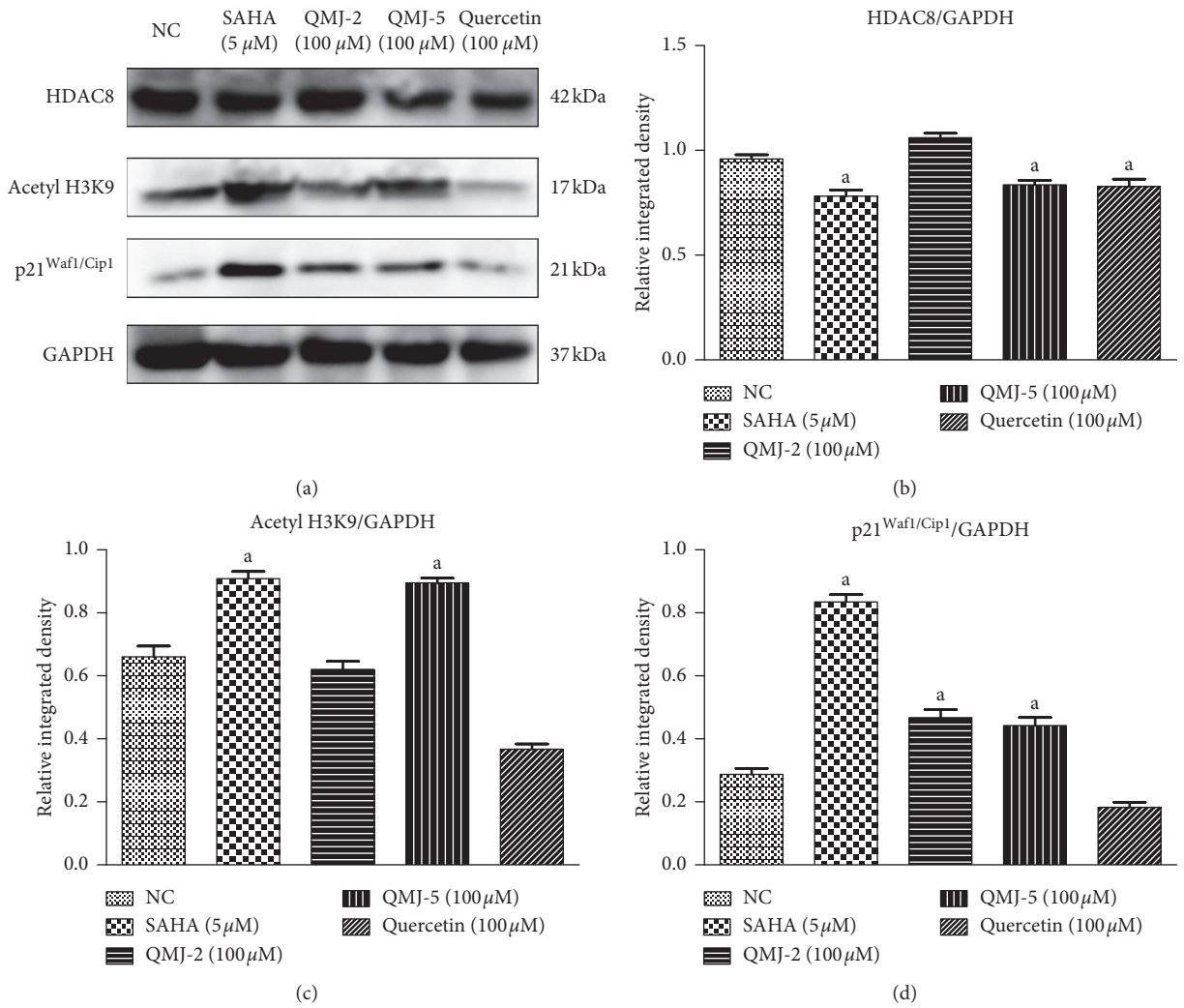


Figure 2: Effect of various compounds incubated for 24 h on HDAC8, acetyl histone H3K9, and p21$^{Waf1/Cip1}$ protein expression in HCT116 cells. All values are expressed as mean ± SEM of three experiments. $^{a}p < 0.05$ vs. normal control (NC).

3.2. In Vivo Studies

3.2.1. Toxicity Study. Both QMJ-2 and QMJ-5 along with quercetin were found to be safe and well tolerated at a dose of 2000 mg/kg *p.o.* There was no mortality observed in the experimental animals at the tested dose. Further studies were carried out using 1/20th of the administered dose.

3.2.2. ACF Formation and Adenocarcinoma Incidence. The incidence of colorectal cancer is detected through the formation of ACF in colonic mucosa. In the present study, ACF formation was observed in all the groups that had been administered with the toxicant DMH. ACF count of the excised colon in DMH control, 5-FU-, quercetin-, QMJ-2- and QMJ-5-treated groups were found to be 25 ± 2.1, 10 ± 1.3, 19 ± 2.6, 16 ± 2.1, and 15 ± 2.6, respectively. A significant ($p < 0.05$) reduction in the ACF count was observed in 5-FU-, QMJ-2- and QMJ-5-treated groups when compared to that of the DMH control group. Incidence of colon adenocarcinoma was observed in all the treatment groups, whereas a significant ($p < 0.05$) reduction in adenocarcinoma was found only in 5-FU- and QMJ-5-treated groups. Table 4 shows the results of ACF formation and adenocarcinoma incidence in various treatment groups.

3.2.3. Colon Weight/Length Ratio and Organ Index. A significant ($p < 0.05$) increase in colon weight/length ratio was observed in DMH control when compared with the normal control group. Treatment with 5-FU and other test compounds significantly ($p < 0.05$) reduced the colon weight/length ratio when compared with DMH control. Among the test compounds, QMJ-5 was comparatively better in reducing colon weight/length ratio compared to other groups (Figure 6). An increase in the kidney index was observed in the DMH control group which was significantly reduced in 5-FU, QMJ-2 and QMJ-5 treatment groups. A significant decrease in the spleen index was observed in quercetin and QMJ-2 treatment groups compared to normal control. Significant reduction in the liver index was observed in QMJ-2 and QMJ-5 treatment groups when compared with

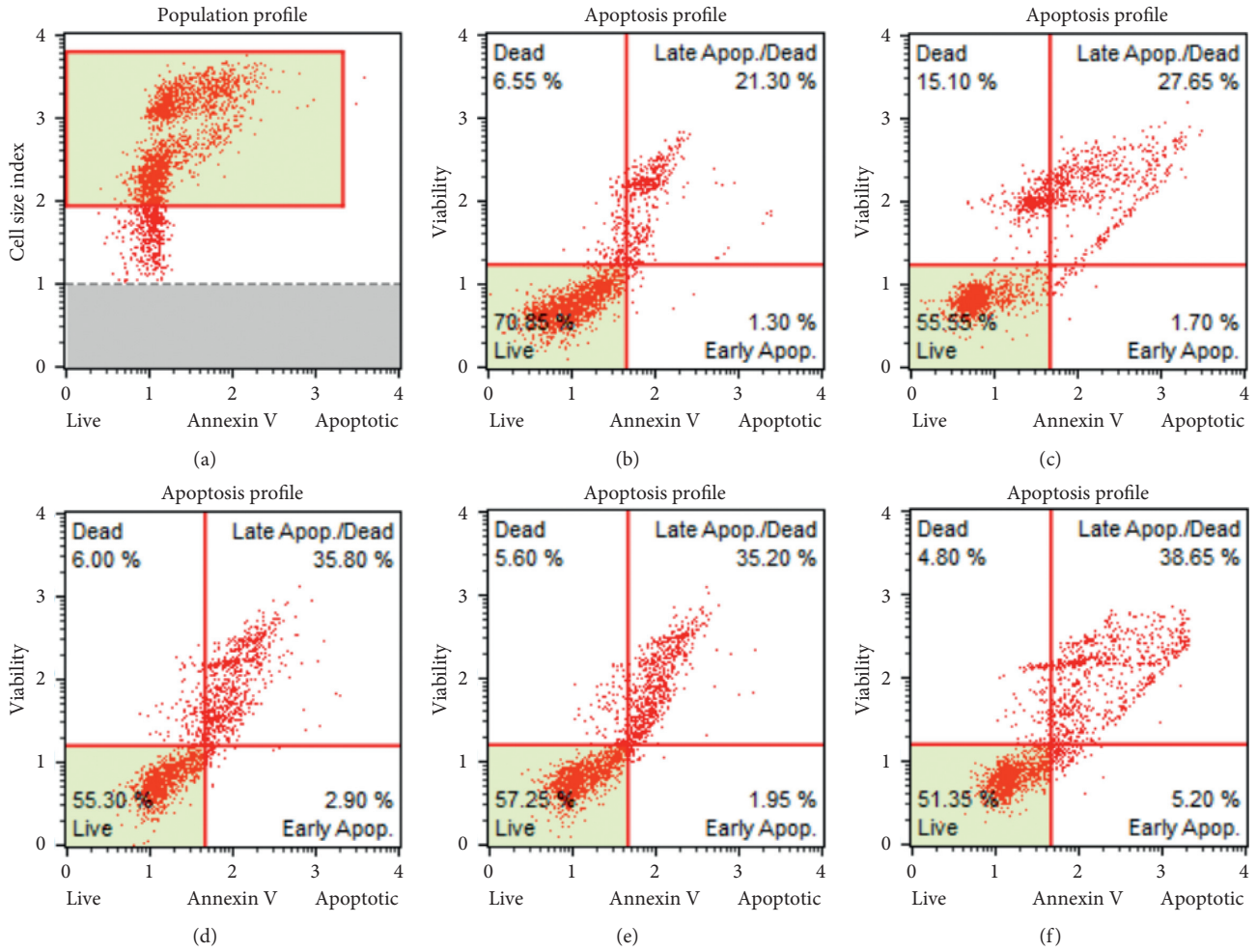


Figure 3: Apoptotic profile after treatment with various compounds in HCT116 cells was determined as % live,% early, and late apoptosis and % dead cells by MUSE cell analyzer: (a) normal control; (b) normal control; (c) SAHA (5 μM); (d) quercetin (100 μM); (e) QMJ-2 (100 μM); (f) QMJ-5 (50 μM).

the normal control and a significant reduction in 5-FU, quercetin, QMJ-2, and QMJ-5 was observed when compared with DMH control (Figure 7).

3.2.4. Histopathology. Histopathological examination of the colon tissues in the normal control group showed the presence of villi without any signs of dysplasia or aberrant crypt. However, in the DMH control group, the presence of aberrant crypt was prominent with an abnormal architecture of the colonic mucosa. However, reduction in the aberrant crypt and a restoration towards the normal morphology of colon were observed in the treatment groups. Figure 8 shows the representative histopathological image.

4. Discussion

Colorectal cancer (CRC) is one of the lethal forms of malignancy and is a global burden both in terms of morbidity and economic expenditure. Various factors are associated with the pathogenesis of CRC among which numerous studies have highlighted the role of epigenetics. The epigenetic enzyme HDACs are overexpressed in colorectal cancer leading to cellular proliferation and differentiation [24]. Among the various HDAC enzymes, HDAC8 is an emerging target in cancer research. Genomic analysis of colon cancer cells shows an increased expression of HDAC8 compared to normal cells [25]. Studies have reported that HDAC8 inhibits apoptosis in colon cancer cells by repressing Bcl-2-modifying factor (BMF) transcription [26]. Interestingly, the deacetylase activity of HDAC8 is exhibited by the enzyme without being associated with any multiprotein complex [27]. These features provide rationale for developing inhibitors against HDAC8 that could be beneficial towards CRC. Natural molecules, especially polyphenols, have been widely studied and are found to be valuable for the treatment of cancer. Among the diverse polyphenolic compounds, flavonols exhibit beneficial property in cancer. The structural feature of flavonols including C2=C3 unsaturation and the presence of the 3-hydroxy group is a crucial determinant for their antitumor property. Exploiting this feature, the polyphenol quercetin and a series of 3-hydroxyflavone analogues were evaluated for their HDAC8 inhibitory potential and anticancer property against colorectal cancer.

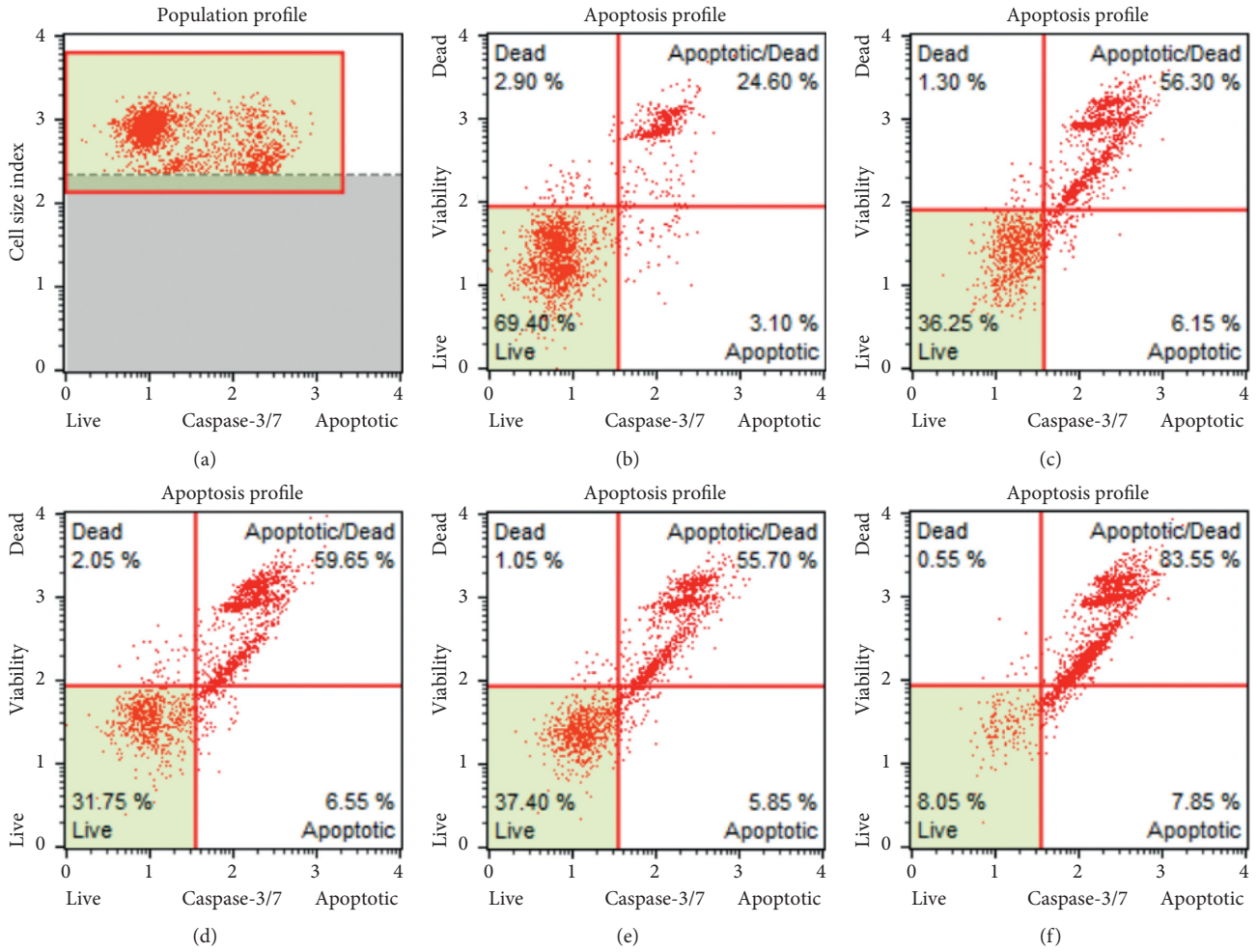


Figure 4: Apoptosis induction through the activation of caspase 3/7 after treatment with various compounds in HCT116 cells was determined as % live, % early, and late apoptosis and % dead cells by the MUSE cell analyzer. (a) Normal control; (b) normal control; (c) SAHA (5 μM); (d) quercetin (100 μM); (e) QMJ-2 (100 μM); (f) QMJ-5 (50 μM).

The screening of probable anticancer compounds is facilitated based on cytotoxic studies that are reliable and often used to determine their potential in inhibiting the growth of cancer cells [28]. MTT assay was used to determine cytotoxicity where it was found that, out of all the compounds synthesized and tested against colon cancer cells, two analogues QMJ-2 and QMJ-5 were found to be most promising. Furthermore, both the analogues exhibited cytotoxicity at two-fold lower concentration when compared to that of the inhibitory concentration observed in normal (Vero) cell line.

Studies have reported the role of epigenetic alterations in understanding the pathophysiology of colon cancer [29]. Among the epigenetic enzymes that are involved, HDACs play a crucial role in the development of colon cancer. In the present study, whole cell HDAC inhibition assay was performed in colon cancer cells. It was observed that, QMJ-2, QMJ-5, and quercetin had a dose-dependent inhibition on HDAC enzyme. Since both the analogues inhibited HDAC enzyme nonspecifically, we further explored their potential to inhibit human HDAC8, 1, and 6 enzymes. HDAC8 and 1 belong to class I, whereas HDAC6 belongs to class IIb superfamily of HDAC enzymes [30]. It was evident from our study that QMJ-2 and QMJ-5 inhibited HDAC8 and HDAC1 enzymes with an IC_{50} value of less than 50 μM. Furthermore, both the compounds inhibited HDAC6 enzyme at a higher concentration suggesting their specificity towards class I HDACs. In addition, QMJ-5 inhibited HDAC8 enzyme at a lower concentration compared with HDAC1 enzyme suggesting its specificity towards HDAC8. The nonspecific HDAC inhibitor SAHA and the polyphenol quercetin also inhibited various HDAC enzymes as evident from our study. Although SAHA inhibited different HDAC isoforms at a lower concentration, it was not specific. Studies have reported that HDAC inhibitors repress histone deacetylation leading to the hyperacetylation of histone protein and several other genes [31]. In the present study, we observed a reduction in the expression levels of HDAC8 after treatment with the synthesized compound. Moreover, an increase in the acetylation levels of histone H3K9 was observed among the treatment groups, suggesting their ability to alter the chromatin state which would further lead to the expression of tumor suppressor genes.

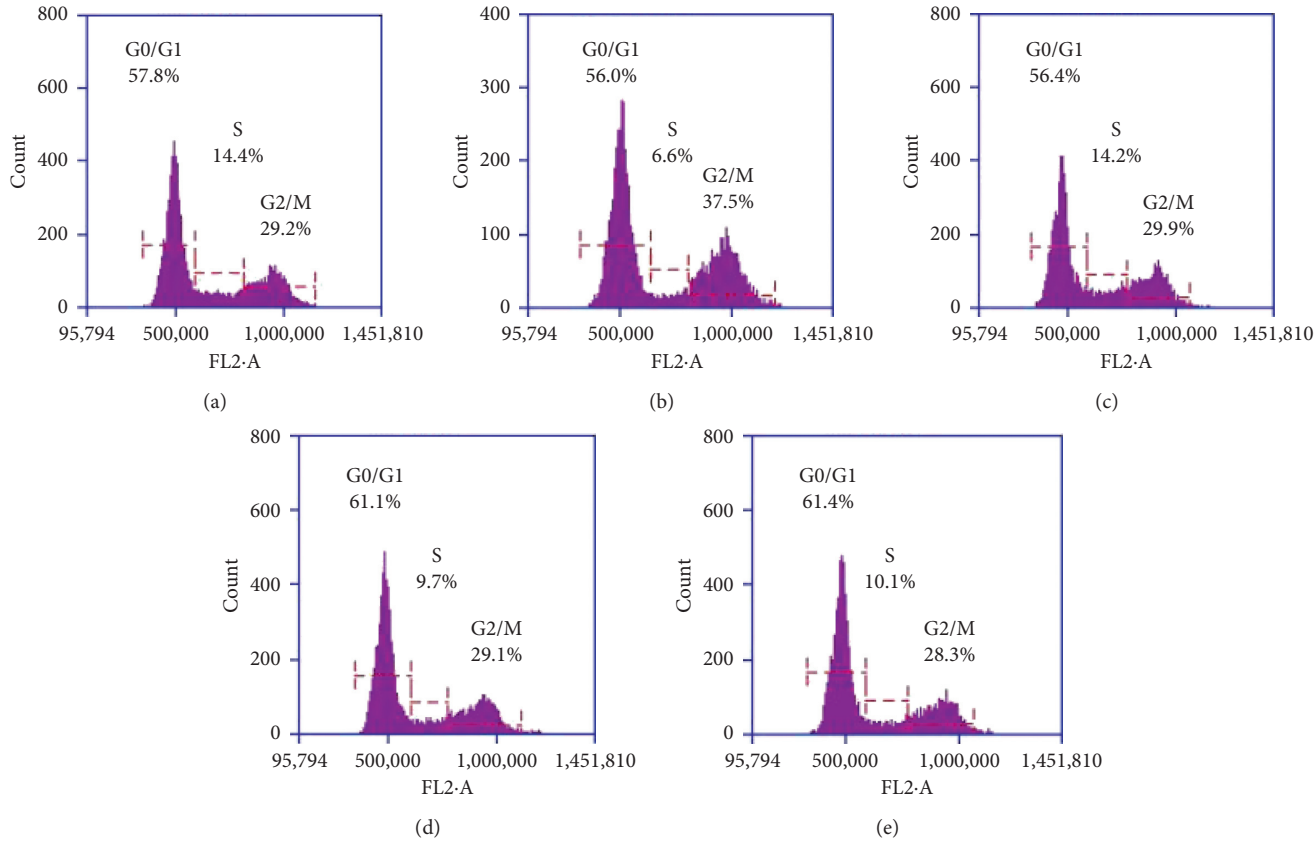


Figure 5: Effect of various compounds on different phases of HCT116 cells was estimated using flow cytometer. Histogram plots display the different phases of the cell cycle and the percentage of cells in each phase. (a) Normal control; (b) SAHA (5 μM); (c) quercetin (100 μM); (d) QMJ-2 (100 μM); (e) QMJ-5 (50 μM).

Table 4: Effect of various treatments on the ACF count and adenocarcinoma incidence in colon tissue.

Sl. no.	Groups	ACF/5 cm^2	Adenocarcinoma
1	Normal control	0	0
2	DMH control	25 ± 2.1	17 ± 1.7
3	5-FU	10 ± 1.3[a]	8 ± 1.1[a]
4	Quercetin	19 ± 2.6	15 ± 1.1
5	QMJ-2	16 ± 2.1[a]	14 ± 1.3
6	QMJ-5	15 ± 2.6[a]	11 ± 0.8[a]

All values are expressed as mean ± SEM of six animals. [a]$p < 0.05$ vs. DMH control.

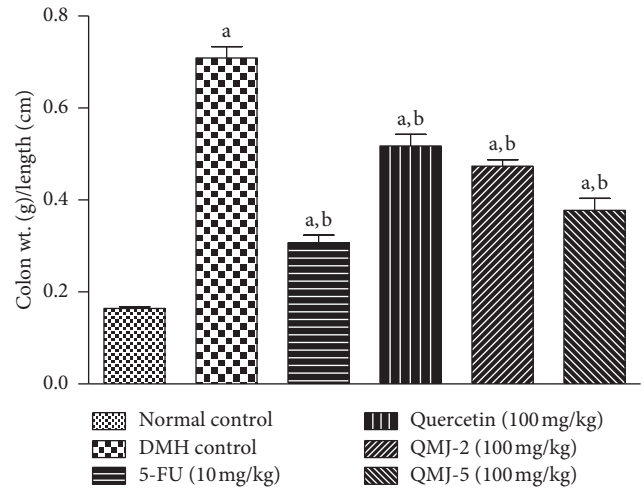


Figure 6: The bar graphs represent colon weight/colon length ratio. All values are expressed as mean ± SEM of six animals. [a]$p < 0.05$ vs. normal control; [b]$p < 0.05$ vs. DMH control.

To study the plausible mechanism of cell death induced by the analogues in cancer cells, apoptotic assay was performed. In the event of apoptosis, a membrane phospholipid, phosphatidylserine translocate from the inner leaflet of the cell membrane to the outer membrane of the cell [32]. Annexin V, a specific marker binds with phosphatidylserine and facilitates in the determination of apoptosis. It was found that, QMJ-2 and QMJ-5 were able to induce apoptosis in colon cancer cells evident from the percentage of cells in the early and late stages of apoptosis. Furthermore, to determine the downstream signaling pathway of apoptosis, activation of caspase 3/7 was assessed. Caspases are a family of cysteine protease that plays a primary role during apoptosis [33]. The executioner caspase (3, 6, and 7) conducts the mass proteolysis of target proteins eventually causing apoptosis. It was found that, the analogues activated caspase 3/7 leading to the induction of the mitochondrial pathway of apoptosis as the mechanism of cell death.

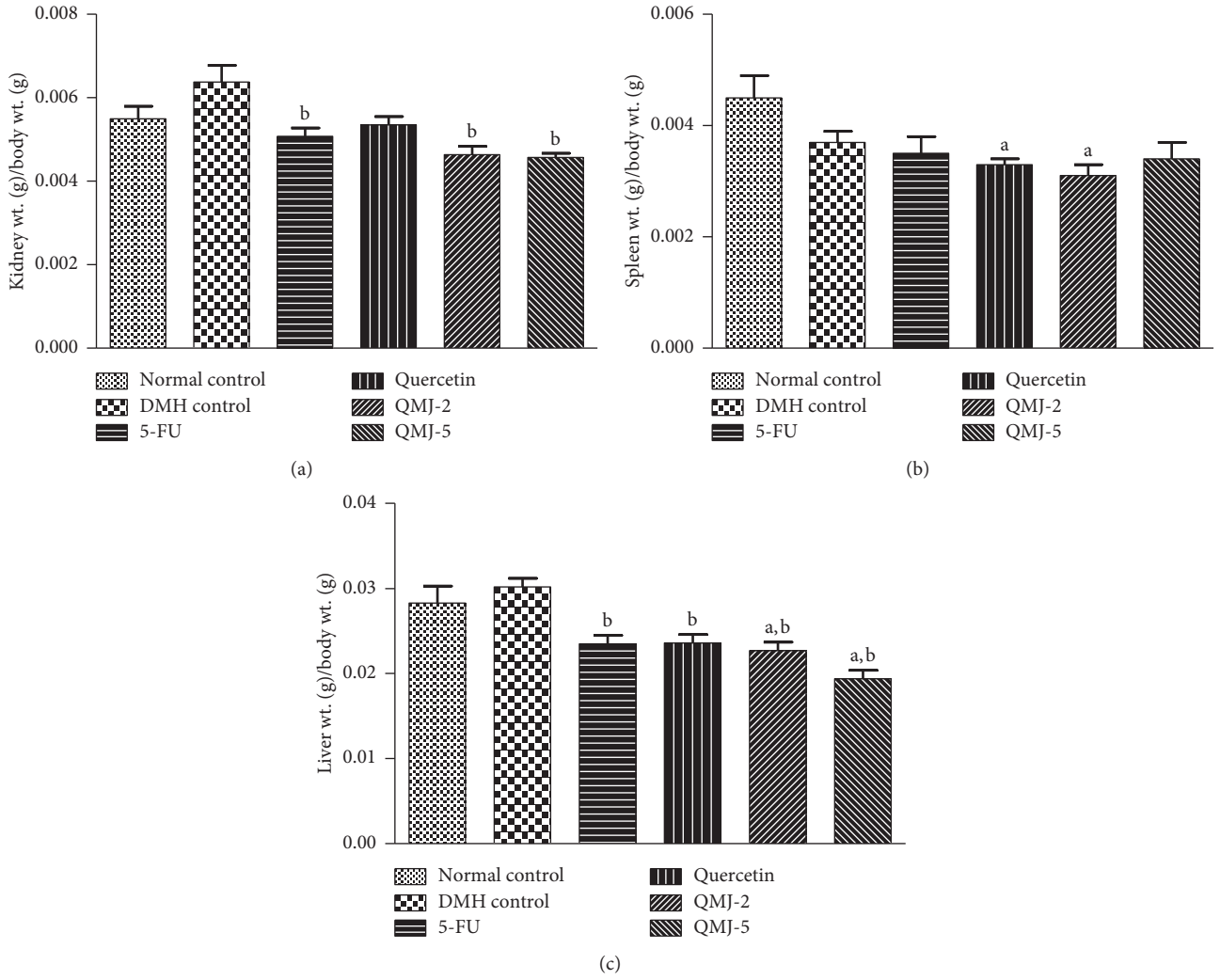


FIGURE 7: The bar graphs represent the effect of various treatments on the major organ index. All values are expressed as mean ± SEM of six animals. [a] $p < 0.05$ vs. normal control; [b] $p < 0.05$ vs. DMH control. (a) Kidney index; (b) spleen index; (c) liver index.

Cell cycle analysis is a useful tool that provides information on various phases (G_0/G_1, S, and G_2/M) of cell cycle depending upon the DNA content. The initiation of cell cycle progression is controlled at the G_1 phase where, any damage to the DNA would retard the progression and allow the cell to repair before it could enter the S phase [34]. However, in conditions where, there are no further signs of cellular repair, the mitochondrial pathway of apoptosis gets activated mediating through the accumulation of phosphorylated p53. In the present study, cell cycle analysis revealed that both QMJ-2 and QMJ-5 arrested the cell cycle of colon cancer cells at the G_0/G_1 phase. The cyclin-dependent kinase inhibitor p21 regulates cell cycle progression and plays an important role in the prevention of tumor development [35]. Studies have reported that HDAC inhibitors induce the expression of *p21*^{Waf1} gene leading to cell cycle arrest [36]. We observed an increase in the expression level of p21$^{Waf1/Cip1}$ in SAHA, QMJ-2, and QMJ-5 treatment groups suggesting their ability to induce the expression of p21$^{Waf1/Cip1}$ leading to cell cycle arrest.

Previous reports suggest that compounds with HDAC inhibitory potential induce apoptosis and arrest cell cycle through the mitochondrial pathway [37]. Moreover, studies have demonstrated that polyphenols induce cell cycle arrest and apoptosis through the inhibition of histone deacetylase enzyme [38]. Similar results were obtained from our study where QMJ-2 and QMJ-5 inhibited HDAC8 expression and increased the levels of acetylated histone H3K9. The compounds also arrested the cell cycle at the G_0/G_1 phase through the expression of p21$^{Waf1/Cip1}$ protein and induced apoptosis through the activation of caspase 3/7.

Promising results from *in vitro* studies supported the need to assess the *in vivo* potential of the analogues in DMH-induced colorectal cancer in rats. DMH is an extremely precise colorectal carcinogen that along with its metabolite azoxymethane (AOM) hastens the development of colorectal cancer in rats [39]. The formation of aberrant crypt

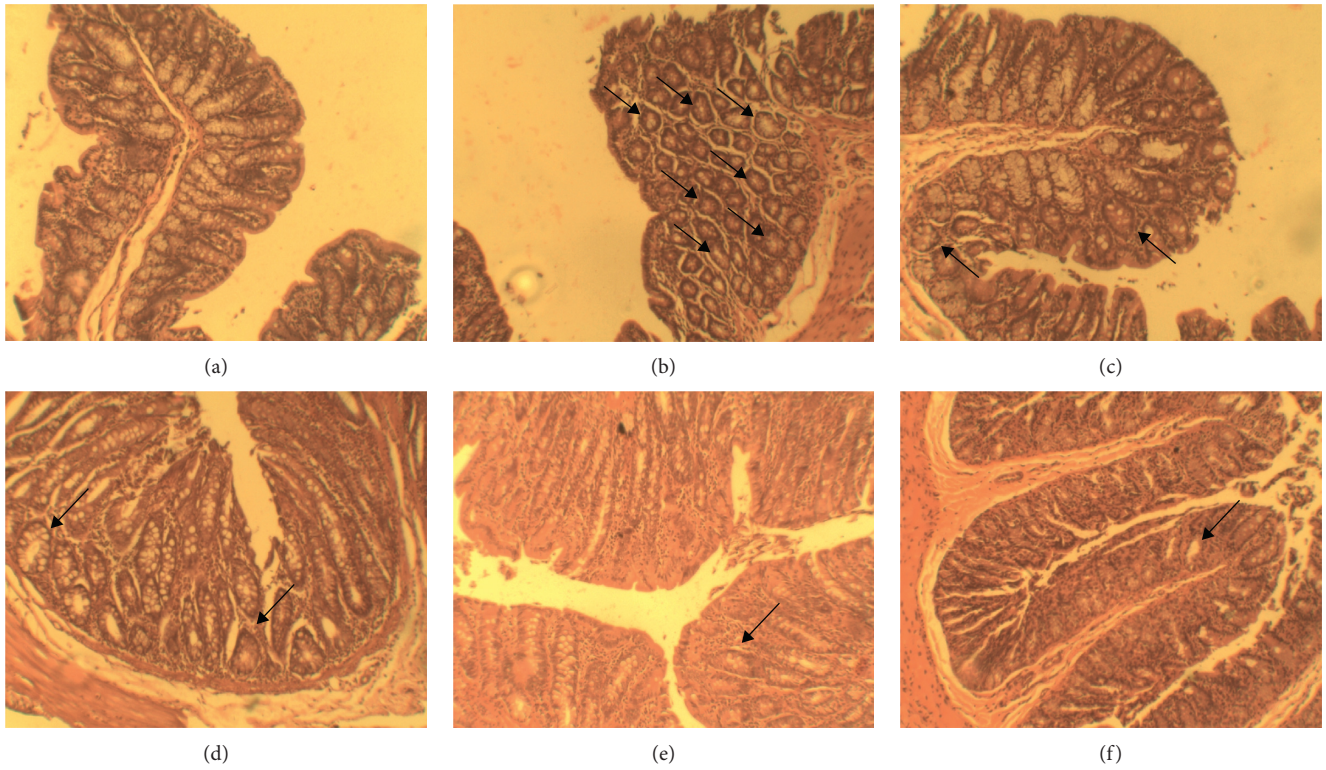


Figure 8: Histopathological changes of colon tissues from different treatment groups at 40x magnification. Arrows indicate aberrant crypt foci. (a) Normal control; (b) DMH control; (c) 5-FU; (d) quercetin; (e) QMJ-2; (f) QMJ-5.

foci (ACF) is the initial lesion observed in colon tissues, and a reduction in the ACF count is an essential indicator of recovery from colorectal cancer [40]. In the present study, histopathological analysis indicated the formation of ACF and abnormal mucosal architecture in DMH control group. Histopathology of various treatment groups revealed the reduction in ACF formation and restoration of normal colon morphology. It was observed that both QMJ-2 and QMJ-5 significantly reduced the ACF formation compared with that of DMH control group, suggesting their role in protecting the colon mucosa. The incidence of adenocarcinoma is another key-determining factor for the development of colorectal cancer. A marked reduction in the adenocarcinoma formation was observed in the QMJ-5-treated group when compared with that of the DMH control group further supporting their protective effect. Administration of DMH causes alteration in the colonic mucosa and the formation of adenocarcinoma, leading to an increase in colon weight and shortening in the length of colon [41]. Similar results were obtained from our study where an increase in colon weight/length ratio was observed in the DMH control group compared to normal. QMJ-2 and QMJ-5 reduced the ratio which demonstrated their protective effect against colorectal cancer. Organ index studies exhibited that there was no significant change in the kidney index of QMJ-2 and QMJ-5 treatment groups compared to normal. However, there was a significant reduction in the liver index of animals treated with QMJ-2 and QMJ-5 suggesting further studies to determine the mechanism. Since the available HDAC inhibitors are effective against leukemia, newer HDAC inhibitors effective against solid tumors is the need of the hour. From the present study, it was evident that QMJ-2 and QMJ-5 inhibited HDAC enzyme *in vitro* and had a protective role against DMH-induced colon cancer *in vivo*.

5. Conclusion

The present study revealed that both analogues QMJ-2 and QMJ-5 and the polyphenol quercetin were found to be cytotoxic and inhibited HDAC enzyme, where QMJ-5 showed greater specificity towards HDAC8. Induction of apoptosis by QMJ-2 and QMJ-5 in colon cancer cells was mediated through the activation of caspase 3/7 along with the cell cycle arrest at the G_0/G_1 phase through the expression of $p21^{Waf1/Cip1}$. The synthesized analogues reduced the formation of ACF and adenocarcinoma in the animal model of colorectal cancer. Thus, the present study identified the potentials of novel 3-hydroxyflavone analogues as HDAC8 inhibitors with anticancer property against colorectal cancer providing a lead for new drug development.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

C Mallikarjuna Rao, B. S. Jayashree, and Subhankar Biswas were involved in designing the experiment. Synthesis and characterization were carried out by Subhankar Biswas under the supervision of B. S. Jayashree. Experiments and data analysis were carried out by Subhankar Biswas and Neetinkumar D. Reddy. Subhankar Biswas prepared the manuscript. All authors have read and approved the final manuscript.

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Review Article

Therapeutic Properties of Stingless Bee Honey in Comparison with European Bee Honey

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Both honeybees (*Apis* spp.) and stingless bees (*Trigona* spp.) produce honeys with high nutritional and therapeutics value. Until recently, the information regarding potential health benefits of stingless bee honey (SBH) in medical databases is still scarce as compared to the common European bee honey (EBH) which is well known for their properties as therapeutic agents. Although there have been very few reports on SBH, empirically these products would have similar therapeutic quality as the EBH. In addition, due to the structure of the nest, few studies reported that the antimicrobial activity of SBH is a little bit stronger than EBH. Therefore, the composition of both the types of honey as well as the traditional uses and clinical applications were compared. The results of various studies on EBH and SBH from tissue culture research to randomised control clinical trials were collated in this review. Interestingly, there are many therapeutic properties that are unique to SBH. Therefore, SBH has a great potential to be developed for modern medicinal uses.

1. Introduction

Honey is an important natural food product since ancient times and is known for its nutritional and therapeutic values. It is produced from liquid plant exudates which is gathered, modified, and stored by different types of bees [1]. Among all, European honeybees (*Apis mellifera*) and stingless bees (Figure 1) are the two most common bees. The European honey bee is a member of a group of bees in the genus of *Apis* whereas stingless bee can be classified into two genera, namely, the *Melipona* and the *Trigona* [2]. Both have important role in flower pollination [3–5]. Honey produced by stingless bees is known with various names such as Meliponine honey, stingless bee honey (SBH) pot-honey, and also *Kelulut* honey (in Malaysia). It is one of the valuable bee products and is attributed with some medicinal properties by ancient peoples [6]. Since the 20th century, various studies on the chemical and biological properties of honey had been conducted due to their extraordinary antibacterial, bacteriostatic, anti-inflammatory, wound, and sunburn healing effects [7].

In the modern time, due to its outstanding medicinal value, honey has been both exported and imported globally. Like other food supplements, the therapeutic effects of honey

(a) (b)

Figure 1: (a) Stingless bee. (b) Stingless bee honey in the nest.

also depend on its quality [8]. Sensorial, chemical, physical, and microbiological characteristics are generally used to determine the quality of honey. Even within same species, the quality parameters of honey produced varies and dependent on other factors such as the maturity achieved in the bee nest or hive during the harvesting season, climatic and geographic factors, and other elements that affects the floral abundance [6]. For example, in the United States only more than 300 different types of honey can be found, each with unique flavour and appearance, depending to its floral sources. Currently, researchers have established that the main reason behind the diverse color, flavour, and also functional properties of honey is mainly due to its phenolic composition rather than variation in other components, such as carbohydrates and proteins [9].

Most well-researched natural honeys are the European bee honey (EBH) such as Manuka honey, jelly bush honey, African jungle honey, and Malaysian Tualang honey. As compared to the benefits of EBH (Manuka honey) which has been internationally recognised [10], the potential health benefits of stingless bee honey (SBH) are only recently gaining a lot of attention [11–13]. Despite that, a variety of phenolic compounds such as gallic acid, caffeic acid, catechin, and apigenin have also been reported in both honeys [14, 15]. Honey exhibits significant antioxidant, anticancer, and antiatherogenic activities which may be attributed partly to these compounds [16–18]. For example, SBH has been proven to show peculiar antioxidant activities and exhibits outstanding ability in reducing inflammation and infection [19]. Therefore, in this, we describe the health effects of stingless bee honey in comparison with honey produced by the European honey bee. The importance of polyphenols in honey as well as their potential mechanisms in treating certain diseases is also discussed.

2. Physicochemical Properties of Stingless Bee Honey and European Bee Honey

Alongside water and sugars as the major contents, both SBH and EBH are also known to be rich in vitamins, enzymes, amino acids, and minerals, with almost 200 different compounds were reported in both honeys. Usually, honeys are acidic due to its low pH (~pH 4), and are made up of 80% sugars and 17% water, while the remaining 3% is contributed by various enzymes, acids, and minerals [20]. However, the composition of honey differs according to the floral source and origin [21]. For example, in Thailand [22], it was reported that the composition and quality of Thai SBH differs from EBH.

Fructose is reported as the most abundant sugars found in both honeys with approximately 31–39% of various sugars in honey [23]. Other than fructose and glucose, many studies had reported the presence of various disaccharides and oligosaccharides in honey. Inulobiose, kestose, and nystose are some of the fructooligosaccharides identified in Malaysian Tualang honey (*Apis dorsata*) while New Zealand honey contains isomaltose and melezitose [7, 24] as well as raffinose has been found in Italian honey [25]. Minerals or trace elements present in honey are potassium, zinc, phosphorus, calcium, sodium, magnesium, sulphur, copper, iron, and manganese [26].

Because of its unique flavour and high nutritional value, the price of honey is relatively higher than other sweeteners. Adulteration of honey is a serious problem which currently has a significant impact on economy as well as irrefutable nutritional and organoleptic ramification. Lack of knowledge regarding composition and physicochemical characteristics of SBH worldwide has led to its adulteration and falsification [27]. Full data or detail information on the physicochemical properties of honey is important to decrease the possibility of adulteration. The data obtained from various studies are being used to develop new regulatory standards of SBH [27]. The different physicochemical characteristics of EBH, namely, Tualang honey and Manuka honey and SBH are summarized in Table 1.

3. General Nutritional Properties and Dietary Values of Honey

The high nutritional and therapeutic value of honey has been well documented from long time ago. Before cane sugar is being used widely, honey has always been the choice of natural sweetener. Honey is a concentrated solution of reducing sugars such as fructose and glucose and nonreducing sugars such as sucrose and maltose. Among all, fructose and glucose represent the largest proportion of honey

Table 1: Different physicochemical characteristics of European bee honey and stingless bee honey.

Physicochemical properties	European bee Honeys		Stingless bee honeys
	Tualang honey, *Apis dorsata* [28–30]	Manuka honey, *Apis mellifera* [31, 32]	*Trigona* spp. [6, 8, 27]
Appearance	Dark brown	Light dark brown	Amber brown
Moisture content (%)	23.30	18.70	25.00–31.00
pH	3.55–4.00	3.20–4.20	3.15–4.66
Total reducing sugars (%)	67.60	75.80	54.90–87.00
Glucose (%)	29.50	35.90	8.10–31.00
Fructose (%)	29.60	40.00	31.11–40.20
Sucrose (%)	0.60	2.80	0.31–1.26
Maltose (%)	7.85	1.20	ND
Calcium (%)	0.18	1.15	0.017
Potassium (%)	0.51	1.00	0.07
Sodium (%)	0.26	0.0008	0.012
Magnesium (%)	0.11	1.00	0.004
Specific gravity	1.34	1.39	ND
Electrical conductivity (mS/cm)	0.75–1.37	0.53	0.49–8.77
Hydroxymethylfurfural HMF (mg/kg)	46.17	400.00	8.80–69.00
Ash content (g/100 g)	0.19	0.03	0.01–0.12

ND: not detected.

composition. As the results, honey tastes sweeter than sucrose as sweetening power of fructose is 1.3 while sugar cane-derived sucrose is only 1 [33], making it a better substitute as sweetener, with higher nutritional value as compared to commercial sugar. Despite its reported high fructose level, 48.1% of SBH contained lower levels of reducing sugar when compared to *Apis mellifera* honey (EBH) standards [8, 34].

Honey is one of the outstanding sources of energy due to its high sugar concentration. The energy input represented by honey is approximately 300 kcal per 100 g [35]. The high calorific value of honey makes it suitable for athletes as it contains readily absorbed glucose which will be converted into energy in a short time [33]. Other than it is used as a source of energy, honey is also important for bones and teeth. It helps in absorption of calcium and magnesium retention which may contribute to stronger bone and better dental calcification. This is due to the presence of non-digestable carbohydrate such as raffinose that produces short-chain fatty acid (SCFA) as the by-product from the fermentation process in the caecum and colon. The SCFA helps to lower the intestinal pH and creating a favourable environment that increases mineral such as calcium solubility and absorption [36].

4. Polyphenols of Stingless Bee Honey and European Bee Honey

Flavonoids and phenolic acids are the most common group of polyphenols that are previously detected in both honeys. As for the flavonoid groups, only the flavonols (such as myricetin, kaempferol, 8-methoxy kaempferol, quercetin, isorhamnetin, quercetin-3-methyl ether, quercetin-3, 7-dimethyl ether, pinobanksin, rutin, and galangin), flavones (such as genkwanin, luteolin, apigenin, tricetin, and chrysin), and flavanones (such as pinocembrin and pinostrobin) were previously detected in honey. Meanwhile, among the phenolic acid group, the hydroxybenzoic acids such as methyl syringate, gallic acid, syringic acid, benzoic acid, and 4-hydroxybenzoic acid and hydroxyl-cinnamic acids such as chlorogenic, vanillic, caffeic, p-coumaric, and ferulic acids are present in various honey samples [37]. Common polyphenols detected in both honeys, each with different potential therapeutic effects, are summarized in Table 2.

Honey is known for its antioxidant activity. A prior investigation indicated that the total antioxidant activity of honey is primarily provided by its phenolic composition, rather than vitamin C and other components [12]. The antioxidants that occur naturally in honey are flavonoids, phenolic acids, enzymes (e.g., glucose oxidase and catalase), ascorbic acid, carotenoid-like substances, organic acids, Maillard reaction products, amino acids, and proteins [40, 50] Several *in vivo* studies strongly suggested that long-term consumption of diets rich in these types of polyphenols significantly ameliorates the adverse effects of several liver-, heart-, kidney-, brain-, and pancreas-associated diseases as well as those of genetic disorders such as tumors and cancer [38, 51].

5. Traditional Uses of Stingless Bee Honey and European Bee Honey

Natural honey has been used to prevent and treat variety of ailments since years ago [52]. For example, newborn babies were fed with EBH as a supplement [53], meanwhile EBH also has been used by Ayurvedic physicians as alternatives for medicines, and it was recommended to satisfy the immediate calorie demand for the patients [52].

A study by Reyes-González et al. [54] reported that the SBH is also known for its medicinal value and uses in food. According to the natives, after being extracted, the honey is often consumed along with a hot drink, or even alone. As medication, SBH is employed for treating various sicknesses

Table 2: Common phenolic compounds with their potential health benefits found in both European bee honeys and stingless bee honeys.

Compound	Molecular formulae	Potential health benefits	References
Gallic acid	$C_7H_6O_5$	Antioxidant Anti-inflammatory Cardioprotective activity Antimutagenic Anticancer	[38]
Caffeic acid	$C_9H_8O_4$	Cardiovascular diseases treatment Anti-inflammatory effects Anticancer Antidiabetic	[39] [40] [41, 42] [43]
Catechin	$C_{15}H_{14}O_6$	Cardiovascular diseases treatment Antidiabetic potential Anti-inflammatory	[44] [45]
Apigenin	$C_{15}H_{10}O_5$	Anti-inflammatory Antimutagenic Treating cardiovascular diseases	[46] [37]
Chrysin	$C_{15}H_{10}O_4$	Improves cognitive deficits and brain damage Anticancer	[47]
Cinnamic acid	$C_9H_8O_2$	Improves cognitive deficits and brain damage effect Antimicrobial effect	[47] [48]
Kaempferol	$C_{15}H_{10}O_6$	Cardiovascular diseases treatment	[39]
p-Coumaric acid	$C_9H_8O_3$	Anticancer activity Improves cognitive deficits and brain damage effect	[41, 43] [47]
Quercetin-3-O-rutinoside (rutin)	$C_{27}H_{30}O_{16}$	Antiallergic Anti-inflammatory Antiproliferative Antitumor	[49]

by combining this honey with different ingredients such as lemon, agave mezcal, and pulp of *Crescentia alata*. The combination was used to treat cold, cough, and respiratory illness such as bronchitis. Besides that, the SBH is extensively used as a fundamental part of medicine by the Maya traditional doctors as remedy for high fever, treatment for wounds and burns, and also the cure for poisonous stings [55]. Despite being known as functional food, honey is also credited with many therapeutic values.

6. Therapeutic Effects of Stingless Bee Honey and European Bee Honey and Their Polyphenols

Microbial resistance towards modern antimicrobial drugs is rising and had become the topic of interest among the scientists in which scientists are developing novel drugs with less or no microbial resistance, and also have broad-spectrum inhibition activity. Despite the traditional uses of honey as therapeutic agents, honey is recently acknowledged in modern medicine development [56] due to its valuable nutritional quality. It also portrays potential properties against reactive oxygen species (ROS), acts effectively as anti-inflammatory and antibacterial agents against bacteria and fungi and a potential substitute in reducing coughs and wound curing [56]. The common therapeutic properties of most honeys are more likely based on their floral origins. Since few years back, the role of honey in wound healing has been widely studied and proven to be the most effective therapeutic effects of honey [57]. Previously, honey has been used to treat wound infection and promotes wound healing by the Russians during World War 1. Mixture of honey and cod liver oil has shown to be effective by the Germans, in treating ulcers and burns [58]. In addition, honey is world widely known for its roles in the treating of famous ophthalmological diseases such as keratitis, conjunctivitis, corneal injuries, blepharitis, and chemical and thermal burns to eyes [59, 60].

Honey contains various polyphenols, which differs according to the origin and bee species [48]. Various polyphenols, of which some are also detected in honey, have been proven to curb the development of many diseases. They perform this action via several specific mechanisms such as regulation of a specific gene expression or altering metabolic pathways by means of promoting or blocking specific pathways [37]. However, differences in honey samples may affect the type of polyphenols found in honey. As one type of honey might not contain all of the polyphenols described and the protective effects of polyphenols are varied, it is advisable to consume variety of honey samples. The therapeutics effects of SBH and EBH such as antidiabetic, wound healing, anticancer, treatment of eye disease, and effects of fertility as proven by many scientific studies will be described as below. The therapeutics effects of both honeys are summarized in Table 3.

6.1. Antidiabetic. Hyperglycemia, deranged lipid profiles, and inadequate insulin production by the pancreas are the

Table 3: Summary of therapeutic properties of European bee honey and stingless bee honey from previous studies.

Properties	Honey types and bee species	Therapeutic effects	Reference
Antidiabetic	Nigerian honey (*Apis* spp.)	Increased high-density lipoprotein (HDL) cholesterol Reduced hyperglycemia, triglycerides (TGs), very low-density lipoprotein (VLDL) cholesterol, non-HDL cholesterol, coronary risk index (CRI), and cardiovascular risk index	[61]
	Gelam honey (*Apis dorsata*)	Increased expression of phosphorylated JNK and JKK-β. Reduced expression of TNF-α, IL-6, IL-1β, and Akt phosphorylation	[62]
	European bee honey (*Apis* spp.)	Expression of TNF-α, IL-6, IL-1β, and Akt phosphorylation No effect on glucose level at low dosage Increased blood glucose at high dosage	[63]
	European bee honey and stingless bee honey (*Apis cerana indica*, *Apis mellifera*, *Apis dorsata*, *Apis florae*, and *Trigona iridipennis*)	Higher percentage of inhibition against α-amylase and α-glucosidase enzyme (*Trigona sp*.)	[64]
	Stingless bee honey (*Geniotrigona thoracica*)	Prevent increased of fasting-blood glucose (FBG), total cholesterols (TC), TGs, and LDL levels Increased HDL and serum insulin levels Decreasedchanges of histopathological and oxidative stress expression level, inflammation, and apoptosis markers in pancreatic islets Increased expression level of insulin	[65]
Wound healing	Multifloral honey, West Bengal (*Apis mellifera*)	Close resemblance of D-spacing and collagen diameter to normal skin collagen (scanning electron microscope observation)	[66]
	Multifloral honey, Iran (*Apis mellifera*)	Increased Oedema and necrosis Less infiltration of polymorphonuclear and mononuclear cells Improve wound contraction Increased epithelialisation	[67]
	Multifloral honey, Ibadan, Nigeria (*Apis mellifera*)	Increased concentrations of glycosaminoglycan and proteoglycan Increased granulation tissue in electroscalpel (ES) wound Increased fibroelastic tissue in honey treated wounds of ES group and honey treated wound cold scalpel	[68]
	Tualang honey (*Apis dorsata*)	High tensile strength of colon anastomosis and fibroblast count High inflammatory cells	[69]
	European bee honey (*Apis* spp.)	High hydroxyproline level in jaundiced animals treated with honey High bursting pressure	[70]
	Stingless bee honey (*Trigona* spp.)	Prevent growth of rifampicin-resistant *S. aureus* and maintaining the sensitivity of *S. aureus* towards rifampicin	[71]
	Stingless bee honey (*Apis mellipodae*)	Showed effective effects in inhibiting growth of *S. aureus* and other pathogenic bacteria	[11]
	European bee honey (*Apis* spp.)	Stimulates healing process, clears infection, stimulates tissue regeneration, and reduces Inflammation	[58, 72, 73]
Anticancer	European bee honey (*Apis mellifera*)	Increased number of viable HepG2 cells in the human hepatoma cell (HepG2) treatment Improvement of the total antioxidant status Caspase-3 activity is time and dose-dependent	[74]
	Multifloral honey (*Apis mellifera*)	Increased rate of incidence, the efficacy to multiply, and the tumor size	[75]
	Stingless bee (*Trigona* spp.)	Reduced the total number of ACF and aberrant crypt and multiplicity of crypt No changes in the level of blood profile parameters, liver enzymes, and kidney functions	[76]
	Trigona incisa, *Timia apicalis*, *Trigona fusco-balteata*, and *Trigona fuscibasis*	Increased cytotoxicity effects towards HepG2 cell line, while propolis crude extracts exhibit high cytoxicity effects towards all the human cancer cell lines	[77]

Table 3: Continued.

Properties	Honey types and bee species	Therapeutic effects	Reference
Treatment of eye diseases	Honeydew honey(*Apis mellifera*)	Bacterial flora in the conjunctival sac of patients with cataract and scheduled for vitrectomy was successfully eradicated after 7 days	[78]
	Australian and New Zealand honey (*Leptospermum* sp.)	Reduced formation of the whole colony-forming units in the eyelids and conjunctivae in patients with dry eye syndrome after one and three months of therapy	[79]
	Tualang honey (*Apis dorsata*)	No difference between the conventional treatment with Tualang honey eye treatment for chemical eye injury	[80]
	European bee honey (*Apis* spp.)	Corneas manifested an immediate regression of the corneal oedema	[81]
	Stingless bee (*Trigona* spp.)	Retardation of the cataract progress in 20% of the rats in the group that received honey for the opacification treatment	[82]
	Stingless bee honey (*Meliponula* spp.)	Reduced the infection time for eye diseases caused by *Staphylococcus aureus* and *Pseudomonas aeruginosa*	[83]
Fertility	Tualang honey (*Apis dorsata*)	Increased intromission and ejaculation percentage in rats Increased rate of fertility and mating	[84]
	Honey Uremia, Iran (*Apis* spp.)	Reduced apoptosis and necrosis rate of the testicular cells in cells affected by noise stress and thereby increased cell viability	[85]
	Tualang honey (*Apis dorsata*)	Beneficial effects on level of corticosterone, pregnancy outcome, and adrenal histomorphometry	[86]
	Tualang honey (*Apis dorsata*)	Reduced cortisol and increased progesterone level of stress-induced female rats	[87]
		Increased testicular, epididymal weights, epididymal sperm count, motility, viability in nondiabetic, and sperm quality	[88]

characteristics of chronic metabolic disorders, diabetes mellitus [60]. Numerous studies have reported the antidiabetic effects of honeys particularly from EBH. EBH from Nigeria, for example, had shown increment in high-density lipoprotein (HDL) cholesterol level, while hyperglycemia, triglycerides (TGs), very low-density lipoprotein (VLDL) cholesterol, non-HDL cholesterol levels, coronary risk index (CRI), and cardiovascular risk index (CVRI) were reduced in alloxan-induced male diabetic Wistar rats [61]. Pretreatment of EBH known as Gelam honey produced by *Apis dorsata* on pancreatic hamster cells has been reported to modify the inflammation-induced insulin signalling pathways [62]. Promising antihyperglycemic effects of EBH in the diabetic rabbit model has been reported as blood glucose levels, and other related parameters were significantly reduced in this study [63]. Apart from its wound healing effects, the EBH known as Tualang honey has great antioxidant activities towards the organs of chemically induced diabetic rats such as pancreas. The hypoglycemic effect of Tualang honey in diabetic animal model might also be contributed by the protective effects against oxidative stress of the pancreas [89].

α-Amylase and α-glucosidase are the two main enzymes that are involved in elevation of blood glucose. The inhibition of these two enzymes indicates a good antidiabetic effect as it helps to reduce the levels of blood glucose. In a comparative study, the antidiabetic properties of EBH and SBH were analysed using *in vitro* α-amylase and α-glucosidase enzyme inhibition assays, whereby SBH was found to exhibit the highest inhibition of both enzymes, indicating a better antidiabetic properties as compared to other EBH honeys used in this study [64]. The complex starch molecules will be converted into simple sugars by these enzymes; therefore, a competitive inhibition between α-amylase and α-glucosidase with the phytochemicals in the honey could prevent the rise of blood sugar level at a faster rate [64]. SBH also showed remarkable antidiabetic effects *in vivo* as reported by Aziz et al. [65], where administration of this honey to diabetic male rats did not increase the level of fasting blood glucose, total cholesterols, triglyceride, and low-density lipoprotein.

Honey and other medicinal plants which are reported to contain many bioactive compounds [90] were used traditionally and are still being used as alternative to treat diabetes [91]. Evidences from scientific studies showed that dietary polyphenols are useful in treatment of diabetes mellitus. Out of many polyphenols found in both honeys, only few of them, such as quercetin, apigenin, luteolin, catechin, rutin, and kaempferol, are detected to exhibit antidiabetic properties. This is achieved via several mechanisms to reduce blood glucose levels [37]. These include several important mechanisms such as α-amylase and α-glucosidase and gluconeogenic enzymes inhibition [92, 93], enhancement of pancreatic b-cell protection and glucose uptake [94, 95], and reduction of oxidative stress [95].

The potential role of honey polyphenols in inhibiting α-amylase and α-glucosidase enzymes that facilitate carbohydrate breakdown has been confirmed. Quercetin successfully inhibits the α-glucosidase enzymes and reduces maltose-induced postprandial hyperglycemia in patients diagnosed with type 2 diabetes mellitus (T2DM) [96]. Meanwhile, another study proved the inhibition of α-amylase and α-glucosidase by luteolin and luteolin-7-0-

glycoside [97]. The level of blood glucose in an animal's body is controlled by pancreatic β-cells, so any changes in this process will lead to diabetes mellitus. Therefore, to control diabetes mellitus, pancreatic β-cells must be protected. Honey polyphenols such as quercetin also helps to protect pancreatic β-cells in numerous studies. For example, quercetin administration at a dose of 10–15 mg/kg for ten days in streptozotocin-induced rats resulted in increment of pancreatic β-cell numbers [98].

6.2. Wound Healing. Honey therapy has been used to treat septic wounds, surgical wound, or wounds of abdominal wall and perineum due to its excellent wound healing properties. Previously, it is also being used in treating abrasion, amputation, and burns [58, 73, 99]. In general, the oedema, inflammation, and exudation that commonly occur in all types of wounds were reduced by honey in order to improve the wound healing effects. The growth of epithelial cells and fibroblasts was also stimulated by honey [67, 100].

In Iran, the EBH was topically applied on wounds created on rabbits. As the results, the oedema and necrosis seems to lessen, and infiltrations of polymorphonuclear and mononuclear cell become fewer. The wound contraction also seems to improve, with better epithelialisation, and lower concentrations of glycosaminoglycan and proteoglycan [67]. Moreover, application of the EBH on wounds made on the animal model showed faster healing activity as compared to nitrofurazone or sterilized petrolatum [101].

In another study, EBH, ampicillin ointment, and saline were used to treat full-thickness skin wounds created on buffalo calves. The healing efficacy is superior to EBH-treated wound in comparison with ampicillin and saline treatments where least inflammation, most rapid fibroblastic and angioblastic activity, and epithelialisation were observed [101]. Similarly, a study by Sarkar et al. [66] investigated the effect of EBH on collagen homeostasis restoration in diabetic animal whereby a full-thickness wound was created on streptozotocin-induced rat. Topical application of normal saline, EBH, and povidone iodine on wound was compared. The findings showed that the EBH might be predominantly helpful in synthesis, glycation, deposition regulation, and collagen quality alike normal skin. Honey application was also proven to accelerate diabetic healing process [66].

Moreover, a recent study had shown enhanced healing of electroscalpel-induced wound of Wistar rats by EBH treatment, as compared to silver sulfadiazine which is being used as positive control [68]. Another *in vivo* study also proved that oral administration of EBH to measure the healing of colonic anastomosis in rats, had shown increment of the tensile strength measured by bursting pressure, increased fibroblast count and lowered mean of inflammatory cells count in rats supplemented with honey after the surgical procedure in comparison with the control group [70, 102]. The increase in tensile strength in EBH-treated wound might be caused by the increase in collagen concentration, produced by fibroblasts.

A few *in vitro* studies revealed the substantial antimicrobial activity of SBH, which could also suggests the possible wound healing activity of this honey [71, 103, 104]. In addition, combination of SBH and other substances such as antibiotic ampicillin or garlic extract, rather than these substances alone, showed more effective effects in inhibiting the growth of *S. aureus*, which is the most common pathogenic bacteria causing wound infection [11, 71]. Honey is useful as wound dressing as it helps to stimulate the healing process and can clear the infection quickly for it portrays better cleansing activity. Besides, it has proven anti-inflammatory activity and plays a great role in stimulating tissue regeneration [58, 73, 99].

6.3. Anticancer. Honey, as described by many scientific evidences, may be considered as a great chemopreventive agent. Chemoprevention may be described as the usage of natural or synthetic compounds in order to decrease the risk of cancer development [104].

Scientific evidence has proven that superoxide anion radical and inflammation can cause somatic mutation which will eventually evolve to initiate cancer. Due to its excellent anti-inflammatory activity, the anticancer effect of honey was also being investigated. According to Ahmed and Othman [105], as honey is known with its apoptotic, antiproliferative, antioxidant, anti-inflammatory, estrogenic, and immunomodulatory activity, these might be considered as the possible mechanisms of how honey prevent the progress of the cancer formation.

In one experiment, the human hepatoma cell (HepG2) is treated with the EBH. The results showed that the viability of the cells is greatly reduced in a dose- and time-dependent manner. Hepatic injury may be initiated by oxidant molecule such as nitrogen oxide, through reactive oxygen species (ROS) and lipid peroxidation products, and these molecules may also cause inhibition of apoptosis by various pathways [74]. As expected, the level of radical nitrogen oxide in the culture supernatant was reduced by honey treatment. Therefore, it can be concluded that the anticancer effect of honey might be due to its antioxidant activity, which helps in curbing the initial formation of cancer. In another study, a rat model was induced with mammary cancer; however, oral administration of EBH was proven to prevent the mammary cancer induced with 7, 12-dimethylbenz[a]anthracene (DMBA). An 18-week laboratory test reveals that EBH consumption had significantly lowered the rate of incidence, the efficacy to multiply, and the tumor size in rats of DMBA-induced mammary cancer. In conclusion, the antioxidant activity of EBH might also be the reasons of the protective effect against DMBA-induced mammary cancer [15, 75].

Meanwhile, SBH, which is also known for its antioxidant activity, prevents the induction of colon cancer by azoxymethane (AOM) in rats. Aberrant crypt foci (ACF) act as a biomarker in identifying the colon cancer development. To observe the effect of SBH on ACF, SBH was administered orally (1183 mg/kg body weight) and had proven to reduce the total number of ACF and aberrant crypt and crypt multiplicity. Therefore, SBH is neither harmful nor toxic to the animal [76]. An *in vitro* study was also conducted to screen for the cytotoxic activity of different stingless bee products

against five human cancer cell line, namely, BT474 (ductal carcinoma and lung undifferentiated cancer), HepG29 (liver hepatoblastoma), KatoIII (gastric carcinoma), and SW620 (adenocarcinoma), whereby the crude extracts of SBH showed great cytotoxicity effects towards HepG2 cell line, while propolis crude extracts exhibit high cytotoxicity effects towards all the human cancer cell line [77].

Polyphenols with anticancer effects that can be found in both honeys are quercetin, apigenin, chrysin, and luteolin [37]. The mechanisms that are involved in cancer prevention by these polyphenols include inhibition of cell proliferation [106], modulation of cancer signalling pathways [107], and induction of tumor cell apoptosis [108]. Uncontrolled cell proliferation had caused the cancer cells to increase at a faster rate; therefore, if the uncontrolled cell proliferation can be inhibited or reduced, cancer prevention is more likely to be successful. Polyphenols, which are also known for their antioxidant properties, are very helpful in preventing cell proliferation. For example, chrysin which is an important honey flavone helps to control the cell proliferation by activating p38-MAPK via accumulation of p21Wafi/Cip1 in C6 glioma cells of rats [37, 109]. Meanwhile, apigenin prevents proliferation of pancreatic cell as it helps to reduce levels of cyclin A, cyclin B, and the phosphorylated forms of cdc2 and cdc25, thereby arresting the G2/M phase of the cell cycle [37, 110].

6.4. Treatment for Ocular Diseases. A study demonstrated that the bacterial flora in the conjunctival sac of patients with cataract and scheduled for vitrectomy was successfully eradicated after continuous administration of 25% sterile honeydew honey (EBH) for 7 days [78]. Similarly, Albietz and Lenton [79] pointed to the fact that the EBH significantly reduced formation of the whole colony-forming units in the eyelids and conjunctivae in patients with dry eye syndrome after one and three months of therapy.

Recently, Bashkaran et al. [80] compared the anti-inflammatory and antioxidant effect of the EBH with a corticosteroid preparation (prednisone) in the treatment of alkali burn in rabbit eyes and confirmed the anti-inflammatory effects of this honey on experimental animals, with no significant difference between the two treatments. In a clinical study, in which 16 patients with oedema of the corneal epithelium who had not been indicated for a surgical procedure were subjected to local therapy with the EBH. The result indicated that all corneas manifested an immediate regression of the corneal oedema with EBH treatment [81]. Previously, a preliminary study was conducted by Vit [82], where SBH drop was applied on selenite-induced rats and had resulted in reducing the rate of the cataract progress, in 20% of the rats in the group that received honey for the opacification treatment. Despite that, SBH was also proven to reduce the infection time for eye diseases caused by *Staphylococcus aureus* and *Pseudomonas aeruginosa*, via *in vivo* studies using rabbit as the animal model [83].

Polyphenols inhibit the angiogenesis and inflammatory cytokines and also eye diseases by suppressing formation of reactive oxygen species (ROS) and upregulate antioxidative enzymes [111]. Polyphenols with anticataract properties are mainly flavonoids, phenolic acids, carotenoids, and vitamins [112, 113]. Quercetin and catechin are the specific polyphenols that portray promising effects against ocular diseases [84, 114]. Both quercetin and catechin are previously detected in SBH and EBH [14, 37] and could also be found in fruits and vegetables [84, 114]. Quercetin (3,3′,4′,5,7-pentahydroxyflavone) can inhibit hydrogen peroxide-induced cataracts while catechin derivatives inhibit cataracts in rats induced by *N*-methyl-*N*-nitrosourea [114].

6.5. Effects on Fertility. Honey has been shown to portray positive effects on fertility by means of enhancing the hormones related to fertility [84]. Sexual dysfunction and impaired fertility are among the adverse effects that have been associated with cigarette smoking, especially in males. A laboratory study reported that reproductive toxicity induced by cigarette smoke was alleviated by the oral consumption of EBH at 1.2 g/kg/day, which raised the successful intromission and ejaculation percentage in rats, thus resulting in increased fertility and mating rates [84]. Besides that, noise stress is one of the stress factors, which is known to hinder male reproductivity. Noise stress has negatively impacted the cells of testicular tissue by promoting the growth of apoptotic and necrotic cells. However, with EBH and vitamin E treatment, it was observed that the cells of mature male Wistar rats which have been exposed to noise stress are enhanced and found healthy. This suggests that EBH and vitamin E have good effects on the testis parenchyma as EBH and vitamin E reduced apoptosis and necrosis in cells affected by noise stress and thereby increased cell growth and activity [85].

In one study, the intake of Tualang honey (EBH) produced by *Apis dorsata* to restraint-stressed pregnant rats at 1.2 g/kg daily resulted in favourable condition on several parameters, especially in the level of corticosterone, outcome of pregnancy, and adrenal histomorphometry [86]. It is reported that alteration of gonadotropin levels in female rats was significantly restored with EBH administration at 1 g/kg. Regularly, diabetic rats suffer from low sperm quality; however, SBH administration to diabetic rats portrays improvement in sperm quality, with additional protective effects on spermatogenesis process even in diabetic condition. In nondiabetic rats, administration of SBH helps to increase the count of epididymal sperm count, the motility, and viability of the sperm. This could suggest for potential property of the fertility enhancer in the SBH. In conclusion, the SBH could be a great alternative in order to prevent sperm and testis damage in diabetic rats [88].

7. Conclusion

This current review of the SBH in comparison with EBH revealed a significant role of the SBH as a therapeutic agent in various health issues such as antidiabetic, wound healing, anticancer, treatment of eye diseases, and also in fertility.

Studies have proven that the SBH has excellent potential and portrays beneficial effects as antimicrobioal, anticancer agent, improving hypertension, lipid profiles, and with some studies showing better antidiabetic effects than the EBH *in vivo*. In addition, other therapeutic properties are also at par or even significantly better from the much-researched EBH. In order to provide a major comprehensive understanding on the potential uses and benefits of the SBH, more systematic studies need to be carried out. Previously, studies on SBH were done using tissue cultures, animal models, and clinical trials to demonstrate the biotherapeutic activities. However, the information on its beneficial effects is still scarce. With regard to its benefits to human health, more scientific studies and clinical trials on human subjects need to be conducted to relay a better understanding in evaluating the potential of stingless bee honey as a therapeutic agent.

There are a plethora of areas to study for researchers who are interested in the biotherapeutic effects of the SBH. In terms of quality control, methods to authenticate pure SBH need to be developed. A rapid and destructive analysis technique is required to avoid possible adulteration by irresponsible manufacturers. In return, it is expected that a quality standard can be established by the identification of its bioactive component. Since SBH is rich in antioxidants, these substances might account for some of the potential health benefits portrayed by them. Therefore, innovative efforts should be taken to fully explore and utilize these benefits. Honey-based products should be diversified, such as making supplement capsules or tablets which contain probiotics isolated from the SBH that can aid in gastrointestinal health. These properties should also be made readily in the form of topical creams or gels for wound healing or other purposes.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Phenolic Rich Fractions from Mycelium and Fruiting Body of *Ganoderma lucidum* Inhibit Bacterial Pathogens Mediated by Generation of Reactive Oxygen Species and Protein Leakage and Modulate Hypoxic Stress in HEK 293 Cell Line

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Ganoderma lucidum (*G. lucidum*) fungus (Family Ganodermataceae) is widely used as a traditional medicine in China, Japan, and many Asian countries on account of its numerous medicinal properties such as antioxidant, anticancer, antimicrobial, energy enhancing, and immunostimulatory. This broad spectrum of therapeutic effects exhibited by *G. lucidum* is ascribed to its abundance in several classes of chemical constituents, namely, carbohydrates, flavonoids, minerals, phenolics, proteins, and steroids which possess substantial bioactivities. The aim of the current study was to prepare phenolic rich fractions (PRFs) from aqueous extract of the Indian variety of *G. lucidum* mycelium and fruiting body. These fractions were assessed for their antioxidant capacity by TPC (total phenolic content), TFC (total flavonoid content), FRAP (ferric reducing antioxidant power), and ABTS [2,2-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid] assays. Quantification of flavonoids and nucleobases present in the fractions was carried out by high-performance thin layer chromatography (HPTLC). The antibacterial activity of the fractions was evaluated against *Escherichia coli*, *Salmonella typhi*, and *Staphylococcus aureus*. The antibacterial mechanism of action of the PRFs was established to be generation of reactive oxygen species and leakage of proteins within bacterial cells. Additionally, the protective effect of the PRFs in counteracting hypoxia was observed in HEK 293 cell lines.

1. Introduction

Modern medicine is witnessing a paradigm shift, with the focus being majorly centered on herbal sources as an alternative to synthetic medicines. Herbs comprise innumerous active metabolites which are capable of a vast array of pharmacological and therapeutic effects. Such metabolites include alkaloids, flavonoids, phenolics, terpenoids, carbohydrates, and esters. [1]. Among these bioactive constituents, phenolic compounds extracted from medicinal herbs and dietary plants have been reported to possess antioxidant, anticarcinogenic, anti-inflammatory, etc. effects [2]. Natural sources of medicinal importance encompass Kingdom Fungi which comprises multiple species of highly diverse and ubiquitous organisms that abundantly contain bioactive primary and secondary metabolites. These bioactive metabolites, either alone or in conjugation with other compounds, have been proven to promote human health owing to their multifarious pharmacological effects [3]. One such important medicinal mushroom among fungi is *Ganoderma lucidum* (*G. lucidum*) which has been documented in traditional Chinese medicine and traditional Tibetan medicine to be a highly effective therapeutic. Ancient oriental medical literature has reported the use of *G. lucidum* for its significant revigorating and energy-enhancing properties [4].

G. lucidum is a wood-rot basidiomycete classified within the family Ganodermataceae of Polyporales order. Its constituent bioactive compounds and their associated therapeutic properties against a number of health disorders

have globally established *G. lucidum* as a pharmacologically and commercially important medicinal mushroom [5]. For instance, polysaccharides like ganoderans A, B, and C were reported to exhibit anticancer, anti-inflammatory, antimicrobial, and immunomodulatory effects [6]. Triterpenes present in *G. lucidum* were found to enhance its medicinal properties like antioxidant and lipid-lowering effects [7, 8]. HPTLC analysis performed on aqueous, ethyl acetate, and methanolic extracts of *G. lucidum* fruiting body and mycelia proved the presence of antioxidant compounds viz. ascorbic acid, gallic acid, rutin, and quercetin [9]. A study performed on different solvent (acetone, chloroform, methanol, and aqueous) extracts of *G. lucidum* mycelium showed encouraging results against pathogenic bacterial growth [10], which was mainly attributed to its phenolic contents. Similar study done on hexane, dichloromethane, ethyl acetate, and methanolic extracts of *G. lucidum* shed light on its antioxidant and antimicrobial efficacy [11].

In light of the aforementioned therapeutic potential of *G. lucidum*, the present study was undertaken as a novel attempt to characterize phenolic rich fractions (PRFs) from Indian high-altitude variety of *G. lucidum*. Aqueous extracts as well as diethyl ether, ethyl acetate, and residual phenolic fractions were prepared from *G. lucidum* mycelia and fruiting body. All these extracts and fractions were characterized by various antioxidant assays and high-performance thin layer chromatography (HPTLC). The extracts and fractions were also evaluated for potential bioefficacy, i.e., antibacterial activities against three major bacterial pathogens, namely, *Escherichia coli* (*E. coli*), *Salmonella typhi* (*S. typhi*), and *Staphylococcus aureus* (*S. aureus*), and protective effect against hypoxia stress on the human embryonic kidney HEK 293 cell lines.

2. Materials and Methods

2.1. Chemicals and Reagents. All chemicals and reagents used in the experimental procedures were purchased from Sigma-Aldrich (USA) and belonged to HPLC grade. Water used for maceration, preparation of extract, and in other purposes was of Millipore grade (Merck, USA).

2.2. Bacterial Strains and Cell Lines. The microbial strains used were *E. coli* (ATCC 9837), *S. typhi* (clinical isolate from All India Institute of Medical Sciences, Delhi, India), and *S. aureus* (ATCC 12600). Cell line used was the human embryonic kidney HEK 293 cell line.

2.3. Preparation of Aqueous Extracts of G. lucidum Mycelium and Fruiting Body. G. lucidum mycelium (GLM) and fruiting body (GLF) powder were commercially procured from M/s Aryan Enterprises, Delhi, India (batch numbers GLMP-1603 and GLFP-1603, respectively). Aqueous extracts of GLM and GLF were prepared by maceration. Briefly, in separate experimental settings, 500 grams each of GLM and GLF powder were dissolved in 5 L of water, accompanied by intermittent stirring. A small quantity of absolute ethanol was added to prevent contamination, and the vessel was covered and kept undisturbed at room temperature. After 2 days, the aqueous phase was filtered and centrifuged, and the supernatant was collected. The supernatant was concentrated by freeze-drying in a lyophilizer to obtain pure extracts (GLMaq and GLFAq) which were preserved at 4°C for further use.

2.4. Preparation of Phenolic Rich Fractions from Aqueous Extracts of GLM and GLF. Phenolic rich fractions were prepared from GLMaq and GLFaq by solvent fractionation technique using diethyl ether and ethyl acetate. In this method, GLMaq and GLFaq were dissolved in water to obtain a sample concentration of 200 g/L. Then, diethyl ether was added to the solution and kept on intermittent shaking. The fraction was separated using a separating funnel, following which two distinct layers, i.e., organic layer and aqueous layer, were formed. After complete fractionation, the upper organic layer was separated and the aqueous phase was introduced to a rotary evaporator to remove traces of diethyl ether. Furthermore, the process was repeated by mixing the aqueous layer with ethyl acetate, to obtain the second phase of fraction. Similarly as above, the resultant upper organic layer was separated, and traces of ethyl acetate were removed in a rotary evaporator to leave behind the residual phenolic fractions. All the three fractions obtained were then appropriately labeled (diethyl ether fractions: GLMdee and GLFdee; ethyl acetate fractions: GLMea and GLFea; and residual phenolic fractions: GLMph and GLFph), lyophilized, and preserved at 4°C for further use. All the aforementioned six phenolic rich fractions were collectively termed as "PRFs."

2.5. Determination of Antioxidant Capacities

2.5.1. Total Phenolic Content (TPC). Total phenolic content (TPC) was determined according to a method reported by Kumar et al. [12]. In this assay, different concentrations of gallic acid, ranging from 1 mg/ml to 1 μg/ml, were taken to plot the standard curve. TPC values of GLMaq, GLFaq, and PRFs were expressed as microgram gallic acid equivalent (GAE) per gram of extract (μg GAE/g of extract). Absorbances were measured at 725 nm using a microtiter plate reader. All readings were taken in triplicates.

2.5.2. Total Flavonoid Content (TFC). Total flavonoid content (TFC) was determined according to a method described elsewhere [12]. Briefly, different concentrations of rutin ranging from 1 mg/ml to 1 μg/ml rutin were taken as standards. TFC values of GLMaq, GLFaq, and PRFs were expressed as microgram rutin equivalent per gram of extract (μg rutin/g of extract). Absorbances were measured at 510 nm, and analysis was performed in triplicates.

2.5.3. Ferric Reducing Antioxidant Power (FRAP) Assay. FRAP of the various GLMaq, GLFaq and PRFs, was determined using a method described previously [13]. Here, different concentrations of trolox ranging from 400 μM to

12.5 μM were taken as standards. FRAP values of GLMaq, GLFaq, and PRFs were expressed as μM trolox equivalent per g of extract (μM trolox/g of extract). The absorbances were measured at 593 nm, and analysis was performed in triplicates.

2.5.4. ABTS Assay. ABTS (2,2-azino-bis(3-ethylbenzo thiazoline)-6-sulfonic acid) free-radical scavenging assay was carried out as described previously [13]. Different concentrations of trolox ranging from 800 μM to 50 μM were taken as standards. ABTS free-radical scavenging values of GLMaq, GLFaq, and PRFs were expressed as μM trolox equivalent per gram of extract (μM trolox/g of extract). Absorbances were recorded at 734 nm, and analysis was performed in triplicates.

2.6. Characterization by HPTLC

2.6.1. HPTLC Analysis of Nucleobases. Separation and quantification of nucleobases, namely, thymine (T), uracil (U), adenine (Ad), cytosine (C), and guanosine (Gs), present in GLMaq, GLFaq, and PRFs was accomplished by HPTLC (Camag assembly, Switzerland) using a mobile phase described by Mishra et al. [14]. This mobile phase comprised dichloromethane, methanol, and formic acid in a ratio of 8 : 2.25 : 0.8. Separation was brought about on glass-backed silica gel 60 F_{254} HPTLC plates. The plate was developed at room temperature in a CAMAG twin-trough vertical development chamber, wherein the solvent front was maintained at 85 mm. Following development, the plate was subjected to densitometric scanning at a wavelength of 254 nm in the absorption mode, with deuterium as the light source. The quantities of the nucleobases as identified in the various extracts were calculated from the corresponding peak areas generated by winCats software (version 1.4.4.6337).

2.6.2. HPTLC Analysis of Flavonoids. Separation of flavonoids, namely, quercetin (Q), gallic acid (G), ascorbic acid (As), and rutin (R), present in GLMaq, GLFaq, and PRFs was conducted using HPTLC (Camag assembly, Switzerland). The mobile phase used here was ethyl acetate : dichloromethane : formic acid : glacial acetic acid : methanol in a ratio of 10 : 10 : 1 : 1 : 2 [9]. Analytical parameters for separation and quantification of flavonoids were retained as described in Section 2.6.1.

2.7. Antibacterial Efficacy

2.7.1. Preparation of Bacterial Inoculums. Glycerol stocks of three bacterial strains: *E. coli*, *S. typhi*, and *S. aureus* were revived by incubating them in nutrient broth for 16 to 18 h at 37°C, in an incubator-cum-shaker (Orbitek, India), at 90 rpm. Pure culture obtained was plated on a nutrient agar medium using the streak plate method, and the plates were preserved at 4°C for further experimentation.

2.7.2. Screening of Fractions for Antibacterial Activity. The inhibitory activity of GLMaq, GLFaq, and PRFs against the aforementioned bacterial pathogens was determined by Kirby–Bauer disk diffusion methodology [15]. Here, 100 μg of the GLMaq, GLFaq, and PRFs prepared in nutrient broth were loaded onto disks made out of Whatman No.1 filter paper and were appropriately dried under sterile conditions. These disks were placed upon nutrient agar medium plates swabbed with bacterial strains. The plates were incubated at 37°C for 16 to 18 h in an incubator-cum-shaker (Orbitek, India), at a shaking speed of 90 rpm, following which potential zones of inhibition around the disks were observed. Disks loaded with 5 μg of kanamycin were taken as positive control for all the three bacterial pathogens under consideration.

2.7.3. Determination of Minimal Inhibitory Concentration. 16 to 18 h-old bacterial colonies cultured upon the nutrient agar medium were diluted in 0.8% physiological saline to prepare a 0.1 McFarland suspension. Then, the bacterial cultures were inoculated in test tubes containing 5 ml nutrient broth. Different concentrations ranging from 10 to 100 μg of the specific GLF and GLM aqueous extracts and PRFs showing sufficient zones of inhibition were added to the nutrient broth. The tubes were then incubated at 37°C for 16 to 18 h in an incubator-cum-shaker (Orbitek, India), at a shaking speed of 90 rpm. The lowest or minimal concentration of a sample showing inhibition of bacterial growth was adjudged to be its minimal inhibitory concentration (MIC) for a given pathogen [15]. Kanamycin and pure nutrient broth medium were used as the positive and negative controls, respectively.

2.7.4. Generation of Reactive Oxygen Species within Bacterial Cells. Levels of ROS generated within a bacterial pathogen's cellular environment upon exposure to specific GLF and GLM aqueous extracts and PRFs screened for potent antibacterial activity were measured using 2',7'-dichlorofluorescein diacetate (DCFDA) [16]. Briefly, the bacterial cell density was adjusted to 10^5 CFU/ml using 0.8% physiological saline, and the cultures were inoculated in nutrient broth tubes. Various concentrations of the samples close to their respective MIC values and above were added to these nutrient broth tubes. The tubes were then incubated at 37°C for 3 h. Following this, the cultures were centrifuged at 4°C for 15°min at a speed of 500° ×g. The supernatant obtained was treated with 50 μM DCFDA and incubated for 1 h at 37°C, in dark. A control group not treated with DCFDA was taken as control. The amount of ROS generated in the microbial samples was measured using a Cary Eclipse fluorescence spectrophotometer at an excitation wavelength of 485 nm and emission wavelength of 528 nm. All readings were taken in triplicates.

2.7.5. Protein Leakage in Bacterial Cells. The protein leakage induced within the bacterial pathogen's cellular environment upon exposure to specific GLF and GLM aqueous

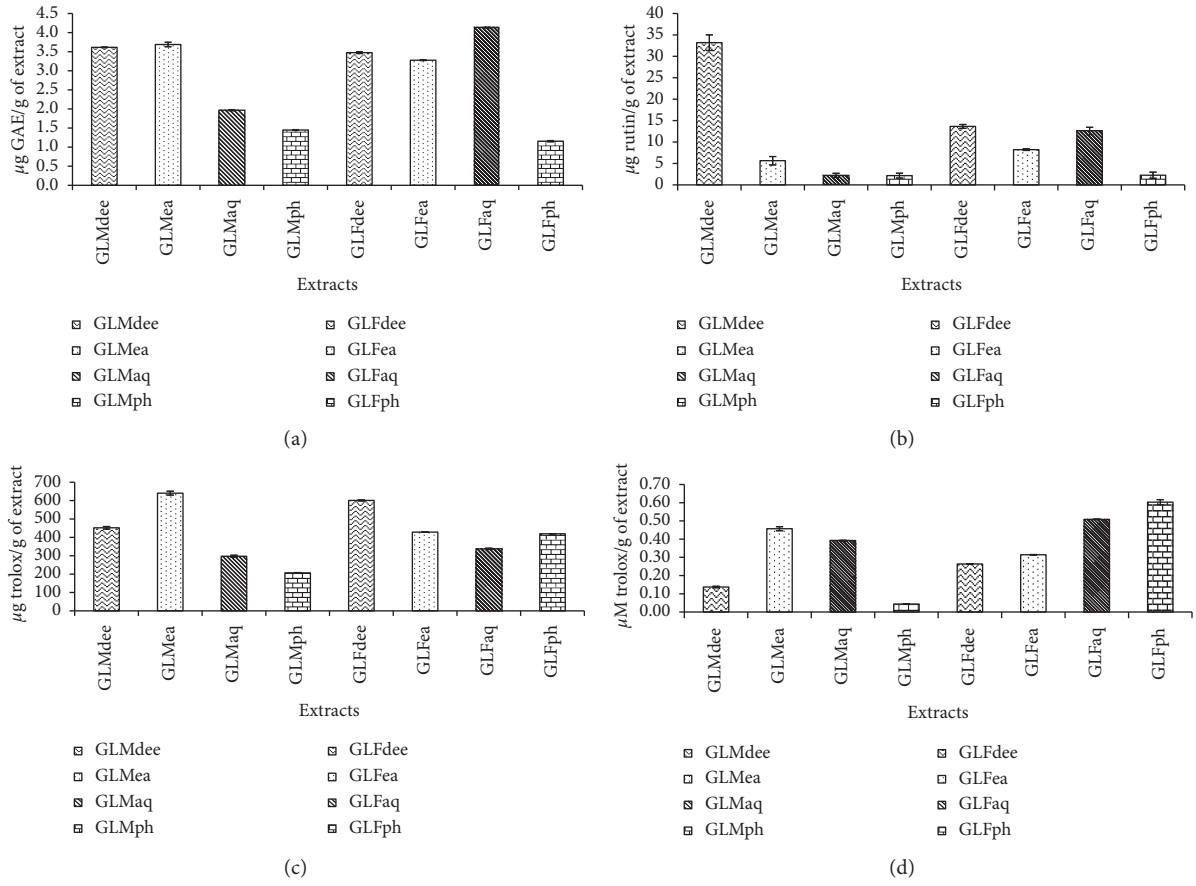

Figure 1: Total phenolic content (TPC) (a), total flavonoid content (TFC) (b), ferric reducing antioxidant power (FRAP) (c), and 2,2-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) free-radical scavenging potential (d) of aqueous extracts and PRFs of *Ganoderma lucidum* mycelium and fruiting body.

extracts and PRFs screened for potent antibacterial activity was evaluated by Bradford's method using a protocol reported elsewhere [16, 17]. According to this method, 16 to 18 h cultures of the bacteria cultured in nutrient broth were washed with 0.8% physiological saline by centrifugation at 10,000 rpm for 20 min, followed by resuspension in saline. The bacterial cells were then treated with GLF and GLM fractions, with concentrations ranging from their respective MIC values and above. After incubation for 3 h, each bacterial suspension was centrifuged at 12,000 rpm for 15 min, and the supernatant obtained was analyzed for protein content using Bradford assay. Concentration of protein was estimated at a wavelength of 595 nm using bovine serum albumin (BSA) as standard. All readings were taken in triplicates.

2.8. Protective Effect against Hypoxia. The ameliorative effect of GLMaq, GLFaq, and PRFs on restoring cellular viability of human embryonic kidney (HEK) 293 cell lines under hypoxic stress conditions was evaluated. Herein, the HEK 293 cell lines were maintained in high glucose DMEM (Dulbecco's minimal essential medium) (pH 7.2), supplemented with antibiotics (gentamycin sulphate (100 mg/L) and penicillin (100 mg/L)) and enriched with foetal bovine serum (FBS) (10%, v/v) as described by Kirar et al. [18]. Cells were cultured in 96-well microtitre plates (Nunc, Denmark), maintaining a cell density of 10^5 cells/cm^2 and were kept in an incubator (Galaxy 170R, New Brunswick) with 21% O_2, i.e., normoxic condition. Experiments were conducted on cells that were 70 to 80% confluent.

To assess the restorative action of the GLM and GLF extracts and PRFs, the experimental HEK 293 cells were divided into four groups for each type of the aforementioned sample. The grouping pattern was as follows: (i) normoxia control (N), (ii) normoxia supplemented with the GL extract or fraction under study (N + sample), (iii) hypoxia control (H), and (iv) hypoxia supplemented with the GL extract or fraction under study (H + sample).

Cells placed under normoxic conditions were maintained according to the parameters as described in preceding paragraphs, whereas, for subjecting to hypoxia stress, the HEK 293 cells were retained in a low oxygen environment (1% O_2, 5% CO_2, and 94.5% N_2) for 24 h in a Galaxy 48R incubator (New Brunswick). For supplementation studies, different concentrations, i.e., 50, 75, 100, and 125 μg/ml of the GLM and GLF samples were used. Cellular viability of the cells was evaluated using MTT assay [19]. The dose of a particular GL sample facilitating maximum restoration of cellular viability of the HEK 293 cell lines under hypoxic insult was inferred as its respective optimum dose.

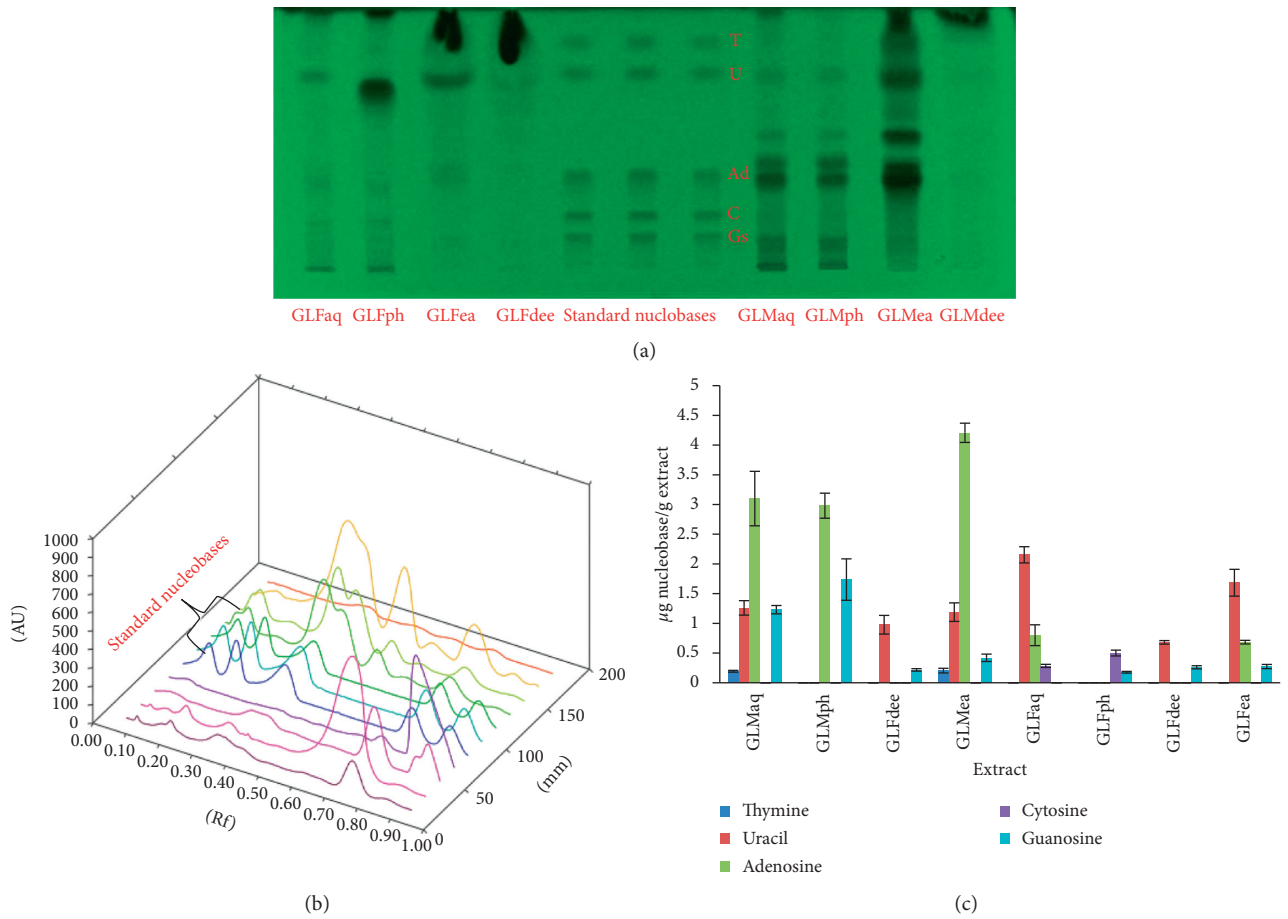

Figure 2: HPTLC chromatogram (a), 3D spectra (b), and quantities (c) of nucleobases identified in aqueous extracts and PRFs of *Ganoderma lucidum* mycelium and fruiting body (T: thymine; U: uracil; Ad: adenine; C: cytosine; Gs: guanosine).

2.9. Statistical Analysis. Statistical analysis for all the assays was carried out using SPSS software V.20 (IBM, USA). Statistical significance was verified at $P < 0.05$, for the data depicting dose-dependent antibacterial mechanisms of action.

3. Results and Discussion

3.1. Preparation of GL Aqueous Extracts and PRFs. Regarding aqueous extracts, GLMaq gave a higher percentage yield by weight (5.7%) than GLFaq (2.8%). This observation is in concurrence with previous reports where aqueous extract of *G. lucidum* mycelium, prepared by accelerated solvent extraction, gave better yield than *G. lucidum* fruiting body [13]. Between the diethyl ether fractions, GLFdee gave a fairly better yield (3.8%) than GLMdee (3.28%). In case of the ethyl acetate fractions, GLFea gave a much better yield (3.7%) than GLMea, which was only 0.64%. The residual phenolic fractions for both GLF and GLM gave exceedingly higher values, yielding 92.4% for GLFph and 92.16% for GLMph.

3.2. Antioxidant Capacity of GLM and GLF Aqueous Extracts and PRFs

3.2.1. TPC Assay. The total phenolic content of the GLMaq, GLFaq, and PRFs as estimated in terms of μg GAE/g extract is shown (Figure 1(a)). GLFaq had the highest TPC content (4.13 μg GAE/g of extract), followed by GLMea (3.69 μg GAE/g of extract) and GLMdee (3.61 μg GAE/g of extract).

3.2.2. TFC Assay. TFC assay carried out for all the GLMaq, GLFaq, and PRFs brought out the highest total flavonoid content in GLMdee (54.89 μg rutin/g of extract), followed by GLFdee (29.64 μg rutin/g of extract) and GLFaq (27.86 μg rutin/g of extract) (Figure 1(b)).

3.2.3. FRAP Assay. FRAP assay proved GLMea to have the best antioxidant potency (640.5 μM trolox/g of extract) in terms of ferric-reducing ability, closely followed by GLFdee (601 μM trolox/g of extract) and GLMdee (452 μM trolox/g of extract) (Figure 1(c)).

3.2.4. ABTS Free-Radical Scavenging Assay. According to this assay, maximum ABTS free-radical scavenging activity was that of GLFph with a value of 0.6 μM trolox/g of extract, followed closely by GLFaq (0.51 μM trolox/g of extract) and GLMea (0.46 μM trolox/g of extract) (Figure 1(d)).

Natural sources like fungi or plants are complex in nature and hence display variation in the types and

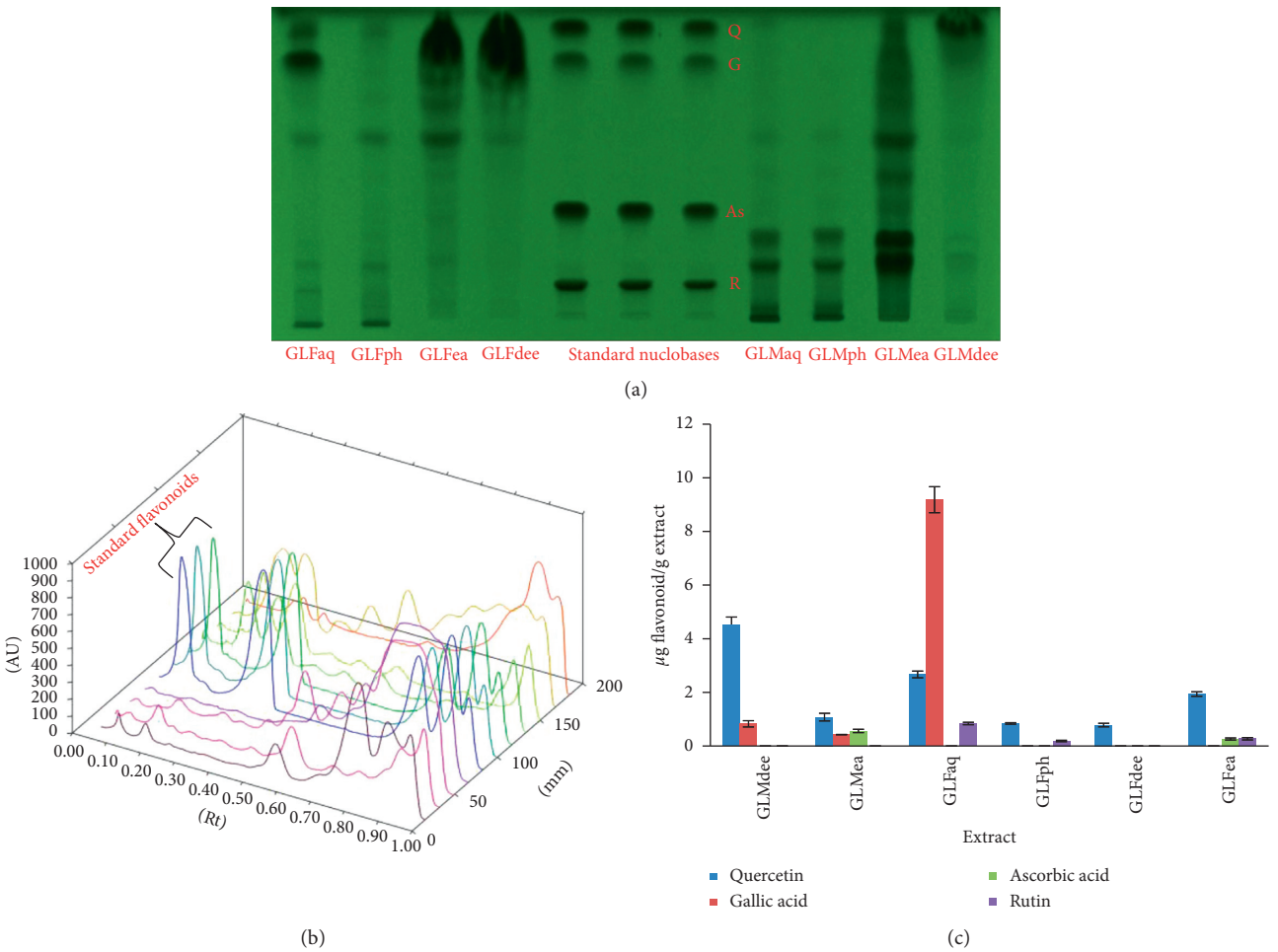

Figure 3: HPTLC chromatogram (a), 3D spectra (b), and quantities (c) of flavonoids identified in aqueous extracts and PRFs of *Ganoderma lucidum* mycelium and fruiting body (Q: quercetin; G: gallic acid; As: ascorbic acid; R: rutin).

quantities of bioactive compounds present in them, which may further be segregated into either primary or secondary class of metabolites [20]. In view of this complexity, it is preferable to evaluate the overall biological or therapeutic activity of a natural extract instead of individually studying the bioactive nature of each constituent metabolite. Thus, in the present study, representative antioxidant assays were carried out to establish the antioxidant potential of GLMaq, GLFaq, and PRFs. Though any particular trend pertaining to the antioxidant capacity of the phenolic fractions was not obtained, it was clearly observed that both the mycelium and fruiting body fractions possessed substantial antioxidant potential. Similar observations were acquired for aqueous, methanolic, and ethyl acetate fractions of *G. lucidum* where both mycelium as well as the fruiting body exhibited appreciable antioxidant activities [13].

Additionally, it can be stated here that the antioxidant potential of the diethyl ether and ethyl acetate fractions of GL mycelium and fruiting body in terms of TPC, TFC, and FRAP potential might be ascribed to the presence of numerous semipolar compounds in them. Such semipolar compounds have previously been reported to possess seemingly high antioxidant potential [13].

3.3. Characterization by HPTLC

3.3.1. HPTLC Analysis of Nucleobases. Characterization of aqueous extracts and PRFs of GLF and GLM by HPTLC clearly proved the presence of two or more nucleobases in all the samples under study, as seen in the chromatogram (Figure 2(a)). GLMea and GLMaq fractions were the richest in terms of nucleobases containing thymine, uracil, adenine, and guanosine. GLFph was the least endowed fraction containing lower quantities of cytosine and guanosine. The peaks derived from corresponding 3D spectra are shown in Figure 2(b). The quantities of nucleobases present in GLMaq, GLFaq, and PRFS were calculated in terms of μg nucleobase per gram of extract (Figure 2(c)). It is worthwhile to mention here that nucleobases like adenine, cytosine, and uracil have been reported to contribute significantly towards antifungal and antibacterial activities [21]. Thus, these results provided a lead to further investigate the antibacterial activity of the GL samples under study.

3.3.2. HPTLC Analysis of Flavonoids. HPTLC investigation of GLM and GLF aqueous extracts and PRFs confirmed the occurrence of one or more flavonoids in all the samples

Table 1: Diameters of inhibition zones (in mean (mm) ± SD) after treatment with aqueous extracts and PRFs of GLM and GLF; MIC values (in mean (μg) ± SD), in parentheses.

	GLMaq	GLMph	GLMdee	GLMea	GLFaq	GLFph	GLFdee	GLFea
S. typhi	8 ± 0.12 (25 ± 1.75)	8 ± 0.55 (30 ± 2.1)	8.5 ± 0.47 (45 ± 3.15)	6.3 ± 0.15 (60 ± 7.12)	8.2 ± 0.76 (65 ± 3.46)	7 ± 0.32 (30 ± 1.08)	—	—
E. coli	7.5 ± 0.3 (35 ± 2.36)	8.6 ± 0.5 (30 ± 4.52)	6.5 ± 0.11 (55 ± 3.81)	6.5 ± 0.15 (55 ± 1.18)	—	7.4 ± 0.1 (40 ± 2.19)	6.5 ± 0.28 (55 ± 2)	—

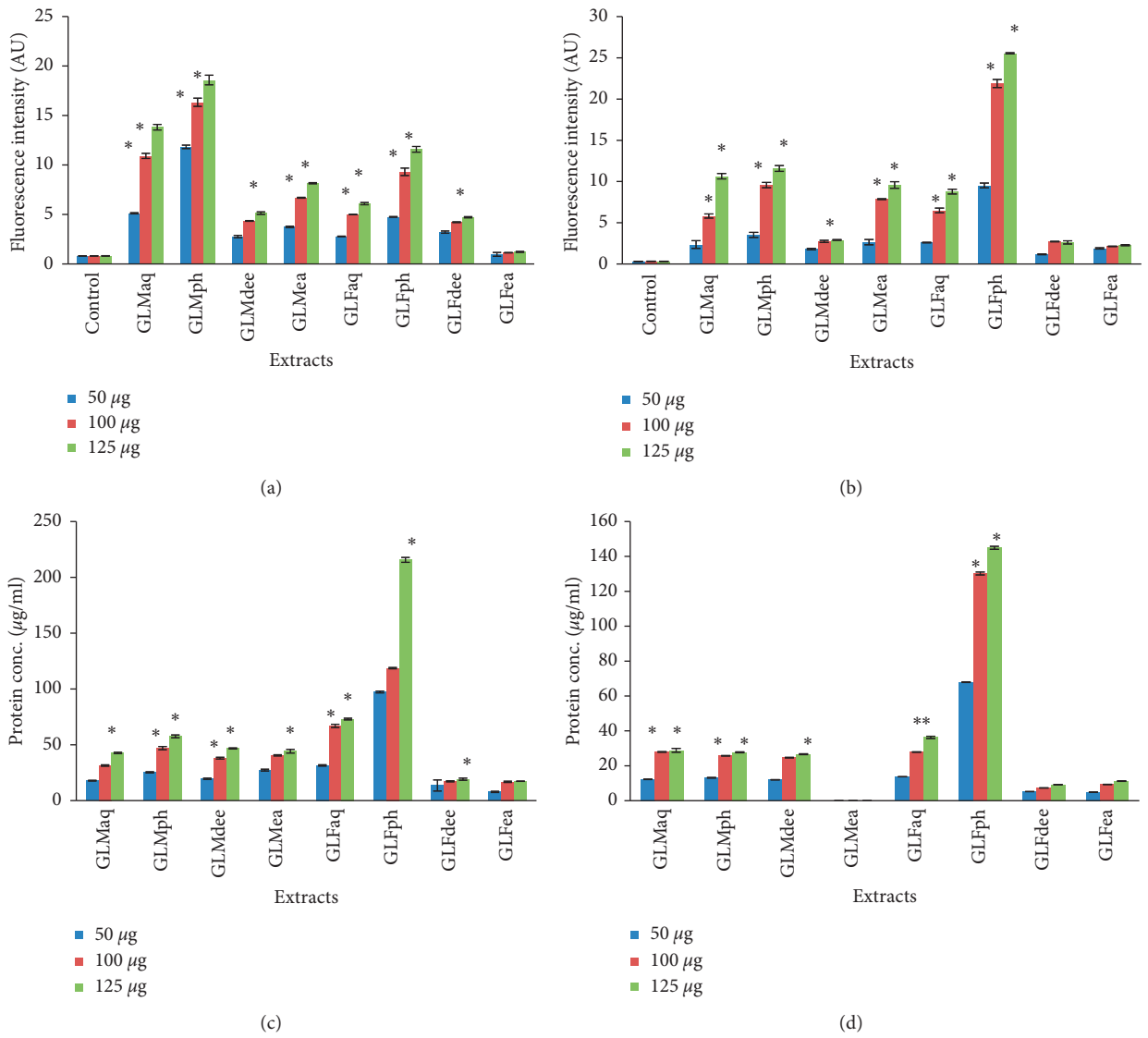

Figure 4: ROS generation in *Escherichia coli* (a), ROS generation in *Salmonella typhi* (b), protein leakage in *Escherichia coli* (c), and protein leakage in *Salmonella typhi* (d) induced by aqueous extracts and PRFs of *Ganoderma lucidum* mycelium and fruiting body (*represents significant change at the level of $P < 0.05$).

except GLMaq and GLMph as depicted in the HPTLC chromatogram (Figure 3(a)). It was observed that GLFaq was the most endowed extract, consisting of quercetin, gallic acid, and rutin. GLMdee had appreciable quantities of quercetin and gallic acid. GLMea contained lesser quantities of quercetin, gallic acid, and ascorbic acid. The peaks derived from corresponding 3D spectra are shown in Figure 3(b). The quantities of flavonoids present in GLMaq, GLFaq, and PRFS were calculated in terms of μg flavonoid per gram of extract (Figure 3(c)).

Further, it can be stated that the appreciable flavonoid contents in GLMdee, GLMea, and GLFaq fractions can be correlated to the significant antioxidant potential of these fractions, as described in previous sections. This is in

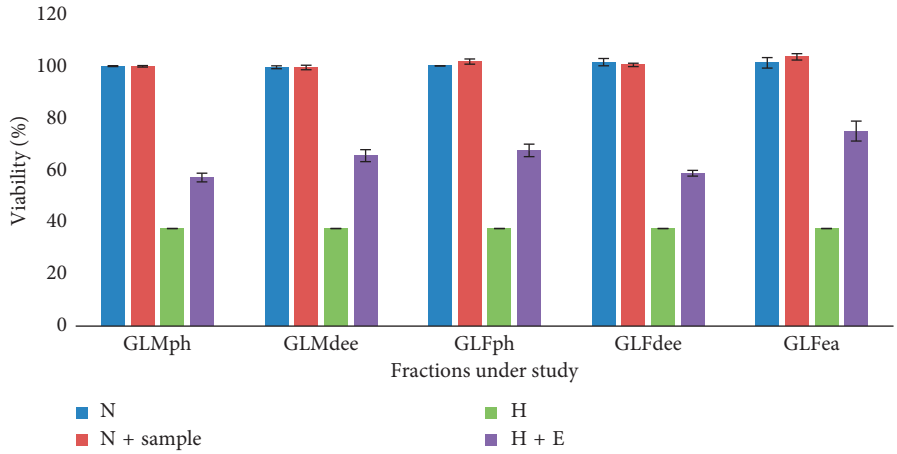

Figure 5: Cellular viability as determined by MTT assay. GLMph (100 μg/ml), GLMdee (75 μg/ml), GLFph (50 μg/ml), GLFdee (100 μg/ml), and GLFea (50 μg/ml) improved cellular viability to 57%, 65.5%, 67.52%, 58.73%, and 75%, under hypoxia. N: normoxia, N + sample: normoxia supplemented with respective phenolic rich fraction of *Ganoderma lucidum* fruiting body or mycelium, H: hypoxia, H + E: hypoxia supplemented with respective phenolic rich fraction of *Ganoderma lucidum* fruiting body or mycelium (numbers in parentheses indicate the corresponding optimal dose for each phenolic rich fraction).

concurrence with the observations provided in earlier studies that higher quantities of flavonoids are responsible for better antioxidant behavior of natural extracts [22].

A previous study [21] had reported the inhibitory action of flavonoids like coumarin, quercetin, and kaempferol against bacterial pathogens, viz., *Staphylococcus aureus* and *Pseudomonas* spp. Owing to the confirmation of similar flavonoids in GLMaq, GLFaq, and PRFs under study, the antibacterial activity of these samples was investigated.

3.4. Antibacterial Efficacy

3.4.1. Screening of GL Aqueous Extracts and PRFs for Antibacterial Activity and Determination of MIC. Aqueous extracts and PRFs of GLF and GLM demonstrated variable degrees of antibacterial activities on the pathogenic bacterial strains being studied. Overall, it was observed that *E. coli* was fairly inhibited by GLMaq, GLMph, GLMdee, GLMea, GLFph, and GLFdee. *S. typhi* was inhibited by almost all the fractions except GLFdee and GLFea. Antibacterial action was much less effective on *S. aureus* as compared to the aforementioned two pathogens. Table 1 specifies the zones of inhibition in millimetre (mm) and corresponding MIC values in micrograms (μg).

3.4.2. ROS Generation by GL Extracts and PRFs in Pathogenic Bacteria. DCFDA is a fluorogenic dye used to measure hydroxyl, peroxyl, and ROS activity within cellular environment on account of its easy permeability within cells. This assay relies on the principle that, after diffusion into the cell, DCFDA gets deacetylated by cellular esterases to a nonfluorescent compound, which is later oxidized by ROS into 2',7'-dichlorofluorescein (DCF). DCF, being a highly fluorescent compound, can be detected by fluorescence spectroscopy with maximum excitation and emission spectra of 495 nm and 528 nm, respectively [17].

Herbal extract-induced generation of free radicals, e.g., ROS, peroxide radicals, and superoxide radicals, within bacterial cells, leading to cellular membrane damage, has been reported to be a crucial antibacterial mechanism of action [17]. Keeping this in view, levels of ROS generated within the bacterial cells were estimated using DCFDA. As can be observed from Figure 4, almost all the GLM and GLF PRFs showed a positive linear trend for ROS generation in both *S. typhi* and *E. coli*, incrementing over a concentration range of 50 to 125 μg/ml of the fractions. However, this trend was not observed in the case of GLFea, where concentration increase did not seem to be very effective in ROS generation. The best results were displayed by both the residual phenolic fractions, i.e., GLFph followed by GLMph.

3.4.3. Protein Leakage Induced by GL Extracts and Fractions within Pathogenic Cells. The antibacterial mode of action of the GLM and GLF aqueous extracts and PRFs was further analyzed by investigating their effect on protein leakage within bacterial cells. Results as depicted in Figure 4 showed that, in the case of *E. coli,* there was a dose-dependent influence of extracts on induced protein leakage over a concentration range of 50 to 125 μg/ml. The maximal protein leakage values were reported for GLFph and GLFaq. However, as per results obtained for *S. typhi*, the results were less effective than those for *E. coli*. GLMea did not have any apparent effect on protein leakage upon *S. typhi* cells. Here, GLFph and GLFaq showed the most substantial effect in inducing protein leakage within bacterial cells.

The better effectiveness of the GLM and GLF aqueous extracts and PRFs in inducing protein leakage within bacterial cells could be attributed to their rich content of flavonoids and nucleobases as verified from HPTLC analyses in the preceding sections. This observation can be corroborated with an earlier study stating that phytoconstituents exert appreciable influence on antimicrobial effect of natural extracts [23, 24].

3.5. Cytoprotective Effect against Hypoxia. It is already established in previous literature that hypoxic stress resulting due to renal diseases damages regulatory mechanisms and causes further progression of kidney disease. Low oxygen tension complicates the pathophysiology, sometimes leading to renal failure [25, 26].

Keeping this in perspective, the cytoprotective potential of GLMaq, GLFaq, and PRFs in imparting ameliorative action to HEK 293 cells, against hypoxia-induced cell death was evaluated in a dose-dependent manner where the concentration of the fractions ranged from 50 to 125 μg/mL. It was observed that exposure to hypoxic stress induced cellular death by reducing cell viability to less than 40% in HEK 293 cell lines. This was in stark contrast to both the groups maintained under normoxia conditions (N and N + sample) where cell viability was ~100%. However, upon supplementation with GLMph, GLMdee, GLFph, GLFdee, and GLFea, cell viability was observed to be substantially improved.

It was discerned that GLFea and GLFph fractions imparted maximum cytoprotective action by revamping HEK 293 cellular viability to 75% and 67.52%, respectively, at optimal doses of 50 μg/mL. This was followed by GLMdee, GLFdee, and GLMph fractions which recuperated viability to 65.5%, 58.73%, and 57%, respectively. The optimal doses of GLMdee, GLFdee, and GLMph fractions were established to be 75, 100, and 100 μg/mL, respectively (Figure 5). The ability of these fractions in modulating cell viability in stress could be accredited to the presence of nucleobases in them as described in preceding sections. This is in line with a previous study that reported the role of nucleobase supplementation in reduction of apoptosis [27].

4. Conclusion

The current study clearly indicates that the aqueous extracts and phenolic rich fractions prepared from *Ganoderma lucidum* fruiting body and mycelia displayed appreciable antioxidant and free-radical scavenging effects, as proven by various assays. Also, the fractions were proven to impart remarkable inhibitory potential against bacterial pathogens by instigating the generation of reactive oxygen species and protein leakage within the bacterial cells. This observation was in coherence with the presence of flavonoids and nucleobases in these fractions, which have been widely reported to possess antimicrobial efficacy. Some of the phenolic rich fractions markedly improved cellular viability of HEK 293 cells under hypoxic stress. These results collectively taken together could widen the prospect of using natural source-derived compounds as therapeutic solutions for the treatment of various diseases.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Niosome: A Promising Nanocarrier for Natural Drug Delivery through Blood-Brain Barrier

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Niosomes (the nonionic surfactant vesicles), considered as novel drug delivery systems, can improve the solubility and stability of natural pharmaceutical molecules. They are established to provide targeting and controlled release of natural pharmaceutical compounds. Many factors can influence on niosome construction such as the preparation method, type and amount of surfactant, drug entrapment, temperature of lipids hydration, and the packing factor. The present review discusses about the most important features of niosomes such as their diverse structures, the different preparation approaches, characterization techniques, factors that affect their stability, their use by various routes of administration, their therapeutic applications in comparison with natural drugs, and specially the brain targeting with niosomes-ligand conjugation. It also provides recent data about the various types of ligand agents which make available active targeting drug delivery to the central neuron system. This system has an optimistic upcoming in pharmaceutical uses, mostly with the improving availability of innovative schemes to overcome blood-brain barrier and targeting the niosomes to the brain.

1. Introduction

Several brain and CNS diseases such as neurological diseases (meningitis, encephalitis, viral, bacterial, protozoan, and fungal and worm infections), neurological disorders (epilepsy, seizures, trauma, Parkinson, multiple sclerosis, dementia, Alzheimer, mononeuropathy, polyneuropathy, and myopathy), and brain tumors (cerebral tumors and glioma) are associated with mortality. These problems needed proper drug delivery for treatment [1]. Several approaches to create novel CNS drug-delivery systems are primarily due to the anatomical and physiological characteristics of the blood-brain barrier (BBB) [2–4]. Neural tissues of the brain are protected in contradiction of neurotoxic agents and variation in blood structure that are important for regular purpose of the neurons that covered through BBB. Most organs in our body, apart from the brain and spinal cord, are perfused by capillaries lined with endothelial cells which need small pores to let the small molecules move fast into the organ interstitial fluid from the circulation [5]. In the brain arteriole, ECs are connected to each other by continuous tight junctions (TJs), known as zonula occludens, which cover the paracellular pathway [6, 7]. This can efficiently block the free polar solutes from paracellular pathways and so cast off admission to brain interstitial fluid. Therefore, the BBB let the small particles to break over the brain through the blood stream such as lipophilic solutes or those that pass in the brain by an active transport apparatus, mainly with crucial nutrients, precursors, and cofactors [8–11]. BBB can be transported into the brain endothelium by several mechanisms, such as BBB peptide transport mechanisms. Previous studies suggest that this mechanism is the principal

attitude for drug delivery to the brain. Generally, there are three systems for drug delivery to the brain [8, 12] including systemic absorption through BBB and nasal and intracerebroventricular (ICV) administration. On the other hand, each one of these methods has several disadvantages which are listed below.

Disadvantages of systemic absorption through the BBB are given below [2, 8]:

(1) Systemically administered therapies may fail to reach therapeutic levels in the CNS.

(2) In some cases, intravenous therapy may cause systemic toxicity.

(3) In neurodegenerative disease, BBB efficiency decreases. It may cause brain vascular damage as well as initiating BBB dysfunction or reducing of blood carriage into the brain which obstacles drug delivery into the brain. This also follows through a chronic medical condition called hypoxia.

Disadvantages of nasal administration are given below [13, 14]:

(1) May cause irritation to the nasal mucosa

(2) Nasal congestion as the result of allergies may obstacle absorption of the drug

(3) Drug delivery efficiency decreases as molecular weight increases

(4) Excessive use of this method causes mucosal damage

Disadvantages of ICV administration are given below [15–17]:

(1) ICV administration requires a device entrenched by neurosurgeons in the subgaleal space under the scalp and associated with the ventricles inside the brain through an outer catheter.

(2) High intracranial pressure throughout drugs' administration using the ICV method; this is the case particularly after higher volumes are directed in excess of a short period. This can cause the patient endure risky and even intolerable pain.

However, as it is mentioned above, systemic absorption through the BBB is easier than the other methods. To suggest an alternative drug-delivery system, two provisions must be deliberated. The drug must be released in a steady rate; it must release in an adequate quantity of the active component at the desire site. The previous methods do not chance these requirements. To accomplish these requirements, nanostructures are a promising approach to improve natural drug delivery through the brain.

The nanostructure could change the characteristics and the behavior of the natural drugs inside the body after administration. It can protect natural drugs from degradation [18, 19] and in delivering them to their target sites [19]. Also, prolongation of blood circulation time [20], enhancement of drug accumulation in the pathological tissues [21], and decreasing toxicity can organize the application of the nanostructure for numerous pharmaceutical uses [22]. On the other hand, drug delivery efficiency can be increased through ligand bindings and applying the natural drug in different surfaces of the body. This is performed by passive diffusion which is contingent on lipophilicity and molecular weight or through active transport systems by interacting with the blood components having the role of a mediator between the blood carrier and the brain. Nanostructures behave differently depending on the surface area and the ligand bindings as well as its mediator [23, 24]. This is typical in treating pathological diseases such as glioblastoma and neurodegenerative diseases. Depending on the biomaterial and morphology of the drug-delivery system, various nanoparticles can be prepared from polymers, metals, nanogel and colloidal systems, and particular and vesicular systems. Vesicular systems include vesicular drug-delivery system that has covering liposomes, ethosomes, transfersomes, bilosomes, and niosomes [25, 26]. Amongst these systems, particularly, liposomes and niosomes are used in treating pathological disease whose sufficiency can be enhanced by targeting permeable components passing through tissues via blood vessels [27–29]. This method has high efficiency compared with the reticuloendothelial system (RES) that could be dysfunctional by removing vesicular particle from the plasma. The important part of applying a successful drug delivery to the brain is performing through increasing the circulation time. This review will focus on niosomes as nanoparticles that are designed for improving their medicinal purposes and consequently to overcome BBB and procedures to progress natural drug delivery efficiency.

2. Structure and Components of Niosomes

2.1. Components of Niosomes. The two major components utilized for the preparation of niosomes exist: lipid compounds (cholesterol or L-α-soya phosphatidylcholine) and nonionic surfactants. Lipid compounds are utilized to provide unbending nature, appropriate shape, and adaptation to the niosomes [30]. The part surfactants assume the main part in the development of niosomes. The accompanying nonionic surfactants for the most part utilized for the arrangement of niosomes are the spans (spans 60, 40, 20, 85, and 80), tweens (tweens 20, 40, 60, and 80), and Brij (30, 35, 52, 58, 72, and 76) [31–33]. Nonionic surfactant-based vesicles or niosomes are the capable drug carriers which require a bilayer structure that are made mostly by nonionic surfactant and lipid compounds (cholesterol or L-α-soya phosphatidylcholine) incorporated in an aqueous phase.

2.1.1. Nonionic Surfactant. Niosomes are multilamellar vesicles prepared from synthetic nonionic surfactants. The nonionic surfactant has a hydrophilic head group and a hydrophobic tail which affect the entrapment efficiency of the drug. As the HLB value of surfactant increases, therefore, alkyl chain rises, thereby, the size of niosomes rises. Therefore, HLB rate 14–17 is not suitable for niosomes formulation [34, 35]. Beyond amount of surfactant, the surfactant structure played main role for stability and

privation vesicle aggregation of niosomes by repulsion of steric or electrostatic force [35]. The effect of surfactant's structure in niosomes formation explains with critical packing parameter (CPP) that definite with the following equation [36]:

$$\text{CPP} = \frac{V}{I_c} \times A_o, \quad (1)$$

CPP is the critical packing parameter, V is the hydrophobic group volume, I_c is the critical hydrophobic group length, and A_o is the area of the hydrophilic head group. The type of micellar structure was predicted by the critical packing parameter value as assumed:

If CPP <1/2 formation of spherical micelles

If 1/2 < CPP <1 formation of bilayer micelles

If CPP >1 formation of inverted micelles

Several sorts of surfactant are applied in preparation for niosomes such as alkyl ethers and alkyl glyceryl ethers, sorbitan fatty acid esters, polyoxyethylene fatty acid esters, and block copolymer (pluronic L64 and pluronic p105). To achieve these structures, some input energy, for example, mechanical (stirring or sonicates) or heat is required.

2.1.2. Alkyl Ethers and Alkyl Glyceryl Ethers. Alkyl ethers are good vesicle-forming nonionic surfactants. They are stable, relatively nonallergic to skin and compatible with other surfactants [37]. Because of their great constancy, they can be applied to encapsulate peptides and proteins [38].

(1) Polyoxyethylene 4 Lauryl Ether (Brij 30). Brij 30 has an HLB value of 9.7 and a phase transition temperature of <10°C [39, 40]. Unlike other alkyl ether derivatives, that reduce vesicle formation in the presence of cholesterol, Brij 30 formed large unilamellar vesicles when combined with 30 mmol/L cholesterol. Nevertheless, it is discordant with benzocaine, tretinoin, and oxidizable medications; meanwhile, with such substances, it causes oxidation leading to discoloration of product. This surfactant does not suit properties to apply for formulation of some drugs and iodides, mercury salts, phenolic ingredients, salicylates, sulfonamides, and tannins (Figure 1).

(2) Polyoxyethylene Cetyl Ether (Brij 58). Brij 52, 56, and 58 are cetyl derivatives of polyoxyethylene that can be used for vesicle formation.

Among them, Brij 58 has developed because of its capacity to arrange inverse vesicles, which are suitable for possible pharmacological requests. The HLB value of Brij 58 remains 15.7 [39] (Figure 2).

(3) Polyoxyethylene Stearyl Ethers (Brij 72 and Brij 76). These are some derivatives of polyoxyethylene ether with worthy vesicle-forming possessions. Especially, Brij 72 and Brij 92 can be used to form multilamellar vesicles with high encapsulation effectiveness which are higher than Brij 76 because of low HLB = 4.9 compared to Brij 76 = 12.4 [39, 41].

2.1.3. Sorbitan Fatty Acid Esters. These are some products of polyoxyethylene esters that are mostly applied in maquillages in water-based products. Sorbitan esters are frequently mentioned to as spans. Their gel transition temperature rises as the length of the acyl chain increases. Hence, sorbitan monolaurate (Span 20) with a C9 chain has a liquid transition at 24°C; sorbitan monopalmitate (Span 40) with a C13 chain has a gel transition temperature of 46-47°C; sorbitan monostearate (Span 60) with a C15 chain has a gel transition temperature of 56–58°C. Vesicles made with these higher molecular weight spans are principal to fewer permeable and more stable to osmotic grades [42]. The molar ratio of cholesterol to span and length of the lipophilic were critical factors for entrapment of drugs into niosomes [35]. Thus, greater encapsulation of acyclovir was described in niosomes that was made using a cholesterol (span 80 ratio of 1 : 3) [43] although high encapsulation of colchicine and 5-fluorouracil was stated in niosomes prepared by cholesterol (span ratio of 1 : 1) [44]. Fang et al. [45] reported that Span 40 was essential in a proniosomal formulation of estradiol to improve its infusion through the skin. A decline in setup efficiency of retinyl palmitate was described as the length of the lipophilic chain increase in the order of Span 40, Span 60, and Span 85.

2.1.4. Polyoxyethylene Fatty Acid Esters. Polysorbates are oily liquids derived from ethoxylated sorbitan esterified with fatty acids. Mutual trade names for polysorbates contain Scattics, Alkest, Canarcel, and Tween. Tweens 20, 40, 60, and 80 are mutual polysorbates which are applied for niosomes' construction [31, 32].

2.1.5. Pluronic L64 and Pluronic p105. Pluronic is a water-soluble nonionic surface-active agent, in which the triblock construction contains polyethylene oxide (PEO) and polypropylene oxide (PPO) segments with the PPO block in the middle and PEO block of equal lengths on either side of the PPO block [46]. Pluronic is arranged in a linear EO-PO-EO triblock copolymer structure. The pluronic L64 surfactant through a structural formulation of $EO_{13}PO_{30}EO_{13}$ and the molecular weight of 2900 g·mole^{-1} also and pluronic P105 surfactant by a structural formulation of $EO_{37}PO_{56}EO_{37}$ and the molecular weight of 6500 g·mole^{-1} were incorporated to form niosomes [47, 48].

2.1.6. Cholesterol. In the niosomes structures, cholesterol is an amphiphilic compound that can cooperate with surfactant to construct hydrogen bonding among hydroxyl groups of cholesterol with hydrophilic head of the surfactant. This results in improvement in the mechanical rigidity of vesicles and membrane cohesion and the leakiness of membrane and finally increases the entrapment efficiency of the niosomes. Cholesterol amount in niosomes influences the structures of niosomes and physical possessions and affects the entrapment efficiency, time circulation, and release of payload. According to the previous studies, it is revealed that the use of cholesterol in preparation of niosomes and its quantities is required to be adjusted depending on the physical and

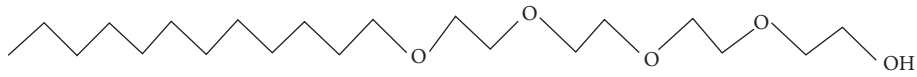

Figure 1: Brij 30.

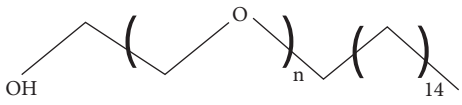

Figure 2: Brij 58.

chemical features of surfactants and the future medicines' type. The interface of cholesterol with surfactant in the bilayer of niosomes is because of hydrogen bonding (Figure 3).

2.1.7. Charge-Inducing Molecule. Some charged molecules are added to niosomes for increasing the steadiness of niosomes through electrostatic repulsion which avoids aggregation and coalescence. The negatively charged molecules applied in niosomes arrangements are diacetyl phosphate (DCP) and phosphatidic acid. Stearylamine (STR) and stearyl pyridinium chloride are the famous positively charged molecules applied in niosomes construction. 2.5–5 molar % concentration of charged molecules is acceptable as high concentration can prevent the niosomes creation.

2.1.8. Hydration Medium. Phosphate buffer at different pH values is frequently used in the hydration medium for the construction of niosomes. The selected pH of the hydration medium is contingent on the solubility of the medicine being encapsulated. Thus, pH 5 phosphate buffer is considered in the preparation of ascorbic acid niosomes [49], whereas pH 7 phosphate buffer is applied in the preparation of aceclofenac niosomes [48].

2.1.9. Structure of Niosomes. Niosome structures are made on the admixture of surfactant and cholesterol with following hydration in water. The bilayer in niosomes is prepared for a nonionic surfactant with its hydrophilic ends exposed on the outside and inside of the vesicle, while the hydrophobic chains express each other within the bilayer. As shown in Figure 4, because of high interfacial tension between water and the hydrophobic tail, monomer units aggregate into vesicle, which forms as closed bilayer structures. In order to achieve these structures, some contribution energy, for example, mechanical (stirring or sonicates) or heat, are essential. Therefore, the vesicle holds hydrophilic drugs within the space surrounded in the vesicle and hydrophobic drugs are entrapped within the bilayer itself, while amphiphilic drugs are consistent with drugs lipophilicity fixed in the space between hydrophilic core and lipophilic tail (Figure 4).

2.2. Comparison of Liposomes and Niosomes. Although the fact that the liposomes and niosomes are practically same, both can be employed as part of the focused and managed sedate conveyance framework; property of both relies on structure of the bilayer and strategies for their planning and both enhanced bioavailability and prevention the body leeway. Niosomes are organized from uncharged single-chain surfactant and cholesterol, while liposomes are organized from double-chain phospholipids, and there are major differences in features which exist between liposomes and niosomes (Figure 5).

2.3. Types of Niosomes. Types of niosomes are classified according to three factors: first, basis of function of niosomes size, second, the method of preparation, and third, based on the vesicle size. So, niosomes can be separated to three clusters including small unilamellar vesicles (SUVs, size = 0.025–0.05 μm), multilamellar vesicles (MLVs, size ≥0.05 μm), and large unilamellar vesicles (LUVs, size ≥0.10 μm), which are described in the following subsections (Figure 6).

2.3.1. Multilamellar Vesicles (MLVs). As shown in Figure 6, MLVs are formed from some bilayers adjacent to the aqueous lipid section individually. The estimated dimensions of these vesicles stay between 100 and 1000 nm in diameter. Multilamellar vesicles, because of simple preparation, are reflexively stable upon keeping for extend phases, and appropriate for lipophilic agents, are widely used.

2.3.2. Large Unilamellar Vesicles (LUVs). The approximate sizes of these vesicles are 100–250 nm in diameter. LUV has a high aqueous part to lipid section proportion, so that the bioactive resources can be captured by membrane lipids.

2.3.3. Small Unilamellar Vesicles (SUVs). The approximate sizes of small unilamellar vesicles are 10–100 nm. Small unilamellar vesicles are consisted of several procedures, such as sonication, high-pressure homogenization, and extrusion methods.

2.3.4. Bola-Surfactant Containing Niosomes. In these kinds of niosomes, bola-surfactant compounds require two hydrophilic heads which can link by one or two long lipophilic spacers. The surfactant use in bola-surfactant containing niosomes is prepared of omega hexadecyl-bis-(1-aza-18 crown-6) (bola surfactant): span-80/cholesterol in 2 : 3 : 1 molar percentage.

2.3.5. Proniosomes. As shown in Figure 7, proniosomes are the niosomes formation that consists of water-soluble carriers and surfactants. The proniosomes are dehydrated niosomes constructions which would be hydrated for earlier

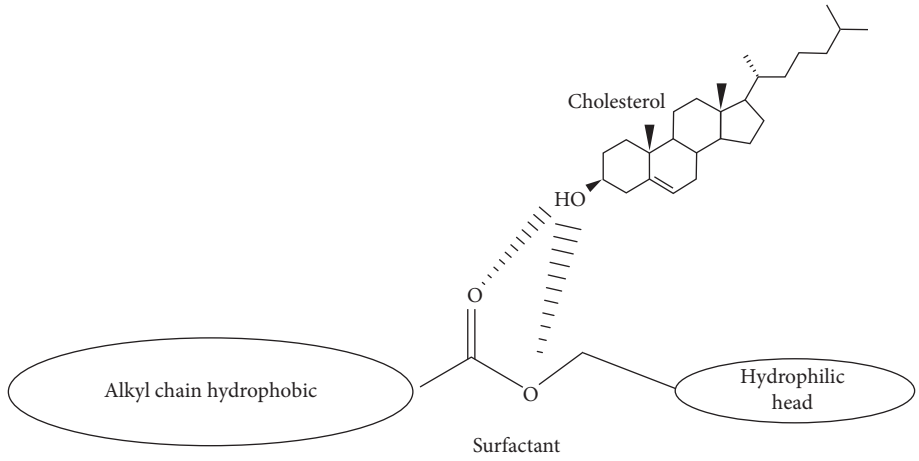

Figure 3: Schematic structural interaction between surfactant and cholesterol.

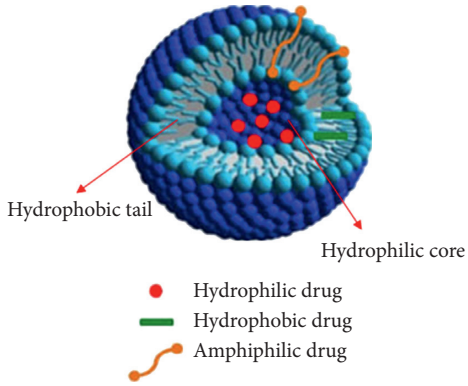

Figure 4: Schematic representation of a niosomes as drug-delivery system [50].

usage. Proniosomes can decrease niosomes problems, for example, aggregation, fusion, and leakage of medication in after a while.

2.3.6. Apsasome. Apsasome includes cholesterol, ascorbyl palmitate, and highly charged lipid such as dihexadecyl phosphate (DCP). It is hydrated by water solvent and sonicated to produce the final product. Apsasome can improve the transdermal drug-delivery systems and decrease the disorders which triggered using reactive oxygen species.

2.3.7. Discome. Large disk-shaped structures or discomes have low cholesterol concentration. It was reported that niosomes were prepared from incubating in cholesteryl poly-24-oxyethylene ether (Solulan C24) at 75°C for 1 h to obtain spherical niosomes. This has caused in the construction of large size approximately 11–60 μm and multilayered vesicular structures. Discomes act as potential drug delivery carriers as sustained release system at the ocular site.

2.3.8. Elastic Niosomes. This type of niosomes could be supple lacking destroying construction, so they have the ability to permit from side to side pores in smaller their size. These vesicles have nonionic surfactants, water, and ethanol. This flexible structure can be used to increase penetration intact skin layers.

2.3.9. Polyhedral Niosomes. This type of niosomes are created by hexadecyl diglycerol ether ($C_{16}G_2$), replacing with any of the nonionic surfactants and polyoxyethylene 24 cholesteryl ether (C_{24}), without cholesterol. These vesicles have unconventional structures which can entrap water-soluble particles. Accumulation of an equimolar volume of cholesterol to the definite surfactant upsurges the curving of the membranes. These conditions result in the formation of spherical vesicles and tubules.

2.3.10. Vesicles in Water and Oil System (V/W/O). Vesicles in water and oil system contain niosomes in water in oil (as external phase) emulsion (v/w/o). This phenomenon is formed by the suspension of niosomes figured from blend of sorbitol monostearate, cholesterol, and solulan C24 (poly-24-oxyethylene cholesteryl ether) to oil phase at 60°C. This results in the formation of vesicle in water in oil (v/w/o) emulsion using cooling to room temperature forming vesicle in water in oil gel (v/w/o gel). This type of niosomes were hired for protein drug delivery and protection from enzymatic degradation after oral administration and controlled release.

2.3.11. Niosomes in Carbopol Gel. In this system, niosomes were prepared from the drug, nonionic surfactant, and cholesterol; then, it is combined in carbopol-934 gel (%1 w/w) base comprising propylene glycol (%10 w/w) and glycerol (%30 w/w).

2.4. Advantages of Niosomes. The application of lipid vesicles and nonionic surfactant vesicles systems for therapeutic goal may suggest advantages as follows:

(i) Niosomes are patient compliance, biodegradable, biocompatible, nonimmunogenic, and low toxicity

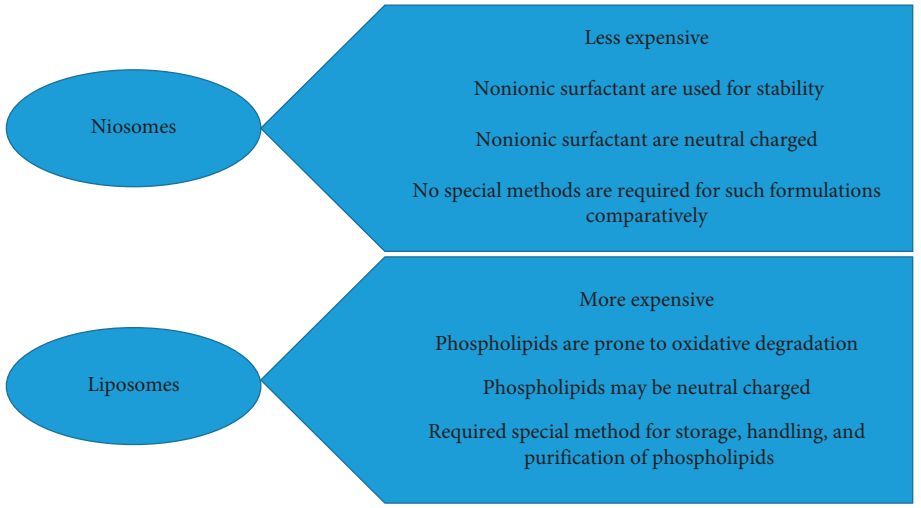

Figure 5: Major differences in characteristics between liposomes and niosomes.

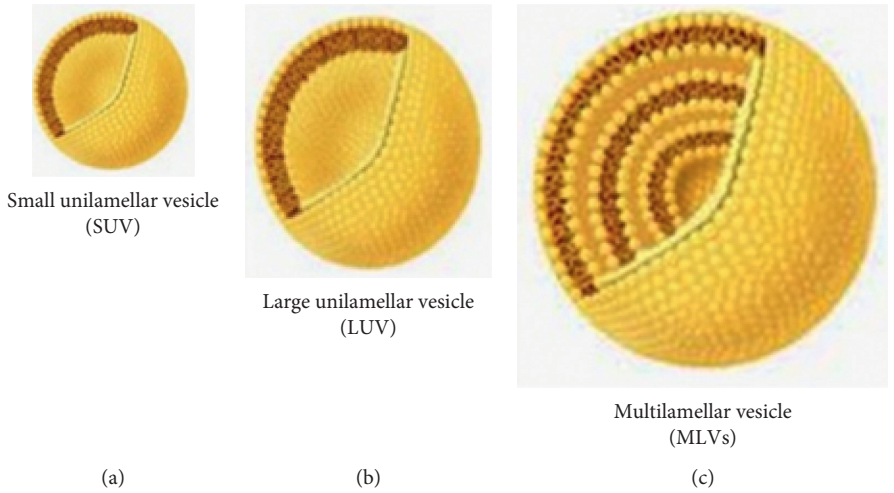

Figure 6: Schematic typical vesicle size of niosomes.

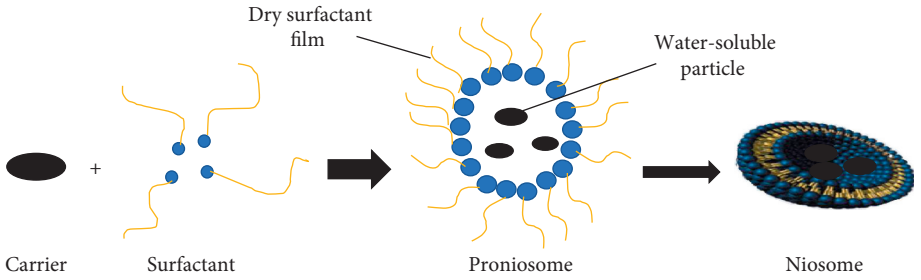

Figure 7: Schematic proniosome and niosomes formation process.

(ii) They are osmotically active and have long storing period

(iii) They perform as a pool to release medication in a steady, organized, and sustained mode

(iv) They provide accommodations for drug molecules with a varied sort of solubility of medication, for example, hydrophilic and lipophilic in addition to amphiphilic medication moieties

(v) Niosomes can rise the stability of the encapsulated medication

(vi) Niosomes can improve the skin penetration of medications

(vii) Niosomes have the capability to overcome BBB and access drug delivery to the brain

(viii) They improve the therapeutic performance of the drug by surface modification and restricting effects to target cells, thereby reducing the clearance of the medication

(ix) Niosomes can expand the oral bioavailability of medications

(x) Surface modification is very simple due to functional groups on their hydrophilic heads

(xi) The characteristics of the vesicle formulation, for example, size, lamellarity, surface charge, concentration, and drug sting, are controllable

(xii) Handling, storage of surfactants, and preparation of noisome do not require special conditions

(xiii) Simple methods are needed for manufacturing and large-scale production of niosomes

2.5. Limitation of Niosomes Drug-Delivery System. Although the used surfactants require further compatibility and low toxicity than other sorts of surfactants, there are not enough studies on the toxicity of niosomes. Previous studies have shown that rise in alkyl chain length of them can result in a reducing in toxicity, while rise in the polyoxyethylene chain length increases the toxicity. The highest restrictions of niosomes in drug delivery are concluded as follows:

(i) The aqueous suspension of niosomes could require inadequate shelf life due to combination, aggregation, permeability of captured medications, and hydrolysis of encapsulated medications

(ii) The preparations of multilamellar vesicles are time-consuming and need distinct tools

2.6. Preparation Methods of Niosomes. The general method of niosomes preparation is by hydration of nonionic surfactants using hydration medium. However, they are prepared by several techniques, such as, transmembrane pH gradient method, lipid layer hydration, reversed-phase evaporation, EER injection, bubbling of nitrogen, sonication, the enzymatic method, the single-pass technique, and microfluidization which are defined here in depth.

2.6.1. Transmembrane pH Gradient Method. Surfactant and cholesterol are ready in chloroform and evaporated under reduced pressure and stream of N_2 to yield a tinny lipid film on the wall of a round-bottomed bottle. The obtained lipid film is hydrated with an acidic compound (usually citric acid). The resulting preparation (multilamellar vesicles) is exposed to freeze-thaw cycles [51–53]. The pH of the sample is then elevated to 7.2 (Figure 8). Bhaskaran and Lakshmi [54] reported that niosomes can be made by this process (entrapment efficiency (EE) = 87.5%).

2.6.2. Lipid Layer Hydration. As shown in Figure 9, surfactant and cholesterol are dissolved in chloroform and evaporated under reduced pressure to produce a thin lipid film on the wall of a round-bottomed flask. The obtained film was hydrated with an aqueous solution of drug at a temperature slightly above the phase transition temperature of the surfactants under moderate shaking conditions [54–57]. Several variables were validated that comprise the mass per batch, angle of evaporation, rotation speed of the vacuum rotary evaporator, and the hydration procedure. The latter variable was developed by various solvents (water, phosphate buffer (PB), and PB/drug) and hydration temperature below and above the gel transition temperature. Sathali and Rajalakshmi prepared terbinafine niosomes by thin film hydration and settled this procedure which, upon sonication, produced small unilamellar niosomes (EE = 85%) [57].

2.6.3. Reversed-Phase Evaporation. The surfactants are dissolved in a mixture of ether and chloroform and added into water phase having the medication emulsified to get w/o emulsion. The resulting mixture is homogenized, and then, organic phase is evaporated [54]. The lipid or surfactant forms a gel first and then hydrates to form spherical stable uniform vesicles [58, 59].

2.6.4. Ether Injection. The mix of surfactant, cholesterol and drug, is dissolved in diethyl ether and over a gauze needle injected gradually into an aqueous phase. The ether solution is evaporated by rotary evaporator above the boiling point of the organic solvent. The large unilamellar vesicles, after evaporation of the organic solvent, are additionally exposed to decrease the size to give single-layered vesicles [58].

2.6.5. Bubbling of Nitrogen. This method is a new procedure for the one-step establishment of niosomes lacking the usage of any organic solvents. Using this buffer, cholesterol and surfactant are spread together (pH 7.4) at 70°C conditions. It presumed by round-bottomed flask with three necks. The first two necks are placed in water-cooled reflux to control the temperature. Due to the sample (cholesterol and surfactant) of homogenized, nitrogen gas was passed from the third neck. Thereby, large unilamellar vesicles were produced. A continuous stream of nitrogen gas bubbles is made and introduced through the dispersion and to give small unilamellar vesicles (Figure 10) [60].

2.6.6. Sonication. In the sonication-mediated procedure, niosomes were prepared by Baillie et al.'s method [61]. The surfactant cholesterol combination is distributed in water phase that contains the drug in flax. The mixture is subjected to probe sonication or bath sonicator for 3 minutes at 60°C until formation of multilamellar vesicles (Figure 11) [62].

2.6.7. The Enzymatic Method. In this strategy, niosomes are produced through an enzymatic route from a mixed micellar solution. Ester bond is sliced by esterases causing breakdown of products such as cholesterol and polyoxyethylene, which

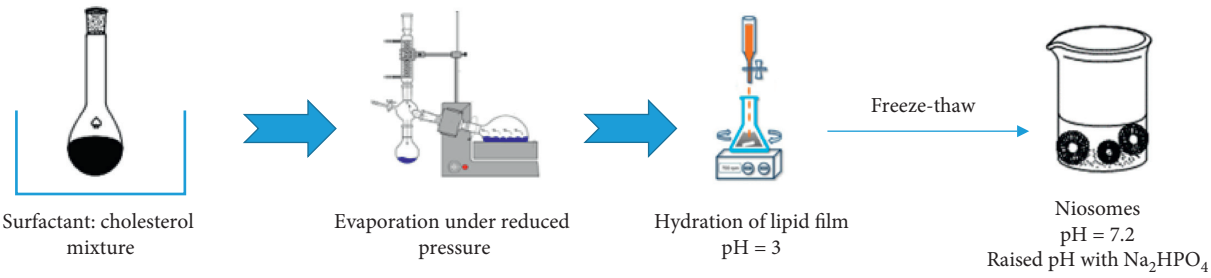

Figure 8: Schematic nonionic surfactant vesicles (niosomes) formation by transdermal pH gradient method.

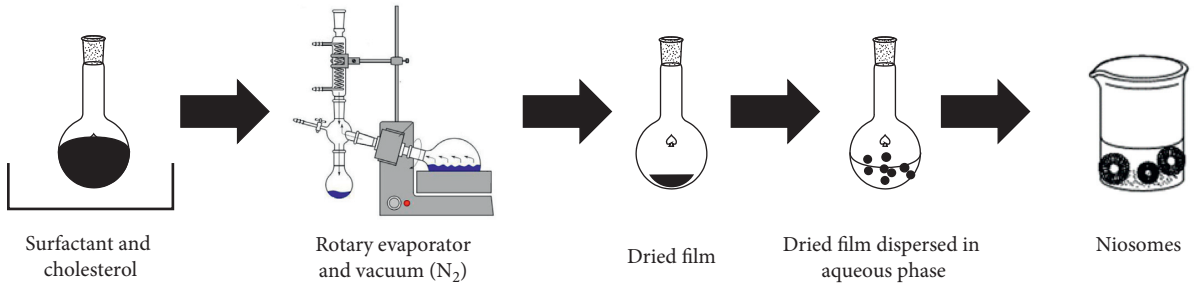

Figure 9: Schematic nonionic surfactant vesicles (niosomes) formation by lipid layer hydration method.

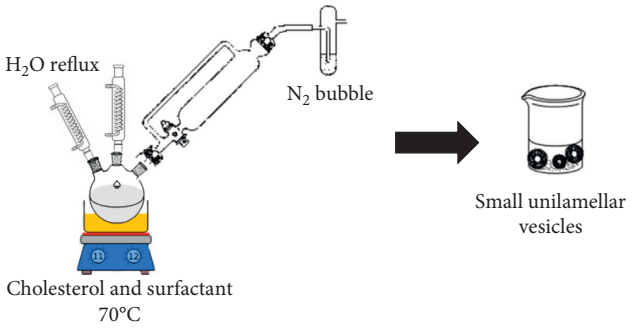

Figure 10: Schematic small unilamellar vesicles (niosomes) formation by bubbling of nitrogen method.

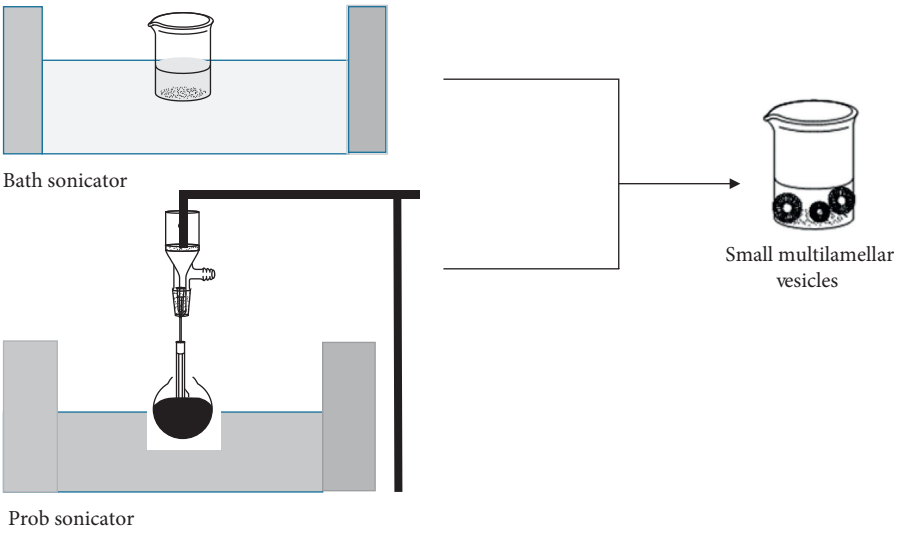

Figure 11: Schematic small unilamellar vesicles (niosomes) formation by sonication method.

are in combination with dicetyl phosphate and other lipids that yield multilamellar niosomes. The surfactants used in this method are polyoxyethylene stearyl derivatives [63] and polyoxyethylene cholesteryl sebacetate diacetate [64].

2.6.8. The Single-Pass Method. It is a patented method including an incessant procedure which contains the extrusion of a solution or suspension of lipids that concluded a porous device and subsequently through a nozzle. It associates homogenization and high-pressure extrusion to provide niosomes with a narrow size supply in the range 50–500 nm [65].

2.6.9. Microfluidization. Microfluidization was a current strategy to give unilamellar vesicles of characterized estimate circulation. Based on the submerged jet principle, in this strategy, two fluidized streams connect at ultrahigh speeds, in correctly characterized smaller-scale channels inside the interface chamber. The impingement of thin-liquid sheet beside a common front was settled such that the energy delivered to the system remains within the area of niosomes establishment. The outcome was a more prominent consistency, reduced size, and well reproducibility of niosomes shape.

2.7. Separation of Unentrapped Drug. Several techniques were developed to achieve the removal of unentrapped solute from the vesicles such as dialysis, gel filtration, and centrifugation.

2.7.1. Dialysis. Dialysis is the main technique used for removal of the unentrapped drug from vesicles. The aqueous niosomal dispersed was evaluated in dialysis tubing against phosphate buffer or normal saline or glucose solution [60].

2.7.2. Gel Filtration. The unentrapped drug is uninvolved by gel filtration of niosomal dispersion through a Sephadex-G-50 column and elution with phosphate-buffered saline or normal saline [60].

2.7.3. Centrifugation. The niosomal suspension was centrifuged, and the above phase was discarded. The pellet was resuspended to give a niosomal suspension free from unentrapped medication [66, 67].

2.8. Characterization of Niosomes

2.8.1. Size and Vesicle Charge. Size and charge of vesicles played main role in their steadiness and drug encapsulation. Size and charge can be determined by a multifunctional zeta potential analysis, in which the size of vesicles was the result of repulsion forces between the bilayers and the entrapped drug. Size of vesicles can be resolute by electron microscopy, molecular sieve chromatography, ultracentrifugation, photon correlation, and optical and freeze fracture electron microscopy [54].

2.8.2. Encapsulation Efficiency. Vesicles were digested with suitable organic solvents such as 50% *n*-propanol or 0.1% triton X-100 and examined with a suitable analytical method [63].

The encapsulation efficiency (EE) percentage is calculated according to the following equation:

$$\text{EE\%} = \frac{\text{amount of drug entrupment}}{\text{total amount of drug}} \times 100. \quad (2)$$

2.8.3. In Vitro Release Study. *In vitro* release studies are performed by release frequency that contains the use of dialysis tubing. The vesicle suspension was combined in an open-end dialysis membrane and placed in a receptor compartment comprising buffer solution with continuous shaking at 25°C or 37°C. Trials are sporadically collected and tested by approved procedures [51, 63, 68].

2.9. Stability. The major complications related to storing of vesicles are photodegradation, aggregation, fusion, and leakage of medication. Ammar et al. [69] reported a stable formulation of tenoxicam as these show high entrapment efficiency (>60%) and retention (>90%) above 30 days. After 30 days, only stable formulations were designated to remain for another 30 days. It was established that there is not significant modification in the size of vesicles after 90 days when equaled with those of newly set niosomes. However, the entrapment efficacy was reduced (10%) after storing [70].

2.10. Therapeutic Applications of Niosomes. Niosomes present an effective drug-delivery system with many pharmaceutical requests (Table 1). Some of them are labeled below.

2.10.1. Protein and Peptide Delivery. Protein delivery after oral administration was restricted via several fences that include proteolytic enzymes, pH, and little epithelial permeability. Niosomes were applied to effectively keep the peptides from gastrointestinal collapse. Pardakhty et al. presented that the oral administration of rh-insulin as niosomal construction based on polyoxyethylene alkyl ethers was secure in contradiction with proteolytic action of chymotrypsin, trypsin, and pepsin. The drug release kinetics was defined by the Baker and Lonsdale equation indicating a diffusion-based delivery mechanism. Niosomes can be established as sustained release oral formulae for transport of peptides and proteins [38, 90].

2.10.2. Transdermal Delivery. Although several drugs were explained for transdermal delivery, niosomes permeation into the skin is still problematic. The flexible noisome construction is an expectant approach to overcome the problem. Transdermal transport of NSAIDs can be the greatest way to escape gastric conflicts. Transfersomes and elastic niosomes are multipurpose kinds of vesicles for transdermal carriage [91]. Manosroi et al. [92] reported anti-

Table 1: Recent studies in drug delivery using niosomes

Application	Surfactant	Method	Drug	Route administration	Reference
Pulmonary delivery	Tween 60	Lipid layer hydration	Ciprofloxacin	Inhaler	[71]
	Span 60	Lipid layer hydration	Clarithromycin	Inhaler	[72]
	Span 60	Sonication	Rifampicin	Intratracheal	[73]
Protein delivery	Brij 92	Lipid layer hydration	Insulin	Oral	[74]
	Span 60	Lipid layer hydration	Insulin	Oral	[75]
	Span 40	Lipid layer hydration	N-acetyl glucosamine	Topical	[76]
	Span 60	Lipid layer hydration	Bovine serum albumin	Oral	[77]
Cancer chemotherapy	Span 60	Lipid layer hydration	Cisplatin		[78]
	Span 60	Lipid layer hydration	5-Flourouracil	Topical	[79]
	Span 80	Sonication	Curcumin		[79]
	Bola surfactant	Lipid layer hydration	5-Fluorouracil	Intravenous	[80]
	Span 60	Lipid layer hydration	5-Fluorouracil	Topical	[81]
Carrier for hemoglobin	Span 60	Lipid layer hydration	Hemoglobin	Intravenous	[82]
Treatment of HIV-AIDS	Span 60	Lipid layer hydration	Lamivudine		[83]
	Span 60	Ether injection	Stavudine		[84]
	Span 60	Lipid layer hydration	Stavudine		[31]
	Span 80	Eether injection	Zidovudine		[85]
Vaccine and antigen delivery	Span 60	Lipid layer hydration	Tetanus toxoid	Parenteral	[86]
	Span 20	Lipid layer hydration	Newcastle disease vaccine		[87]
	Span 60	Lipid layer hydration	Ovalbumin		[88]
	Span 60/span 85	Reversed- phase evaporation	Bovine serum albumin	Topical vaccine	[89]

inflammatory properties of gel comprising new flexible niosomes captured through diclofenac diethylammonium.

2.10.3. Pulmonary Delivery. For asthmatic patients, inhalation treatment is the basis of cure; then, it is restricted by deprived infusion of medication over hydrophilic mucus. Terzano et al. [93] reported that beclomethasone dipropionate as niosomes-based polysorbate 20 was applied for prolonged obstructive pulmonary disease. They reported that the niosomes delivered sustained and targeted delivery, better mucus infusion, and improved therapeutic result.

2.10.4. Carrier for Hemoglobin. Niosomes could be an important transporter for hemoglobin inside the blood. The niosomal vesicles are absorptive to oxygen, and therefore, it performs as a transporter for hemoglobin [94].

2.10.5. Vaccine and Antigen Delivery. Some surfactants have immunostimulatory possessions and have been applied as vaccine adjuvants. The adjuvanticity of niosomes primed from 1-monopalmitoyl glycerol: cholesterol: dicetyl phosphate (5 : 4 : 1) was established in mice that administered a subcutaneous vaccination of ovalbumin or a synthetic peptide comprising a known T-cell epitope and bovine serum albumin [95, 96].

2.10.6. Cancer Chemotherapy. In cancer chemotherapy, targeting with medication transporter system can be allocated into three forms, passive targeting, physical targeting, and active targeting (ligand mediated targeting and physical targeting).

(1) Passive Targeting. Passive targeting facilitates deposition of nanoparticles within the tumor microenvironment, due to particular features inherent to the tumor milieu, not normally existing in healthy tissues [96]. The delivery of nanoparticles was defined by numerous aspects such as tumor microvasculature, nanoparticle size, shape, and surface charge [96].

(2) Physical Targeting. It refers to delivery systems that release a drug only when exposed to a specific microenvironment such as a change in pH or temperature or the use of an external magnetic field.

(3) Active Targeting. It facilitates the active uptake of nanoparticles in the tumor cells themselves. It can engage the versatile molecules to functionalized medication vesicles to identify tumor tissue targets.

By modification of the carrier structure, several modifications are ensued such as change in the molecular size, adjustment of the surface properties, incorporation of antigen-specific antibodies, or attachment of cell receptor-specific ligands. Several ligand-targeting agents were used for brain drug delivery such as low-density lipoproteins, rabies virus glycoprotein (RVG29), transferrin receptor, insulin receptor, propionylated amylose helix, phosphatidylethanolamine, Apo E-reconstituted HDLs, ApoE3 porphyrin-lipid, Angiopep-2.

(4) Active Targeting with Surface Engineered Niosomes, Functionalized with Targeting Ligands. As it is well known, the structure of niosomes is similar to liposome in structure; thus, the surface-functionalized liposome methods can be

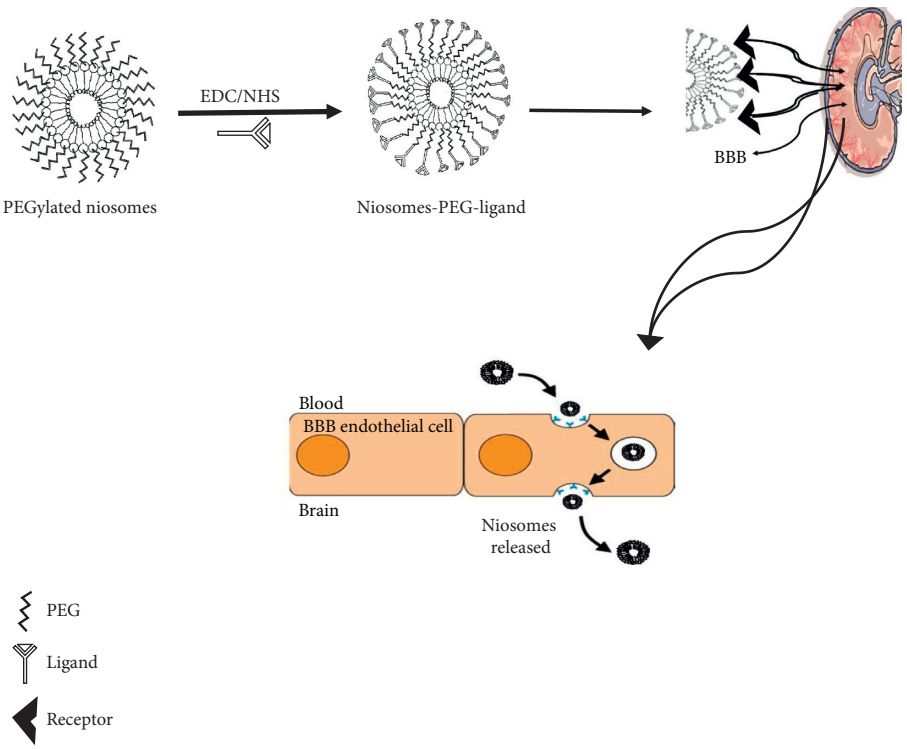

Figure 12: Schematic conjugation of targeting ligand to PEGylated niosomes delivery to BBB.

used to functionalize surface niosomes. Two types of active targeting strategies are widely used for drug targeting to the desired organ/tissue. One of the strategies was that ligands for active targeting have been attached directly to the cholesterol or that ligand was devoted to the distal end of PEG chains in PEGylated niosomes. The other one, the traditional niosomes formulation method, was incorporation of the cholesterol-PEG-ligand conjugate, into the niosomes formulation step [97–99]. Preparation of PEGylated niosomes conjugated with each ligand is shown in Figure 12.

Several studies have been functionalized the niosomes with some of ligands such as glucose-targeted niosomes for transport of vasoactive intestinal peptide (VIP) [90], glucose derivative N-palmitoylglucosamine to develop as probable transporter for brain-targeted delivery of the neuropeptide DynB [100] and Doxorubicin [101]. Also, folic acid and transferrin-targeted niosomes have been made up as possible carrier for CNS drug delivery [102].

3. Conclusion

Nonionic surfactant vesicles were introduced as an innovative and capable method to natural drug delivery. They are mainly composed of nonionic surfactants and cholesterol, and their inside usually comprise a buffer solution at proper pH. They can be made by different approaches, which affect the establishment and the properties of the medication, cholesterol amount, structure, type, and amounts of surfactant. As a drug delivery method, niosomes are osmotically active, less toxic, and chemically stable. Surface modification is comparatively easy on them, due to the functional groups that can add on their hydrophilic heads. Niosomes active targeting to the desire tissue is arbitrated with several therapeutic means as ligand of the distinctive receptor. This system has an optimistic upcoming in pharmaceutical uses, mainly with the increasing availability of new schemes to overcome BBB and targeting the niosomes to the CNS.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Indigofera suffruticosa Mill. (Anil): Plant Profile, Phytochemistry, and Pharmacology Review

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Indigofera suffruticosa Mill. (Fabaceae) is known as anil or anileira and also with other names, due to the production of a blue pigment, which is commonly used for yarn dyeing. It is distributed in some states of Brazil (Pernambuco, Paraíba, Mato Grosso, São Paulo, Bahia, Pará, and others) and is used in the popular medicine as a febrifuge, antispasmodic, diuretic, abortive, analgesic, purgative, or soothing agent against stomach and urinary problems, jaundice, and ulcers and also as an insecticide. In addition, *I. suffruticosa* can be used as animal feed. This review aimed at providing important data on the botanical, distribution, ethnopharmacology, phytochemical, pharmacological, and toxicity of *I. suffruticosa* based on the scientific literature. Information on *I. suffruticosa* was gathered via the Internet (from Elsevier, NCBI, and Sci-Hub) and libraries in the period from February to March 2016. More than 40 chemical compounds have been identified and a few compounds isolated, and the main origins are the essential oils, organic extracts, and aqueous extracts of different parts of the plant. *I. suffruticosa* and its active compounds possess wide pharmacological actions in the literature, such as anti-inflammatory, antibacterial, antifungal, antioxidative, antitumor, antimutagenic, anticonvulsant, gastroprotective, and hepatoprotective activities. Therefore, as an important traditional popular medicine, further studies on *I. suffruticosa* are required for the development of new drugs and therapeutics for various diseases.

1. Introduction

Fabaceae, present in the Brazilian biodiversity, is considered the third largest family of plants, which has about 19,500 species [1], and it is divided into three subfamilies: Mimosoideae, Caesalpinioideae, and Papilionoideae, and it shows a common feature in almost all fruits and vegetables, known as pods [2]. Papilionoideae is a subfamily with greater wealth in the Caatinga. Among diverse species, the *Indigofera* species is taxonomically present [3].

This family is considered of great importance because among the several varieties, many species are used for food purposes and is used as animal feed, latex, resins, raw materials in the manufacture of paints, pesticides, and medicinal drugs, before undergoing processing and purifications (*Dioclea megacarpa*, *Vatairea paraensis*, and *Dipteryx punctata*), and ornamental trees. Examples of species used as food sources are chickpea (*Cicer arietinum*), peas (*Pisum sativum*), beans (*Phaseolus vulgaris*), lentil (*Lens cultivaris*), and soybean (*Glycine max*) [4].

The genus *Indigofera* belonging to the Fabaceae family stands out for being used as fodder [5], green manure, and ground cover [6]. This genus has about 700 species distributed in Asia, tropical Africa, Australia, and North and South America; in Brazil, it is possible to find three species with same popular name "anileira": *Indigofera truxillensis*, *I. hirsuta*, and *I. suffruticosa* [7].

A few decades ago, the investigations of *I. suffruticosa* have focused on their biological activities, including their antitumor [8], anti-inflammatory [9], antimicrobial [10, 11], and antiepileptic [12] properties, but now scientific studies have diversified and deepened their knowledge about this species. These studies evaluated the biological potential of different parts of the plant, with chemical compounds from extractions isolated by using various solvents. This review aimed at providing important data on the botanical, distribution, ethnopharmacology, phytochemical, pharmacological, and toxicity of *I. suffruticosa* based on the scientific literature.

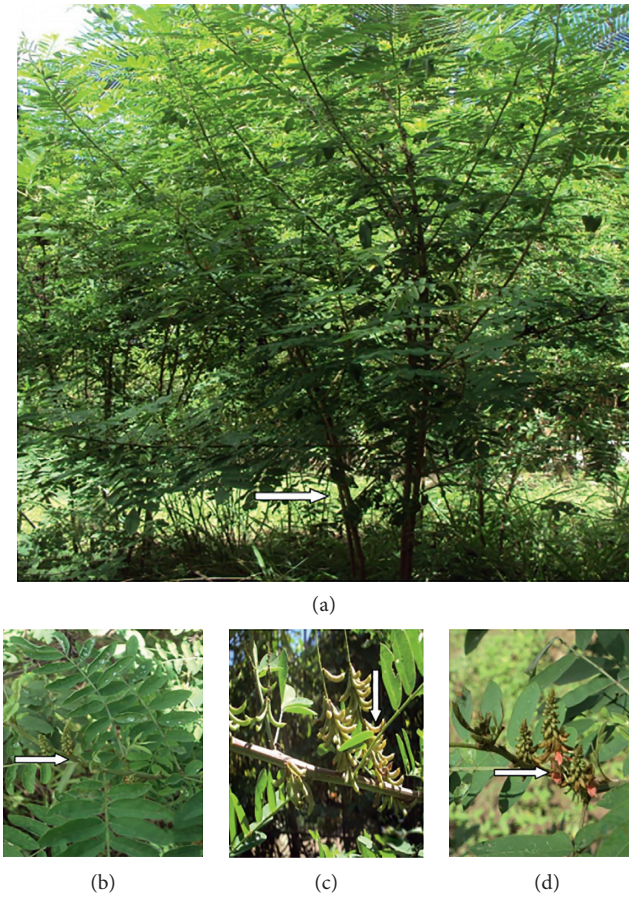
2. Botanical Characterization and Distribution

I. suffruticosa is described as a shrub plant, measuring 1 m to 2 m height, having branches pubescent, stem angular, of grayish color, pinnate leaves composed of 7–15 oblong or oval leaflets, hairless on the face and back, with small flowers, numerous albo-pink or yellow, in axillary racemes, and its fruit is a small sickle pod with 6–10 seeds measuring 25 mm in length [13]. Having strong adaptability, they are considered wild plants that grow in all types of soils, tolerating drought, floods, and high salinities.

I. suffruticosa Mill. (Figure 1) is a species from the Antilla and Central America [14] and more prevalent throughout the tropical America. In Brazil, it is distributed in some states: São Paulo, Sergipe, Bahia, Rio de Janeiro, Minas Gerais, Maranhão [15, 16], Mato Grosso [17], Alagoas [4], Paraíba [18], Ceará, Rio Grande do Norte, Pernambuco, and Pará [19].

3. Traditional Use and Ethnopharmacology

Indigofera suffruticosa is popularly known as "indigo" or "anileira." Such a nickname comes from the German language, meaning "blue pigment," which is extracted through fermentation by hot infusion of its leaves and was used in the textile industries to dye yarns. Currently, this extract is processed by industrial chemical processes, and the use of this plant in the textile industries was abandoned [7]. *I. suffruticosa* may also be related to other popular names such as jiquilite, tzitzupu, indigo fields, anileira guinea, real anileira, caá-chica, caá-chira, timbómrim, timbozinho, and indigueira. The species is widely used in folk medicine to cure many health problems, with uses based on infusions and decoctions of different parts of this plant [20]. They are attributed to this plant's febrifuge, antispasmodic, diuretic, abortive, and analgesic properties against stomach and urinary problems, jaundice, ulcers and purgative, sedative, and insecticidal properties [21].



(a) (b) (c) (d)

Figure 1: (a) Shrub *Indigofera suffruticosa* measuring approximately 1.15 m; (b) leaf and inflorescence; (c) branches with leaves and seeds; (d) branches with flowers, leaves, and inflorescence.

4. Chemical Constituents

Several studies have identified and isolated some chemical constituents of *I. suffruticosa*, including flavonoids, alkaloids, coumarins, triterpenoids, and carbohydrates. Early investigations of the chemical components of *I. suffruticosa* were made by Miller and Smith, 1973, using seed extract, with a highlight of the rich presence of amino acids and possible toxic effects. According to the Natural Products Alert [22] and Chemical Abstracts, the phytochemical profile of this species reveals the presence of alkaloids, polyphenols, terpenoids, steroids, reducing sugars, proteins, and indigoids.

Paiva et al. [23] quantified proteins and natural fibers of this species, showing its use as feed for ruminants. Isolation of 3-nitropropanoic acid glucose esters is featured by having toxic effects due to its conversion to the 3-nitropropanoic acid, a respiratory toxin that inhibits mitochondrial enzymes [24, 25]. In addition, *D*-(+)-pinitol, β-sitosterol, and louisfieserone have been isolated from this plant [25]. Apart from these isolated compounds, Kamal and Mangla [26] identified, characterized, and quantified six rotenoids from different parts of *I. suffruticosa*. Preliminary studies of leaves, seeds, and stems of *I. suffruticosa* demonstrate the presence

of polyphenols (coumarin and chlorogenic acid) and flavonoids (quercetin, rutin, and gallic acid), alkaloids, triterpenoids, and carbohydrates [9, 27].

The main flavonoids identified and isolated from the methanol extract of *I. suffruticosa* leaves include quercetin 7-O-*β*-*d*-glucopyranoside, quercetin 3-O-[*β*-*d*-xylopyranosyl-(1⟶2)-*β*-*d*-galactopyranoside], quercetin 3-O-[*α*-*l*-rhamnopyranosyl-(1⟶6)-*β*-*d*-glucopyranoside], and quercetin 3-O-[*β*-*d*-glucopyranosyl-(1⟶2)-*β*-*d*-glucopyranoside. In addition to these compounds, indigo and indirubin were also isolated [27].

Pentadecanoic acid, 14-methyl-, *n*-hexanedecanoic acid, *z*-[13, 14-epoxy]tetradec-11-en-1-ol acetate, oleic acid, 9-octadecenoic acid[*z*]-, 2-hydroxy-1-[hydroxyl methyl], heptanoic acid, docosyl ester, octadecanoic acid, 7-hydroxy-, 6-octadecenoic acid[*z*]-, and 8-octadecenoic acid [28] were also found.

Chen et al. [29] using aqueous and ethanol extracts of *I. suffruticosa* identified the following different phenolic compounds: syringic acid, p-coumaric acid, vanillin, syringaldehyde, salicylic acid, quercetin, isoliquiritigenin, and formononetin.

The presence of such compounds in the leaf oil of *I. suffruticosa*, (*z*)-3-hexenyl benzoate, methyl hexadecanoate, phytol, linoleic acid, methyl linoleate, *n*-docosane, *n*-tricosane, was also found [30].

5. Pharmacological Activities

5.1. Embryotoxic and Cytotoxic Activity. Leite et al. [10] investigated the cytotoxic potential of aqueous extracts from leaves of *I. suffruticosa* in mouse embryos and found that, at high concentrations of the extract, the growth of the embryos was inhibited, preventing them reaching the final stage of embryogenesis, indicating that their use in high doses in humans can be harmful.

Vieira et al. [8] realized in his studies that aqueous extracts of leaves of *I. suffruticosa* by infusion and maceration in different concentrations (from 6.25 to 50 μg/ml) tested in cell lines of HEp-2 by the MTT method did not produce any cytotoxic effect (>30 μg/ml) when compared with the control and DMEM (Dulbecco's Modified Eagle Medium).

In another study, the indigo ethanol extract purifier of *I. suffruticosa* showed a potent cytotoxic agent, showing the value of 0.89 for the breast tumor cell line (LM2) and lung tumor cell line (LP07), clarifying that the extract has cellular responses such as inducing apoptosis [31].

Carli et al. [32] also observed the cytotoxic effect on cell viability assays with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), having inhibitory concentration (IC_{50}) of 200 μg/ml.

Bioactive compounds of natural origin are essential in antineoplastic therapy, since they show promising effects on carcinogenesis and contribute to new medicinal interests. Therefore, such information has great relevance in understanding the use of this species and its promising future.

Vieira et al. [33] used aqueous *I. suffruticosa* extracts at varying doses (250–1000 mg/ml) in models for embryotoxicity in the development and oviposition of *Aedes aegypti*. In the study, a significant repellent effect was found on oviposition and an embryotoxicity was also observed, slowing the normal growth of the larvae of *Aedes aegypti*.

The use of plant extracts is an alternative method for insect control, which can minimize the harmful effects of some insecticides on nontarget insect species, humans, and the environment, leading to new opportunities for the development of commercial products of natural origin and of great importance.

5.2. Antitumor Activity. Vieira et al. [8] evaluated the effect of aqueous extracts of *I. suffruticosa* processed by maceration and infusion at a dose of 50 mg/kg on sarcoma 180 and found that both forms had significant effect on reducing the tumor (62.6 and 64.5%), respectively.

Cancer is still the leading cause of death in the world, and plants used in traditional medicines may be a potential source of powerful chemopreventive agents because they are enriching source of beneficial secondary components. There are many plant species that have relevant biological data in the scientific literature and even commercial use for this purpose. However, the use of *I. suffruticosa* is still poorly studied, and new studies should investigate the possibility of using as an antitumor drug.

5.3. In Vivo Activity against Ectoparasite (Pediculosis capitis). García et al. [34] used tincture of *I. suffruticosa* to 5% in the population reduction of *Pediculosis capitis* and observed the population reduction of lice mainly in cases of persistence in patients of 55 years. After 2 days of application, the results confirmed the insecticidal activity of *I. suffruticosa* tincture; this treatment seems to be a valuable and effective alternative to existing treatments.

5.4. Antimutagenic Activity. Calvo et al. [35] evaluated the effect of the ethanol extract of the aerial parts of *I. suffruticosa* in trials on Salmonella, which showed mutagenic activity, suggesting that such an action is due to the presence of indigo pigment. Since plants are primary food sources and cure, the natural products need to be evaluated with regard to their toxicity and dosage because the indiscriminate use of homemade preparations of plants can be dangerous to health.

5.5. Antioxidant Activity. Ethanolic extracts of leaves of *I. suffruticosa* stand out with potent antioxidant activity in an experimental model in vitro with the free radical 2,2-diphenyl-1-picrilidrazil (DPPH), and this method is a rapid way to measure the antioxidant capacity of compounds, it is based on the reduction of DPPH in solution, and this action is attributed to the presence of high concentrations of gallic acid in the extract [30]. Plants contain several phytochemicals, such as phenolic compounds, which have promising antioxidant activity, mainly in the prevention of chronic diseases.

5.6. Hepatoprotective Activity. The aqueous extract of *I. suffruticosa* (50 mg/kg, ip) showed the protective effect on

liver tissue of mice bearing sarcoma 180 [36]. Lima et al. [37], using the purified indigo compound from the leaves of *I. suffruticosa*, did not observe a reduction in sarcoma 180 tumor but found that liver cells remained preserved, emphasizing its hepatoprotective effect.

Lima et al. [37] investigated the antitumor action of indica, a compound extracted from the leaves of *I. suffruticosa*, on mice bearing sarcoma 180 cell lines and found that such a compound did not promote a reduction in tumor growth; however, it is found that treatment with indica does not allow to alter the architecture of the liver cells, suggesting a hepatoprotective effect.

Such an effect is considered of extreme importance since liver diseases have become a global public health problem, and much of them are a consequence of the prolonged use of chemicals and drugs.

5.7. Antimicrobial Activity. Santos et al. [38] evaluated the antimicrobial activity of ether, chloroform, and acetone extracts of *I. suffruticosa* against nine strains of *Staphylococcus aureus*, with minimal inhibitory concentration (MIC) ranging from 0.78 to 6.25 mg/ml. The methanol extract of the aerial parts of *I. suffruticosa* showed a significant effect against *Mycobacterium tuberculosis* with an MIC of 125 μg/mL, suggesting the presence of an important bactericidal agent [32].

The endophytic fungi isolated from *I. suffruticosa* also showed activity against different bacteria such as *B. subtilis*, *S. aureus*, *E. coli*, *K. pneumoniae*, and *P. aeruginosa*, with an MIC ranging from 0.39 to 6.25 mg/ml, emphasizing that this species has a potential to inhibit bacterial growth [39].

The aqueous extract of the leaves of *I. suffruticosa* displays significant results against two strains of *Trichophyton rubrum* and *Microsporum canis*, with concentrations of 5 and 10 mg/ml with variation in MIC between 20 and 15 mm, with a similar effect to the standard drug ketoconazole (MIC −20 mm) [11].

The results obtained in these studies suggest that the extracts used had bioactive compounds responsible for the efficacy of the organic material studied. Not only the plant extracts but also those produced by microorganisms can be a source for the industrial manufacture of drugs and useful in the therapy of some microbial infections.

5.8. Anticonvulsant Activity. The *I. suffruticosa* fluid extract (0.06 g/kg for 10 days) in experimental models of shock promotes protective effect in both doses administered orally or intraperitoneally on seizures induced by this model, highlighting the antiepileptic potential of such an extract [40].

At the concentration of 0.06 g/kg, the fluid extract of *I. suffruticosa* was tested in models of chronic epilepsy, which reduced the concentration of inhibitory amino acids (glycine and tannin) and increased the excitatory amino acid (glutamic acid) [41].

In models of seizures, the methanol extract of the leaves of *I. suffruticosa* showed significant activities, and its action is due to the presence of secondary metabolites such as flavonoids and linalool having an action on the GABAergic system [42].

The anticonvulsant effect of the aqueous extract of *I. suffruticosa* was confirmed following behavior and electrophysiological analyses in rats. The systematic analysis of seizure behavior and its potentials was also recorded [36].

The findings of these studies stimulate the continuity of research in the search for new treatments for epilepsy. Although traditional therapy has good efficacy, it has high toxicity, and about 20–30% of patients who use this therapy are unable to control their seizures appropriately and have severe side effects. Therefore, new alternatives that reduce clinical manifestations and chronic conditions become essential.

5.9. Gastroprotective Activity. Luiz-Ferreira et al. [43] explored the gastroprotective effect of chloroformic and methanolic extracts of the aerial parts of *I. suffruticosa*, partitioned with ethyl acetate and administered at a dose of 100 mg/kg, which significantly inhibited the gastric mucosal lesions induced by ethanol and nonsteroidal drugs in rats.

In traditional medicine, several plants are used in the treatment of gastric disorders, and from the evidence presented here, it could be stated that extracts obtained from the aerial parts of *I. suffruticosa* are interesting sources for the development of a phytotherapeutic formulation to treat gastric ulcer.

5.10. Immunostimulatory Activities. Carli et al. [32] investigated the effect of the ethanol extracts of *I. suffruticosa* on immune activity *in vitro* and observed that the extract triggered a high nitric oxide production and stimulated the synthesis and release of TNF-α, thus triggering the activation of macrophages and promoting the production of other molecules that improve or restore the responsiveness of the innate immune system reaction against infections.

In another study, using a purified compound of *I. suffruticosa*, the indigo, also in experimental models *in vitro*, showed an increase in the production and release of nitric oxide and TNF-α [31].

The immune system is of fundamental importance to every living being, and its malfunction can cause various biological damages. Drugs that stimulate the production and action of the components of this system are of great biological relevance, primarily in immunodeficient individuals who are more susceptible to infections. In this way, the good results obtained for this species favor even more effective use in several diseases.

5.11. Anti-Inflammatory Activity. Aqueous extracts of *I. suffruticosa* (250 mg/kg) in inflammation experimental models of mice showed significant anti-inflammatory effect, with similar action to the commercial standard drug, acetyl salicylic acid [9].

In another study, aqueous and ethanolic extracts of leaves of *I. suffruticosa* were used in experimental models of inflammation induced by lipopolysaccharides (LPS) in

macrophages, and it was possible to observe a significant anti-inflammatory effect [29].

The prolonged use of anti-inflammatories promotes several biological damages and the search for new drug therapies that reversibly abuse the harmful effect, and the low cost is still incessant. The studies already published show that *I. suffruticosa* is a strong candidate to be applied in this activity.

5.12. Gynecological Problems/Issues. Yazbek et al. [44] reported that, in Brazilian folk medicine, leaves and root of *I. suffruticosa* have been commonly used to prepare tea for inflammatory diseases of the gynecological tract, mainly organs such as ovaries and/or uterus.

In many places, the main therapeutic resource for the treatment of discomforts and diseases of gynecological origin is still commonly represented by medicinal plants. However, few studies, such as [44], show the promising effect that may resonate on new beneficial therapeutic approaches and on strengthening the practice of women's periodic care.

6. Toxicity

Studies using aqueous extracts obtained by infusing leaves of *I. suffruticosa* in acute toxicity in mice demonstrated the presence of deaths in the tested groups. Some signs of toxicity were noted after a few hours of intraperitoneal administration from a lower concentration to a higher concentration tested dose ($2400\,mg{\cdot}kg^{-1}$): agitation, piloerection, exhaustion, sleepiness, irritability, and spasms. Also, it was found that the LD50 of the acute toxicity of the aqueous extract of leaves of *I. suffruticosa* made by infusion administered in different doses in mice showed no mortality during 72 h of observation [33].

The methanol extract of leaves of *I. suffruticosa* showed a low toxicity with an LD50 of 1600 mg/kg (ip) in mice. The results exhibit a lower significant change in individual behavioral and parameters that slight decrease in spontaneous locomotor activity and an increase in breathing frequency [42].

7. Conclusions

Plants since ancient times have been used as medicine and have been daily providing inspiration for new research, aiming to highlight the diverse potential and expand the library of biologically active molecules.

In recent years, *I. suffruticosa* has attracted the attention of many researchers because of its high therapeutic value in the population. Different extracts of this species have presented significant results in several pharmacological activities, such as antitumor, antioxidant, anti-inflammatory, antimicrobial, antiepileptic, antifungal, anticonvulsant, gastroprotective, and hepatoprotective.

These studies were tested *in vivo* in laboratory animals, and the results presented are not enough for humans to use. Based on the low toxicity presented by the statement and little research of their phytochemicals, new clinical trials should be conducted to fill the gaps in research to establish baseline data for medicinal use, eliminating potential harmful risks and promoting beneficial effects.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Review Article

Use of Flavonoids and Cinnamates, the Main Photoprotectors with Natural Origin

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Many pathological problems are initiated by ultraviolet radiation (UVR), such as skin cancer, the most commonly diagnosed cancer worldwide. The UVA (320–400 nm) and UVB (290–320 nm) wavelengths may cause effects such as photoaging, DNA damage, and a series of cellular alterations. The UVA radiation can damage the DNA, oxidize the lipids, and produce dangerous free radicals, which can cause inflammation, modify the gene expression in response to stress, and weaken the skin immune response. With a minor penetration, the UVB radiation is more harmful, being responsible for immediate damage. Ultraviolet radiation light emitted by the sun is considered necessary for the existence of life but cause radiation problems, especially in the skin. The photoprotective activities of plant extracts and isolated composts were evaluated by many reports, as well as the correlation of these compounds with the antioxidant activity. This review presents plant compounds with interest to the cosmetic industry to be used in sunscreens such as flavonoids and cinnamates.

1. Introduction

Many pathological problems are initiated by ultraviolet radiation (UVR), such as skin cancer, the most commonly diagnosed cancer worldwide that can cause a high mortality rate when it evolves into a worst type, the melanoma. The UVA (320–400 nm) and UVB (290–320 nm) wavelengths that act in the skin cause effects such as photoaging, photosensitivity (DNA damage), appearance of wrinkles, and a series of cellular alterations involving fibroblasts and melanocytes due to UVA radiation, which possesses a greater power to penetrate the skin. The UVA radiation can damage the DNA, oxidize the lipids, and produce dangerous free radicals, which can cause inflammation, break the cellular communication, modify the gene expression in response to stress, and weaken the skin immune response. But both radiations are linked to skin cancer [1–4]. With a minor penetration, the UVB radiation is more harmful, being responsible for immediate damage, causing burns, skin cancer, and cataracts [5, 6].

Continuous sun exposure not only hinders the skin's ability to repair itself but also continues to break down and debilitate the synthesis of new collagen. UV radiation can also lead to degradation of elastin fibers, causing premature decrease in skin flexibility [7].

Skin cancer is a major health problem worldwide. It is a disease caused by the abnormal and uncontrolled growth of the skin cells. These cells are organized in layers, and the type of cancer will be defined according to the affected layer. It is a public health problem and is the most common in the United States; one in five Americans will develop skin cancer in their lifetime. Currently, between 2 and 3 million

nonmelanoma skin cancers and 132000 melanoma skin cancers occur globally each year. In Brazil, 5% of skin cancer cases are melanoma, which has a 75% mortality rate. The most common are the basal cell carcinoma and the squamous cell carcinoma. The cutaneous melanoma is the most aggressive type of skin cancer; it is also rare and deadly [8–12].

All of these effects and new cancer cases (min. 10%) can be prevented or mitigated if people use cosmetic formulations such as sunscreens properly and continuously [1, 13, 14].

The skin is naturally protected from light damage, but when there is excessive exposure to the sun rays, the body may not be able to neutralize them completely, which can lead to many health problems. The use of compounds with antioxidant action could maintain a healthy skin barrier. In addition, they may be useful in photoprotector photostability and may potentially neutralize free radicals induced by UVA radiation [15].

There is of great interest and challenge from the pharmaceutical and cosmetic industries in the development of topical preparations that provide continuous physical, chemical, and biological photoprotection. In addition to that, the damage caused by the synthetic assets has contributed in the development of products whose majority composition is from natural origin, especially vegetable origin, being high quality products with a plurality of benefits [16]. Many costumers' cosmetics are made with natural products as health products. According to Bortolozi [17], Brazil is responsible for 20% of the world consumption of sunscreen, being the world leader.

There is little information about how people in the ancient world protected themselves from the sun. In the 1st century BC, oils, tars, and plant extracts were the various forms used as sunscreens [18].

Rittler, in 1801, discovered the ultraviolet spectrum, and Everard Home, in 1820, discovered the role of the sunlight in skin burns. With that, numerous substances were researched for their ability to absorb this radiation in order to reduce its effects on the skin. Acidified quinine sulfate was the first substance utilized for this purpose by Widmard in 1889, and, in 1891, Hammer incorporated this substance in lotions and ointments, thus creating the first chemical sunscreen in history. Since then, other sunscreens have been appearing, such as esculin, benzyl salicylate, derivatives of *p*-amino benzoic acid, cinnamates, and benzophenone, among others [16].

Only in 1928 did the first sunscreen containing benzyl salicylate and benzyl cinnamate become available in the market in the United States of America. The first patent only came in 1943, with the *p*-amino benzoic acid (PABA). Popularization only occurred in the 1970s, and sunscreens became more effective with the introduction of inorganic titanium dioxide in 1989 and zinc oxide in 1992 [16, 19].

Brassicaceous plants are characterized by a pronounced metabolic flux toward sinapate, produced by the shikimate/phenylpropanoid pathway, which is converted into a broad spectrum of O-ester conjugates. Sinapoylmalate is involved in protecting the leaves against the deleterious effects of UV-B radiation [20].

According to the Skin Cancer Foundation [21], sunscreens help prevent the sun's ultraviolet (UV) radiation from reaching the skin. They protect the skin from UVA and UVB radiations that excessively damage it and increase the risk of skin cancer. They are products consisting of several ingredients and vary in their ability to protect against UVA and UVB.

Sunscreens are used on the skin to minimize or protect from acute and chronic effects of ultraviolet rays, including elimination of free radicals. They are composed of chemical and/or physical sun filters [18, 21].

2. Types of Sunscreens and Mechanism of Action

2.1. The Inorganic Sunscreens. Inorganic sunscreens reflect and scatter UV and visible radiation, while organic sunscreens absorb UV radiation and then reemit energy as heat or light. These synthetic molecules have limited concentration according to regulation concern. Several natural compounds with UV absorption property have been used to substitute for or to reduce the quantity of synthetic sunscreen agents. In addition to UV absorption property, most natural compounds were found to act as antioxidants and anti-inflammatory and immunomodulatory agents, which provide further protection against the damaging effects of UV radiation exposure. Compounds derived from natural sources have gained considerable attention for use in sunscreen products and have bolstered the market trend toward natural cosmetics. This adds to the importance of there being a wide selection of active molecules in sunscreen formulations [22].

The inorganic sunscreens are metal oxides that generally protect the skin by reflecting the radiation. Zinc oxide and titanium dioxide are the inorganic substances most utilized for photoprotection. They present excellent protection against UV radiation and their advantage is that they are chemically inert and hence do not cause allergic sensitization. These substances are recommended for the manipulation of pediatric photoprotection and for people with sensitive skin. These particles are often visible on the skin, and it becomes an inconvenience for consumers of these cosmetics [5, 14]. However, inorganic oxides such as TiO_2 and ZnO exhibit their protecting effect only to a small extent by reflection (and scattering), but mostly by absorption, due to their semiconductor band-gaps [23].

The resolution RDC n. 47 [24], from March 16th 2006, established a maximum concentration of 25% of these inorganic sunscreens in the formulation of products.

2.2. Chemical Filters. Chemical sunscreens are organic compounds. Usually, these compounds protect the skin by absorbing the radiation. Currently, there are organic sunscreens in the market that not only absorb 95% of the (290–320 nm) wavelengths, protecting against erythema and skin wrinkling, but also reflect UV radiation [5, 14]. Many of these molecules are aromatic compounds that have carboxyl groups and, generally, they present an electron-donating

group, such as amine or a methoxyl group, in the ortho or para positions [25].

Different studies show that, despite the fact that these sunscreens should be used on the skin surface, absorption, metabolization, accumulation, and elimination of some organic filters by the organism may occur. This percutaneous absorption can result in harmful processes to the health, such as contact dermatitis, mutations, cancer, estrogenic activity, or photosensitivity reactions [5, 26].

Thus, the photoprotection from naturally occurring substances has gained considerable attention in recent years due to their wide range of biological activities. The development of sunscreens, which include natural products with a broad spectrum of efficacy and low toxicity, is of great interest to the cosmetic industry [16].

The main synthetic organic filters are *p*-aminobenzoic acid (PABA), cinnamates (*p*-octyl-methoxycinnamate), salicylates, benzimidazoles (2-phenylbenzimidazole acid, derivatives 5-sulfonic benzylidene), camphor, and benzophenone [27]. However, recent researches appointed the triazine and benzoltriazole derivatives with biggest potential and have gained importance worldwide [28].

In 1928, in the USA, one of the first sunscreens had benzyl cinnamate in its composition, but its use was limited. Among the currently most used UV chemical filters is the octyl methoxicinnamate [26]. Commercial compounds have several problems, such as low photostability and endocrine disruption properties. Scientific studies show that chemical filters are capable of altering the hypothalamic-pituitary-thyroid (HPT) axis, also affecting the homeostasis of the reproductive axis, as well as some development parameters [29, 30].

Currently, *in vitro* and *in vivo* techniques are used in the determination of the sun protection factor (SPF) and the UVA protection factor (APF) of sunscreens. Among the determination techniques of these *in vitro* factors recommended by the FDA (Food and Drug Administration Agency) and COLIPA (European Cosmetic, Toiletry, and Perfumery Association) the reflectance spectrophotometer with integrating sphere stands out. This technique is mainly applied with opaque materials, such as the cosmetic products used as sunscreens [31, 32].

The *in vitro* techniques to evaluate products as sunscreens present advantages: the practicality to be employed routinely in quality control to assure that each batch of sunscreen is adequate for use; the speed; the affordable cost and reproducibility that guarantee an effective preliminary evaluation of the natural products in the Brazilian flora for future use in the cosmetic industry as photoprotectors [33, 34].

3. Determination of Photoprotection Activity *In Vitro*

The *in vitro* methods are, in general, of two types: methods that involve the measurement of absorption or the transmission of UV radiation through sunscreen product films in quartz plates or biomembranes and methods in which the absorption characteristics of the sunscreen agents are determined based on spectrophotometric analysis of dilute solutions. The efficacy of a sunscreen is usually expressed by the sun protection factor (SPF), which is defined as the UV energy required to produce a minimal erythemal dose (MED) in the protected skin divided by the UV energy required to produce an MED in unprotected skin. The minimal erythemal dose (MED) is defined as the lowest time interval or dosage of UV light irradiation sufficient to produce a minimal, perceptible erythema on unprotected skin. To this determination, oils of various manufacturers can be purchased from local pharmacies. The solubility of oils is determined in different ratios of ethanol and distilled water. It is reported that a maximum of 50% of ethanol could be used in cosmetics. Hence, solubility of oils is detected taking 10% to 50% of ethanol in distilled water. The maximum solubility is observed in 40% ethanol and 60% distilled water solution [35].

3.1. Wavelength Determination and Plant Extracts Absorbance. This analysis is performed in a spectrophotometer in order to determine maximum wavelength (λmax) and maximum absorbance (Amax) of plant extracts dissolved in solvents that are not supposed to absorb ultraviolet radiation. The most widely used are water, 95% ethanol, and hexane. A scan is carried out between the lengths 260–400 nm to verify absorption in the UVA (320–400) and UVB (290–320) regions [36, 37].

3.2. In Vitro Evaluation of Sun Protection Factor by Mansur Method. It is a method to determine the SPF by spectrophotometric reading of diluted solutions of samples and further mathematical treatment by determining the transmittance or absorbance (T = 10–Abs) in the wavelenghts 290–320 nm, with 5 nm breaks, in quartz cuvettes. The solvents used in this technique are usually methanol, isopropanol, or ethanol. The samples are dissolved at a concentration of (0.2 mg/mL) and analyzed in triplicate. The absorbances obtained are added to the equation shown below, which determines the sun protection factor [36, 38–40].

Table 1 shows the sun protection factor (SPF), according to Mansur's method [39], of several plant extracts, where *Aniba canelilla*, *Calendula officinalis*, and *Euphorbia tirucalli* showed the best activities.

$$\mathrm{SFP} = \mathrm{CF} \times \sum_{290\,\mathrm{mm}}^{320\,\mathrm{mm}} \mathrm{EE}\lambda \times \mathrm{I}\lambda \times \mathrm{Abs}\lambda, \quad (1)$$

where CF = correction factor = 10, EEλ = erythematogenic effect of radiation wavelength (λ), Iλ = sunlight intensity at a wavelength (λ), and Absλ = spectrophotometric determination of absorbance of the formulation in solution at a wavelength (λ).

4. Antioxidant Effect and Its Association with Photoprotective Effect of Natural Products

The plant kingdom produces secondary metabolites that are synthesized to develop protective functions against hostile

Table 1: Results for some plant extracts for photoprotective activity.

Scientific name	Popular name	Family	Preparation	SPF ($\mu g \cdot mL^{-1}$)	Reference
Ginkgo biloba L.	Ginkgo	Ginkgoaceae	Ethanol extract	0.82	Pinto et al., n.d. [61]
Hamamelis virginiana L.	Witch hazel	Hamamelidaceae	Ethanol extract	0.68	
Matricaria chamomilla L.	Chamomile	Asteraceae	Ethanol extract	0.56	
Pereskia aculeata Mill	Barbados gooseberry	Cactaceae	Ethanol extract of the leaves	0.69	
Aniba canellila (Kunth) Mez	Preciosa	Lauraceae	Essential oils from leaves	14.08	Fonseca Jr. et al. [62]
Malpighia glabra L	Acerola	Malpighiaceae	Lotion with 10% of dry fruit extract	0.14	Souza et al. [24]
Bromelia laciniosa Mart. ex Schult. and Schult.F	Macambira	Bromeliaceae	Leaf ethanol extract	7.22	Oliveira et al. [63]
Tabebuia aura (Silva Manso) Benth. and Hook. f. ex S. Moore	Caribbean trumpet tree	Bignoniaceae	Lotion with ethanol extract	0.38	Violante et al. [41]
Calendula officinalis L.	Pot marigold	Asteraceae	Lotion with 5% of the flowers oil	14.84	Ramos et al., 2010 [39]
Euphorbiatirucali L.	Aveloz	Euphorbiaceae	Aerial parts ethanol extract	19.82	Ramos et al., 2010 [39]
Byrsonima sericea DC	Murici	Malpighiaceae	Leaf ethanol extract	1.36	Ramos et al., 2010 [39]
Byrsonima sericea DC	Murici	Malpighiaceae	Leaf aqueous extract	0.68	

environments, such as oxidation by solar radiation. These metabolites are constituted by a large number of compounds, and among these, we highlight polyphenols, such as flavonoids and phenolic acids, whose synthesis does not occur in humans. Flavonoids have a basic nucleus in common that can be subdivided into different subgroups depending on the carbon of the C ring on which the B ring is attached and the degree of unsaturation and oxidation of the C ring. They are called isoflavones, neoflavonoids, and other subgroups (Figure 1), having the ability to absorb ultraviolet radiation due to the presence of chromophores in their structure. Rutin (3-*O*-rutinoside-quercetin), part of the flavonols class, can then be used as sunscreen in cosmetic formulations [41–44].

Arct and collaborators; Valenta, Nowack, and Bernkop-Schnürch; and Saija et al. studied the penetration and permeation of flavonoids by in vitro diffusion cell methodologies [44–46]. Bobin et al. evaluated the permeation of rutin, catechin and quercetin in the presence of adjuvants hydrophilic (wetting) and their influence on the permeation of the flavonoids through an artificial membrane [45]. According to the experimental results obtained, these acted as inhibitors of the permeation of flavonoids under study, however, at different levels of inhibition [49].

Flavonoids, tannins, alkaloids, anthraquinones, and polyphenols are secondary metabolites present in plants and considered natural sunscreens, since they have the property of absorbing light in the ultraviolet region [5, 42]. Flavonoids have three different actions: capability of absorbing ultraviolet lights, antioxidant properties, and modulating several signaling pathways. The ultraviolet absorption spectrum of a flavonoid generally shows two maximum peaks of absorption, one between 240 and 280 nm and the other at 300–550 nm, which shows the possibility of using these extracts as sunscreens in both UVB and UVA photoprotection preparations [3, 43].

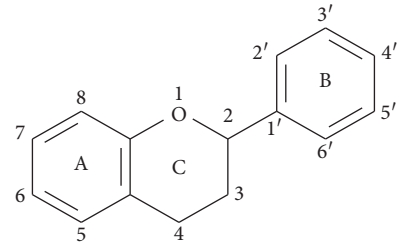


Figure 1: Basic skeleton structure of flavonoids.

The synergistic effect has been shown for the combination of 0.1% rutin and 6.0% benzophenone, which caused a SPF increase from 24.3 ± 1.53 to 33.3 ± 2.89 when the flavonoid was included [45].

According to a study by Filho et al. [46], rutin and quercetin, potent antioxidants, incorporated a water-in-oil emulsion, used in concentrations of 10%, individually associated with titanium dioxide and zinc oxide, obtained synergistic effect with a SPF value about 30.

The hydroalcoholic extract of the leaves of the plant species *Arrabidaea chica*, which belongs to the bignoniaceae family, presented in spectrophotometric analysis in the UVB region (283 nm and UVA region 331 nm) peaks in the concentration 0.10 mg/mL [47].

Propolis is a complex mixture, which includes the presence of flavonoids (flavones, flavonones, and flavonols), aromatic acids and esters, aldehydes, ketones, terpenes, steroids, amino acids, phenylpropanoid, fatty acids, hydrocarbons, and many other compounds in smaller proportions. Its chemical composition varies according to the collection site, due to the vegetation. This complexity gives propolis numerous pharmacological properties, among them is the ability to absorb UV rays. Several studies have been done concerning this property: the synergistic effect of the SPF of glycolic extracts and red and green propolis

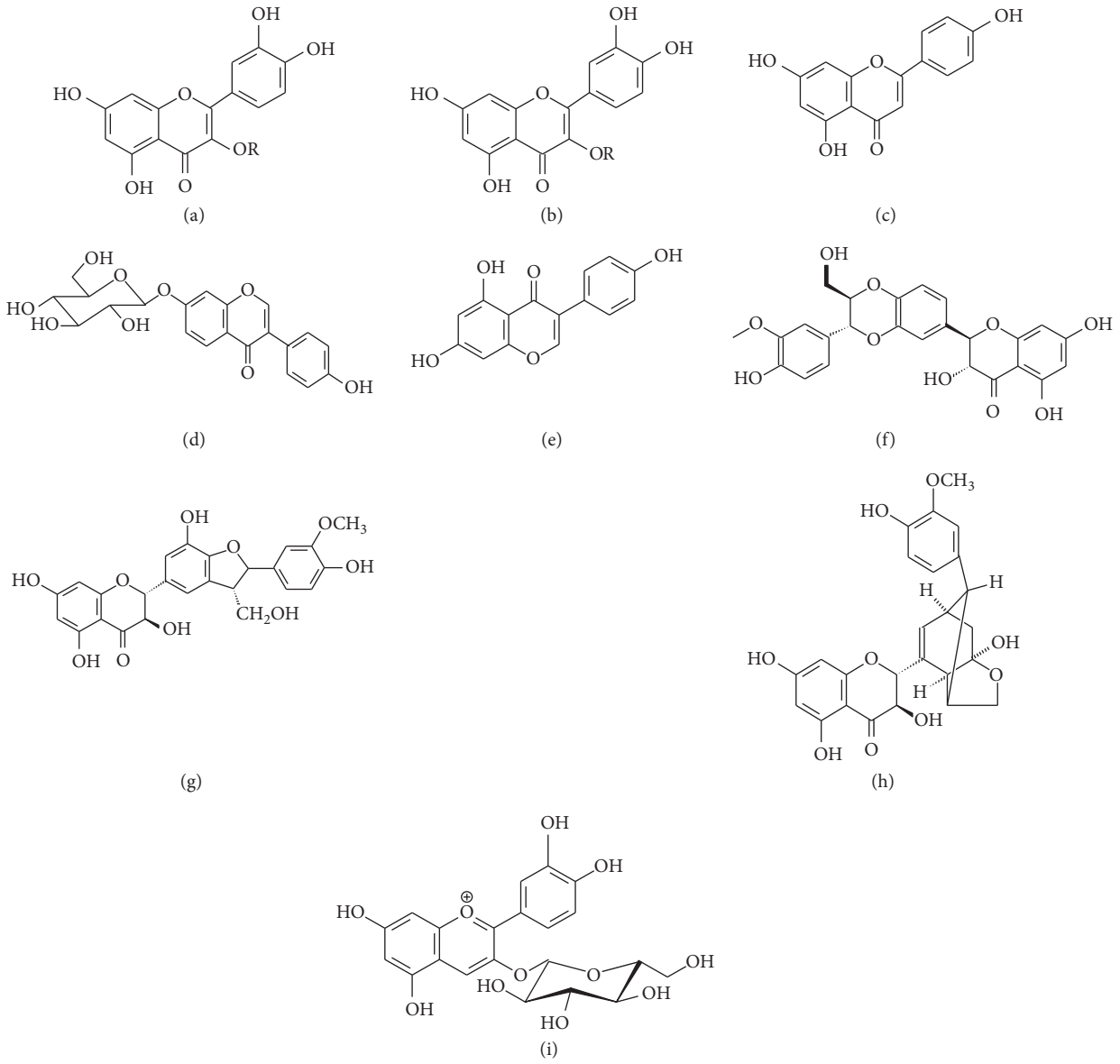


Figure 2: Some photoprotector flavonoids. (a) Quercetin, R= H. (b) Rutin, R= rutinose rutin. (c) Apigenin/cosmosiin. (d) Daidzein. (e) Genistein. (f) Silibinin. (g) Silychristin. (h) Sylidianin. (i) Cyanidin-3-glucoside.

ethanol with UVA/UVB water-soluble sunscreens (association of 2-phenylbenzimidazole-5-sulfonic acid–Eusolex 232®–and 2-hydroxy-4-methoxybenzophenone-5-sulfonic acid, 1,2-propanediol and demineralized water) [48]. Propolis dry extract in 3, 10, and 40% concentrations presented SPF values ranging from 1 to 12 and a synergistic effect in combination with octyl methoxycinnamate [38].

Ethanol extracts of *Aniba canelilla* (Kunth) leaves, according to phytochemical study, revealed the presence of flavonoids and showed to be promising for the use in products for sun protection. However, other studies should be conducted so that the constituents responsible for the effect are characterized [49].

Pycnogenol, the name given to the extract derived from the pine bark of a tree known as *Pinus pinaster*, contains a number of phenolic and polyphenolic flavonoids. Acute and chronic effects of UV radiation exposure were reduced significantly in experimental animals after topical application of pycnogenol. The red clover (*Trifolium pratense*) presents a high content of antioxidant flavonoids like genistein and daidzein. Flavonoids such as genistein (4′,5,7-trihydroxyisoflavone, soybean isoflavone) are potent antioxidants and effectively block UVB-induced skin burns in humans. Silymarin, a mixture of three flavonoids (silybin, silydianin, and silychristin), equol, a metabolite of daidzein produced exclusively by the action of intestinal microflora of mammals, quercetin, and apigenin are alsot flavonoids that have excellent photoprotective properties, but present problems such as chemical instability and should be further studied. In a study using silymarin (SM) and silybin (SB), the photoactive effect by UVA stimulated the damage against primary human dermal fibroblasts. It results in a reduction

in UVA-stimulated ROS generation and SSB production, as well as in the prevention of GSH depletion, a decrease in the activation of caspase-3 and protein level of MMP-1. This data showed that both SM and SB are nonphototoxic and have UVA-photoprotective potential and could be useful agents for UV-protective dermatological preparations [50–52].

The most common anthocyanin found in nature, cyanidin-3-glucoside, has photoprotection against UVA and UVB over the human keratinocytes; some adverse effects of UVB exposure are inhibited when the cells are pretreated with cyanidin-3-glucoside [5, 53]. Figure 2 shows the chemical structures of some flavonoids with photoprotective activity.

Witch hazel (*Hamamelis virginiana* L.), chamomile (*Matricaria recutita* L.), conker tree (*Aesculus hippocastanum* L.), cascara buckthorn (*Rhamnus purshiana* DC), and cinnamon (*Cinnamomum zeylanicum* Nees) have varied chemical compositions. The following compounds are present: flavonoids, tannins, anthraquinone derivatives, and cinnamates. All extracts with 10% of the above-mentioned plants had a good absorption in the UV region, and there was a significant increase in this absorption when individually mixed in a octyl methoxycinnamate solution, potentiating this effect. They showed high stability regarding contamination and degradation [54, 55].

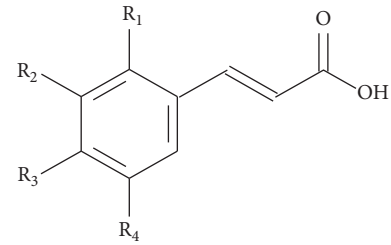
The *Carica papaya* extract, rich in flavonoids and phenolic compounds, displays antioxidant activity and has demonstrated stability when exposed to UVB radiation. The SPF by the Mansur method was 16.047 ± 0.05; adding 1.42 mg of *C. papaya* extract in formulation cream base, the SPF was 2.9 ± 0.01 [56].

A study done [54] with crude extracts of *Guazuma ulmifolia* (West Indian elm), *Maytenus ilicifolia* (espinheira-santa), *Stryphnodendron adstringens* (barbatimão), and *Trichilia catigua* (catuaba) that present total polyphenols in their composition were not considered as potential photoprotectors in the evaluated conditions. Samples were prepared in a protective formula with a concentration of 10 mg/g of total polyphenols of each extract. Only one sample, containing the *S. adstringens* extract, showed a slight increase of SPF, which, however, was not statistically significant.

The *Arrabidaea chica* (Bignoniaceae) contains flavonoids, anthocyanins, and tannins. A formulation with 5% of crude extract in a nonionic cream presented a good absorption both in the UVA and UVB regions, stability, and no toxicity in the animals tested [55].

The leaf ethanol and hydroacetone extracts of *Bauhinia microstachya* var. *massambabensis* were incorporated in sunscreen creamy lotions, generating formulations considered satisfactory in efficacy and safety tests. The formulations showed stability, safety, and no toxicity. Although they do not have a photoprotective capacity, their presence, regardless of the concentration used, increased the SPF of formulations with other synthetic chemicals [57].

Cinnamic acid derivatives are the best candidates for UVB filters in the (310–325 nm) wavelength. A formulation containing a mixture of caffeic acid with caffeic acid phenylethyl ester, or dimethyl caffeic acid, which are polyphenols with photoprotective activity, could provide significant protection values in both the UVA and UVB regions [46, 58].



Cinnamic acid: R1 = R2 = R3 = R4 = H
o- Coumaric acid: R1 = OH; R2 = R3 = R4 = H
m- Coumaric acid: R1 = R3 = R4 = H; R2 = OH
p- Coumaric acid: R1 = R2 = R4 = H; R3 = OH
Caffeic acid: R1 = R4 = H; R2 = R3 = OH
Ferulic acid: R1 = R4 = H; R2 = OCH3; R3 = OH
Synapic acid: R1 = H; R2 = R4 = OCH3; R3 = OH

Figure 3: Chemical structure of major cinnamic acids. Cinnamic acid: R1 = R2 = R3 = R4 = H. *o*-Coumaric acid: R1 = OH; R2 = R3 = R4 = H. *m*-Coumaric acid: R1 = R3 = R4 = H; R2 = OH. *p*-Coumaric acid: R1 = R2 = R4 = H; R3 = OH. Caffeic acid: R1 = R4 = H; R2 = R3 = OH. Ferulic acid: R1 = R4 = H; R2 = OCH3; R3 = OH. Synapic acid: R1 = H; R2 = R4 = OCH3; R3 = OH.

One of the plants with great potential for use as a source of products with photoprotective activity is the carnauba (*Copernicia cerifera*), a Brazilian palm tree that has leaves covered by a waxy coating. This material, after being extracted from the leaves, becomes a raw material for the production of carnauba wax, the most important vegetable wax from the economic aspect with various applications [59, 60]. This product is of great commercial interest for use in organic filters due to its high content of cinnamate derivatives such as aliphatic diesters of *p*-methoxicinnamic acid or hydroxy cinnamic acid. The cinnamates are present in many sunscreens [61]. Figure 3 shows the structure of the major natural cinnamic acids, which are present in plants either free or in the form of esters.

In a study with encapsulation of titanium dioxide with carnauba wax, there was a significant increase in the value of the sun protection factor of this oxide. The authors consider this increase as proof of a previous study that showed that increasing the viscosity of cosmetic preparations containing titanium dioxide was directly related to the increase in SPF [62]. However, a study conducted by Freitas et al. [63] isolated a fraction rich in cinnamic esters from the carnauba wax and showed an excellent absorption in this UV region and also antioxidant activity, showing a promising future for cosmetic formulations such as sunscreens, since it would be a stable organic compound obtained from a natural source.

5. Conclusion

Flavonoids and cinnamates present in many plant extracts have essential characteristics to be sunscreens, such as absorption of ultraviolet rays, as well as antioxidant activity, usefulness in cosmetics, action against free radicals, and

controlling the early ageing caused by various external factors.

There was a synergistic action in several combinations of these compounds or extracts mixed with synthetic filters, thus increasing the sun protection factor (SPF), as well as stabilizing certain formulations. The stability is also something that must be investigated, as well as the toxicity of some extracts, and if it can cause allergic problems and increased costs [9, 10, 11].

5.1. Future Perspective. It is necessary that further research is carried out to increase the spectrum of products from plants that are recognized as potential photoprotectors. In addition, it is important to search for new methodologies to be addressed to this finality.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Clustering, Pathway Enrichment, and Protein-Protein Interaction Analysis of Gene Expression in Neurodevelopmental Disorders

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Neuronal developmental disorder is a class of diseases in which there is impairment of the central nervous system and brain function. The brain in its developmental phase undergoes tremendous changes depending upon the stage and environmental factors. Neurodevelopmental disorders include abnormalities associated with cognitive, speech, reading, writing, linguistic, communication, and growth disorders with lifetime effects. Computational methods provide great potential for betterment of research and insight into the molecular mechanism of diseases. In this study, we have used four samples of microarray neuronal developmental data: control, RV (resveratrol), NGF (nerve growth factor), and RV + NGF. By using computational methods, we have identified genes that are expressed in the early stage of neuronal development and also involved in neuronal diseases. We have used MeV application to cluster the raw data using distance metric Pearson correlation coefficient. Finally, 60 genes were selected on the basis of coexpression analysis. Further pathway analysis was done using the Metascape tool, and the biological process was studied using gene ontology database. A total of 13 genes AKT1, BAD, BAX, BCL2, BDNF, CASP3, CASP8, CASP9, MYC, PIK3CD, MAPK1, MAPK10, and CYCS were identified that are common in all clusters. These genes are involved in neuronal developmental disorders and cancers like colorectal cancer, apoptosis, tuberculosis, amyotrophic lateral sclerosis (ALS), neuron death, and prostate cancer pathway. A protein-protein interaction study was done to identify proteins that belong to the same pathway. These genes can be used to design potential inhibitors against neurological disorders at the early stage of neuronal development. The microarray samples discussed in this publication are part of the data deposited in NCBI's Gene Expression Omnibus (Yadav et al., 2018) and are accessible through GEO Series (accession number GSE121261).

1. Introduction

1.1. Neuronal Development Disorder. Neurogenesis is a process of generating new and functional neurons from neuronal precursors known as NSC (neuronal stem cells) [1, 2]. Functional neurons are generated at the embryonic stage at different stages of development throughout life [3, 4]. With rapid advancement in techniques and curiosity to understand neuronal diseases at the development stage, researchers have explored a wide area of neuronal development diseases and their causes [5–8]. Neuronal stem cells have two major features that are regeneration capacity, that is, ability of self-renewal by process of cell division, and differentiation capacity, that is, process of generating new and specialized cell types [9]. Developed neurons do not carry dendrites and axons, but they play an important role to receive and send signals to other neurons [10]. Significant development has been made to identify genes that are involved in neuronal diseases at the developmental stage [11]. It is important to study different stages of nervous system development and to identify abnormalities that can arise from improper development of brain at its early stage [12]. Significant contribution has been made by scientists to identify neuronal disorders that occur at the early stage of development [13]. Neuronal disorders include abnormalities associated with intellectual disability, attention deficit hyperactivity disorder (ADHD), and cognitive skills disorders, like dyslexia and dysgraphia, and language development

disorders like expression disorder [14–18]. Scientific evidence shows that neurological disorders can be identified at the early stage by the first week or month of a lifecycle [19–21]. It is important to identify which genes are crucial and result in neurological disorders.

We have used high-throughput microarray experiment to identify genes that are involved in the early stage of neurodevelopment. Our aim was to identify genes that were expressed when stem cells were exposed to MCP (monocrotophos), a neurotoxicant, and to evaluate the effective role of resveratrol (RV) and nerve growth factor (NGF) as the neuroprotectant.

1.2. Resveratrol Clinical Perspectives. Resveratrol is a natural phenol and phytoalexin produced naturally by several plants in response to injury [22]. There is exponential evidence since 1939 in the literature that resveratrol is a promising natural compound for prevention and treatment of a wide range of human diseases [23]. Resveratrol is also reported to be effective against neuronal cell dysfunction and cell death, Huntington's disease and Alzheimer's disease [24–27]. Molecular studies show that resveratrol is associated with an induction of genes for oxidative phosphorylation and mitochondrial biogenesis [28]. Effect of resveratrol is known to extend lifespan, and it impacts mitochondrial function and metabolic homeostasis [29]. In the current work, we have mapped effectiveness of resveratrol against injured neurodevelopment samples. In this study, four samples were prepared (control, resveratrol, NGF, and RV + NGF). Datasets of prepared samples were taken to investigate the neuroprotective role of resveratrol against exposure of monocrotophos. In silico expression analysis of different datasets is done to identify genes that are coexpressed.

1.3. Microarray Data Analysis. Microarrays provide a rich source of data on the molecular mechanism of cell function. Each microarray reports expression of thousands of mRNAs [30]. Virtually, almost every human disease is being studied using microarrays experiment, with the aim of finding the novel genes involved in diseases and disease markers and to identify drug targets [31]. Bioinformatics analysis plays an important part of processing the information, embedded in large-scale expression profiling studies, and for laying the biological interpretation of high throughput microarray data [32]. A basic yet challenging task in the analysis of microarray gene expression data is the identification of changes in gene expression that are associated with particular biological conditions [33, 34]. Careful statistical design and analysis are essential to identify genes involved in each biological condition.

A standard workflow is required to utilize computational tools at various steps of microarray analysis. This paper also describes use of different bioinformatics tools for quality control, normalization, coexpression, annotation, and pathway and protein-protein interaction analysis.

1.4. Clustering and Coexpression Analysis. Clustering is a method to identify genes that are coexpressed in each biological condition [35]. Clustering methods uses a distance measure (e.g., Euclidean metric) to compare expression values of pairs of genes for each experiment [36]. When the distance between a pair of genes is small, then the two genes might be clustered. Clusters are analyzed to identify genes that are coexpressed and coregulated.

1.5. Biological Annotation and Interpretation. After extensive analysis of microarray data, one needs to annotate Affymetrix IDs for its significance. Annotation reveals the biological significance of genes like its molecular pathway, diseases involved, gene ontology, and so on [37]. Careful exploration is required to identify genes that are expressed in each condition of microarray experiment. Pathway and process enrichment is a crucial part of annotation, as it leads to the identification of set of genes that are involved in the same pathways [38]. Pathways analysis also highlights the set of proteins that interact with each other; this information is used to categorize protein interaction partners and to study protein-protein interaction network [39].

2. Materials and Methods

2.1. Microarray Data. The MSCs (mesenchymal stem cells) were used to study effect of monocrotophos (MCP) and repairing capability of resveratrol and nerve growth factor. MSCs were exposed to RV, NGF, and RV + NGF, respectively. In total, four samples were generated to identify genes that were coexpressed at the neuronal development stage. Affymetrix gene chip platform (Prime view.CDF) was used to identify gene expression using four samples as described in Table 1.

2.2. Microarray Data Analysis and Annotation. Computational software and tools were used to identify genes that are coexpressed. Figure 1 shows the workflow used for microarray data analysis and annotation. Raw files were used, i.e., chip electronic file (CEL) and chip description file (CDF) for quality control analysis. R and Bioconductor, Affy package, was used for data normalization and data transformation. Gene expression matrix was generated form Affy package, using RMA (robust multiarray average).

Significant analysis of microarray (SAM) [40] and clustering were done using MeV application [41]. The clustering method was used to cluster significant genes obtained from the SAM method. For clustering, the distance metric Pearson correlation coefficient was used, using parameter of k–means algorithm, number of cluster 10, and number of iteration 50. Coexpressed genes were identified by analyzing each ten clusters.

2.3. Pathway Enrichment and Protein-Protein Interaction Analysis. Coexpressed genes identified from clustering analysis were further annotated for biological intervention and pathway analysis. The list of coexpressed genes was searched against pathway and GO database using the Metascape tool (http://metascape.org) [42]. Each gene was studied for its pathway and process enrichment score for

Table 1: Four samples that were used for microarray gene expression analysis.

S.no	Samples	Description	Raw files
1	Control	Control sample	Control.CEL
2	RV	MSCs exposed to RV	RV.CEL
3	NGF	MSCs exposed to NGF	NGF.CEL
4	RV + NGF	MSCs exposed to combined RV + NGF	RV + NGF.CEL

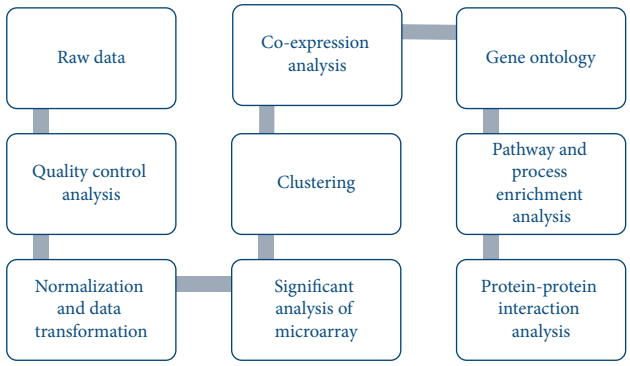

Figure 1: Workflow used for microarray data analysis and annotation.

statistical significance of genes in each biological process. Genes were also clustered according to their pathways. Relationship between genes was also identified by using the network map generated from the Metascape tool and visualized in the Cytoscape tool [43].

Protein-protein interaction analysis is carried out by different protein interaction databases like BioGrid, InWeb_IM, and OmniPath by using the Metascape tool. Molecular complex detection (MCODE) algorithm was used by the Metascape tool to identify densely connected network of protein-protein interaction [44].

3. Results

3.1. Quality Control Analysis. Quality control (QC) is an important aspect of examining microarray data, before any statistical analysis to be done. QC analysis was done using Affy package of R and Bioconductor [45]. Figure 2 shows the chip image of four samples: (a) control sample, (b) NGF sample, (c) RV sample, and (d) RV + NGF sample; it signifies there is no error in chips of all four samples, and they can be used for data exploration and analysis.

3.2. Data Normalization and Transformation. Finding biologically relevant answer from microarray experiment is a primary need of any microarray experiment. Variations in gene expression should be biologically not from any source of errors like biasness in dyes, lasers, samples, and chip spotting during microarray experiment [46]. To analyze microarray data, one needs to remove these biasness and errors in microarray experiment. Normalization is a method to remove these systematic errors that affect gene expression measures [47]. After QC analysis, normalization was done using Affy package of R and Bioconductor. We have used the RMA method of normalization. Figure 3 shows the box plot of four samples after data normalization. Box plot shows statistical values like mean or median and variations between samples [48]. Figure 3 shows that means of all four samples are at position. Data were transformed to logarithm base 2 value of the expression ratio, and expression matrix was written, for further statistical analysis and comparisons.

3.3. Clustering and Coexpression Analysis. Significant analysis of microarray (SAM) was done to identify the number of genes that were statistically significant. Out of 49,495 genes, 49,022 genes were found to be insignificant and 473 genes were statistically significant. K-means clustering was done on significant genes with parameter of $k = 10$. Ten clusters were generated and studied for coexpression of genes. Figure 4 shows cluster 1 (only one cluster is shown but all ten clusters were studied for coexpression analysis) which shows coexpression of Tp53 and B-cell cll/lymphoma2 gene. In addition, Caspase-8, Caspase-10, and dopamine receptor are also coregulated.

Analysis of all ten clusters results in identification of coexpressed genes. Rigorous analysis of clustering shows that 60 genes were coexpressed (AKT1, BAD, BAX, BCL2, BDNF, CASP3, CASP8, CASP9, MYC, PIK3CD, MAPK1, MAPK10, and CYCS). These genes were used for gene ontology, biological function, and pathway analysis. Descriptions including function of these 60 genes were shown in pathway and process enrichment analysis.

3.4. Pathway and Process Enrichment Analysis. Coexpressed genes that were clustered in the clustering step were used for biological annotation and interpretation. The Metascape tool was used to study pathway and process of these 60 genes. Protein-protein interaction network was constructed to identify more proteins that have similar function and belong to same pathway. 60 genes were further clustered into 20 groups on the basis of their enrichment score (enrichment score is the score between observed count and expected count by chance) [49].

In each cluster, one term represents the cluster that is most statistically significant [42]. Figure 5 shows the heat map of enriched terms colored by the p value. Pathway enrichment analysis shows that most of the genes were involved in colorectal cancer, neurotrophin signaling pathway, neuron death, and thyroid hormone signaling pathway. Other clusters indicate genes that were involved in cellular response to organonitrogen compounds, response to nicotine, and head development. Genes that belong to these clusters were further studied in detail for function and pathway analysis.

Top 5 clusters are shown in Table 2; count is the number of genes in each cluster; percentage is the total gene ontology provided in list of genes; Log10(P) is the log base 10 value; and Log10(q) is the log base 10 adjusted p value [42].

Pathway enrichment shows that neuronal development genes are involved in colorectal cancer, neuron death, and other diseases like leukemia and sclerosis [50]. Genes AKT1, BAD, BAX, BCL2, CASP3, CASP8, CASP9, MYC, PIK3CD,

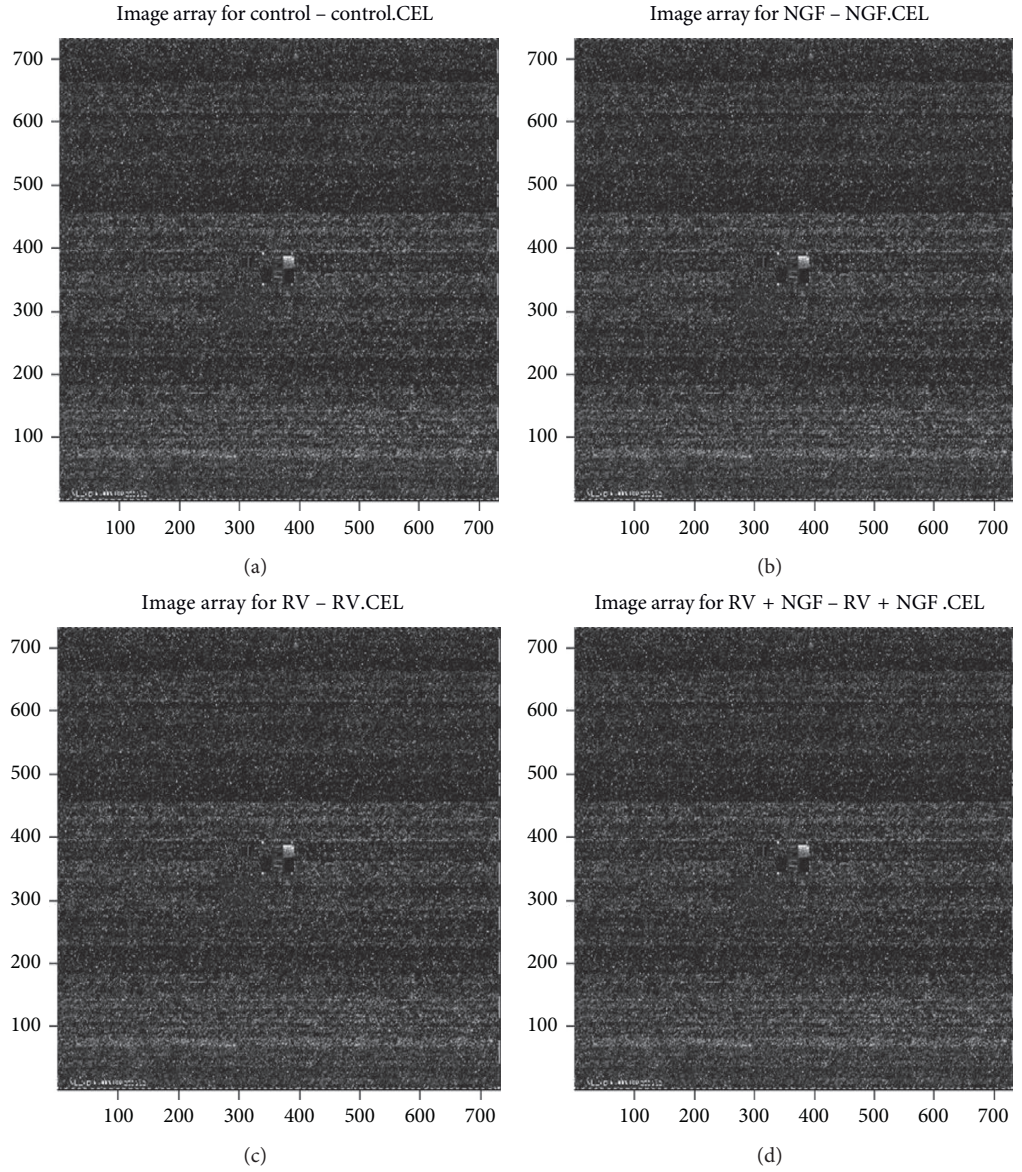

Figure 2: Affymetrix chip image: (a) control sample, (b) NGF sample, (c) RV sample, and (d) RV + NGF sample.

MAPK1, MAPK10, and CYCS are commonly expressed in the cluster of colorectal cancer, neuronal signaling pathway, neuronal death, amytrophic lateral aclerosis, and tuberculosis [51]. Further proteins are identified that show interaction with these proteins on the basis of protein-protein interaction study.

3.5. Protein-Protein Interaction Enrichment Analysis. Protein-protein interaction (PPI) enrichment was done among the list of genes that were clustered in pathway and process enrichment analysis. The Metascape tool predicts PPI network by comparing it with protein interaction databases (BioGrid, InWeb_IM, and OmniPath) [42]. PPI is made between proteins having physical interactions, and PPI network is further subclustered on the basis of the p value score. Figure 6 shows the PPI map between the set of input genes. Three best scoring genes by the p value are identified; these proteins define the functionality of PPI network. Best scoring genes belong to apoptosis (hsa04210) [52], colorectal cancer (hsa05210) [53], and hepatitis B (hsa05161) [54]. PPI network represents involvement of neuronal development genes in diseases like cancer.

The molecular complex detection (MCODE) method was applied to identify closely related protein from PPI network. The MCODE algorithm subclustered PPI network into 3 subclusters. Figure 7 shows MCODE components (red, blue, and green as MCODE 1, 2, and 3). Three dense PPI were made and detail of each cluster is given in Table 3. MCODE prediction validates the results of clustering as previously shown in Figure 4. The same set of proteins was identified by MCODE algorithm as predicted by clustering using the MeV tool. These proteins have the same GO and pathway.

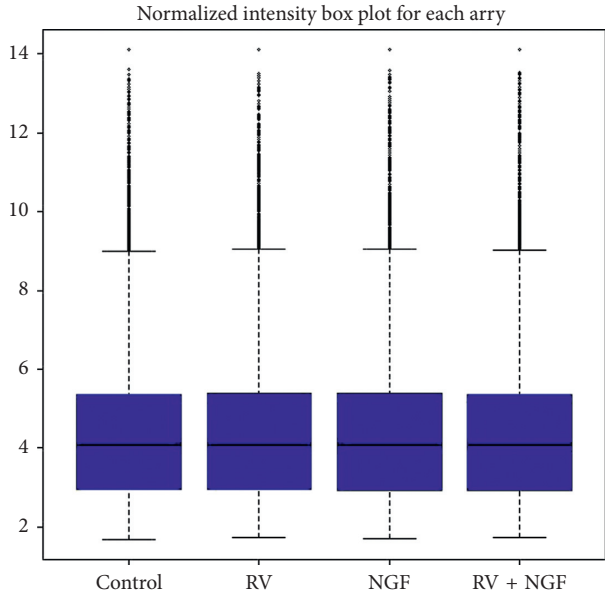

Figure 3: Box plot of microarray samples after normalization.

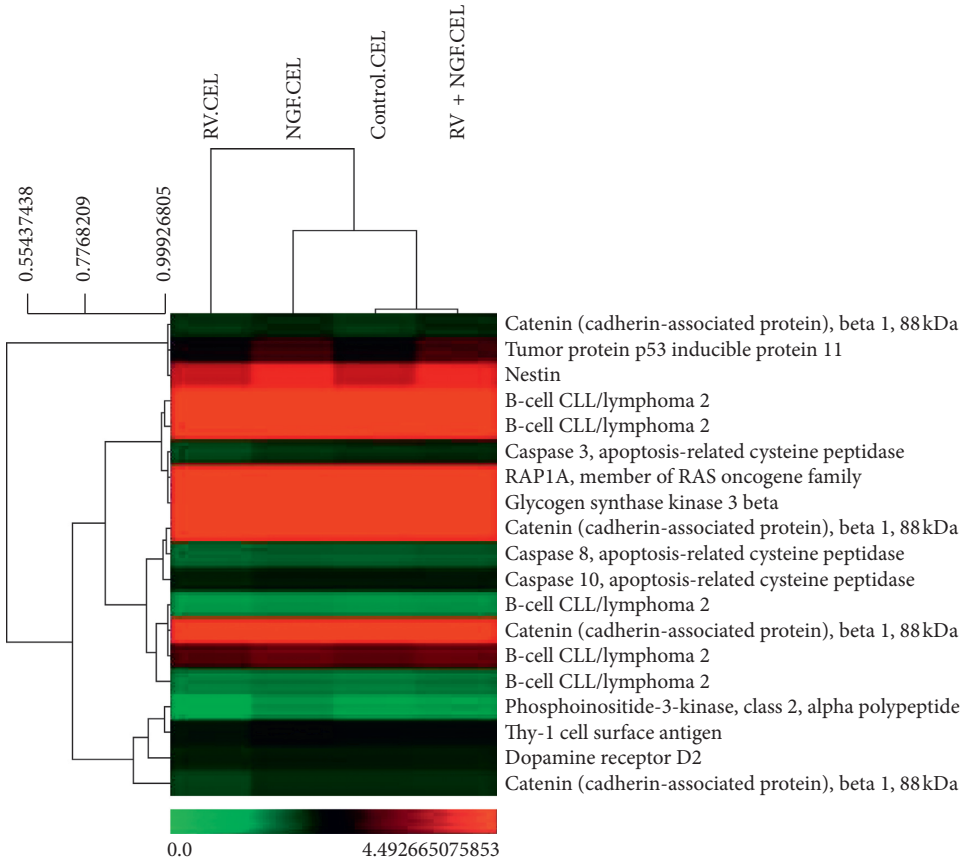

Figure 4: Clustering result of significant genes.

Cluster analysis of MCODE components is done, and details of proteins involved in each cluster and their corresponding pathways are shown in Table 3. Cluster 1 includes proteins CASP3, CASP9, BAX, TP53, BAD, GSK3B, POU5F1, MAPK14, CREB1, SOX2, and KLF4. Gene ontology data show that these proteins are associated with amyotrophic lateral sclerosis (hsa05014) [55], colorectal cancer (hsa05210) [56], and positive regulation of neuron death (GO: 1901216) [57].

Cluster 2 genes are mentioned in Table 3. GO analysis shows that these proteins belong to thyroid hormone

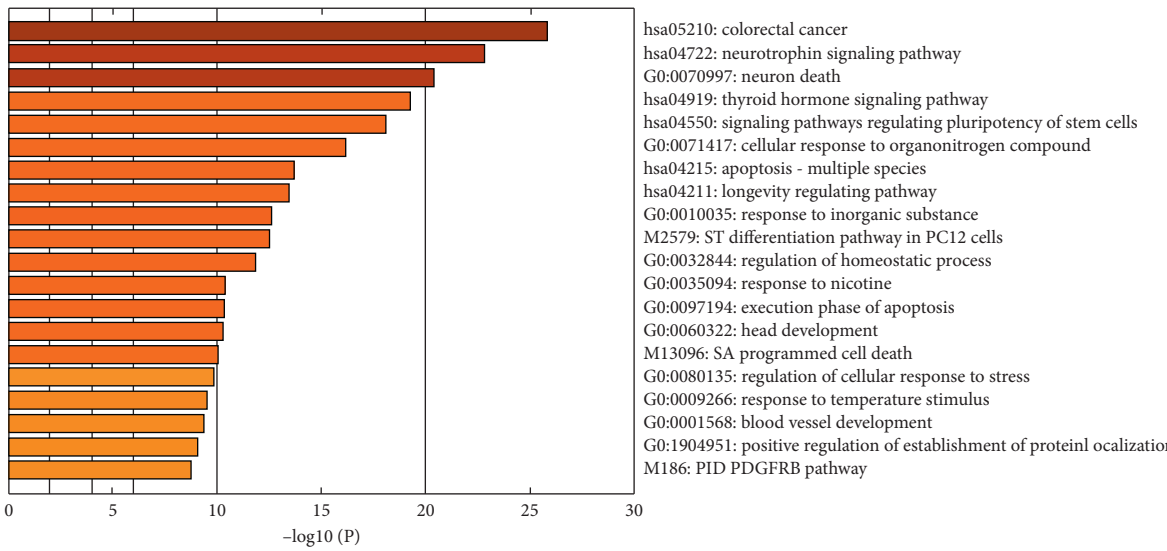

Figure 5: Heat map of enriched terms across input gene lists, colored by p values.

Table 2: Pathway and process enrichment analysis.

S.no	GO	Category	Description	Count	%	Log10 (P)	Log10 (q)
1	Hsa05210	KEGG pathway	Colorectal cancer	15	25	−25.7	−21.4
2	Hsa04722	KEGG pathway	Neurotrophin signaling pathway	16	26.6	−22.8	−19.1
3	Go: 0070997	Go biological processes	Neuron death	19	31.6	−20.3	−16.9
4	Hsa04919	KEGG pathway	Thyroid hormone signaling pathway	14	23.3	−19.2	−15.9
5	Hsa04550	KEGG pathway	Signaling pathways regulating pluripotency of stem cells	14	23.3	−18.0	−14.9

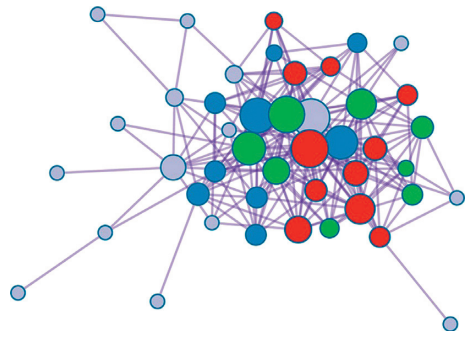

Figure 6: Protein-protein interaction network.

signaling pathway (hsa04919) [55], diseases of signal transduction (R-HSA-5663202) [56], and pathways in cancer (hsa05200) [57]. Cluster 3 proteins are involved in pathways of toxoplasmosis (hsa05145) [55], tuberculosis (hsa05152) [56], and fluid shear stress and atherosclerosis (hsa05418) [55].

MCODE cluster and cluster of MeV software show that some genes were commonly expressed and were coregulated. AKT1, BAD, BAX, BCL2, BDNF, CASP3, CASP8, CASP9, MYC, PIK3CD, MAPK1, MAPK10, and CYCS genes are coregulated. PPI analysis identifies other proteins that have interaction with abovementioned proteins. These proteins are important in neuronal differentiation, and regeneration proteins like ACTB, GSK3B, CREB1, and CTNNB1 have physical interaction with coexpressed proteins [58]. Table 3 also gives the information about proteins and association with diseases. Analysis of disease associated with proteins highlights that some proteins belong to different classes of cancers. 12 proteins (CASP3, CASP9, BAX, TP53, BAD,GSK3B, MTOR, BCL2L11, SIRT1, CASP8, AKT1, and C TNNB1 proteins) are involved in diverse types of cancers like lung cancer, breast cancer, ovarian cancer, colorectal cancer, and leukemia [59].

While other proteins (GSK3B, POU5F1, MAPK14, CREB1, SOX2, KLF4, PRKACA, MAPK10, STAT1, ACTB, TUBB3, MYC, GAPDH, AKT1, and CTNNB1) are related with process of aging, neuronal diseases, cardiovascular diseases, abnormal brain development, mental retardation,

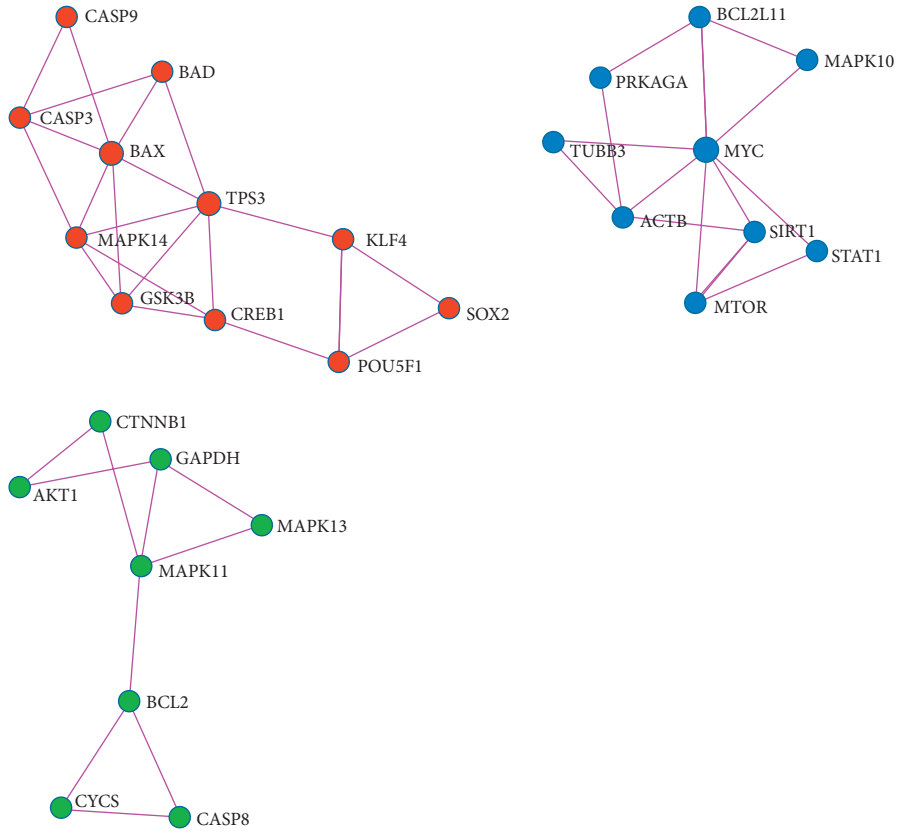

Figure 7: MCODE protein-protein interaction network. Colors show the different components of MCODE (red color: MCODE 1, blue color: MCODE2, and green color: MCODE 3).

schizophrenia, and mycobacterial and viral infections [60–62].

Key findings of the pathway and disease association study are identification of proteins involved in neurological diseases and also expressed at the early stage of neuronal development. SOX2 protein was expressed in optic nerve hypoplasia and abnormalities of the central nervous system [63], STAT1 was expressed during mycobacterial and viral infections [64], TUBB3 was related with fibrosis and cortical dysplasia and brain deformities, AKT1 was expressed in breast cancer, colorectal cancer, ovarian cancer, and schizophrenia [65] and CTNNB1 was expressed in colorectal cancer, hepatocellular carcinoma, ovarian cancer, and mental retardation [66]. The study shows that proteins (SOX2, STAT1, AKT1, and CTNNB1) can be used as markers for neurological disease at the early stage of neuronal development, and they can be potential drug targets for therapeutic development.

4. Conclusion and Discussion

Microarray experiment is designed to investigate the genes that are expressed at the early stage of neuronal development. Neurodevelopmental microarray gene expression data are used to identify genes that are expressed in neuronal disorders, at its initial stage of progress [67]. Four samples were prepared, viz, control, resveratrol, nerve growth factor, and RV + NGF and hybridized to Affymetrix chip (Prime view). Gene expression matrix was constructed, and computational analysis was done. The protocol is designed to study biologically significant genes. Microarray data analysis workflow includes quality control, data normalization, clustering, pathways enrichment, and PPI study. Clustering analysis identifies genes that are coexpressed. These sets of coexpressed genes are used for pathway and process enrichment analysis. Gene ontology and pathway study reveal proteins that share common pathways and function. Further protein-protein interaction network is constructed to identify more number of proteins, which have physical interaction with coexpressed proteins. PPI network is subclustered to predict closely related proteins. Gene ontology information of these proteins is used to identify function and disease associated with proteins. 12 proteins CASP3, CASP9, BAX, TP53, BAD, GSK3B, MTOR, BCL2L11, SIRT1, CASP8, AKT1, and CTNNB1 proteins are predicted that are involved in various types of cancers like lung cancer, breast cancer, ovarian cancer, colorectal cancer, and leukemia [60, 61, 62]. Some proteins like SOX2, STAT1, AKT1, and CTNNB1 proteins are associated with neurological disease like abnormal brain development, mental retardation, schizophrenia, and mycobacterial and viral infections [63–66]. These genes can be used as markers for neurological disease, for detection of abnormalities at the early stage of neuronal development [67]. Predicted proteins can also act as potential drug targets for the drug development process. Further work is required for wet lab

Table 3: Cluster details of MCODE protein-protein interaction and pathways.

Cluster	Symbol	Pathways	Diseases
1	CASP3	Caspase-3	Cancer
	CASP9	Caspase-9	Aging, cancer, chemdependency
	BAX	BCL2-associated X and apoptosis regulator	Colorectal cancer, somatic, T-cell acute lymphoblastic leukemia
	TP53	Tumor protein p53	Breast cancer, colorectal cancer, hepatocellular carcinoma, etc.
	BAD	BCL2-associated agonist of cell death	Cancer, hematological, infection
	GSK3B	Glycogen synthase kinase 3 beta	Cancer
	POU5F1	POU class 5 homeobox 1	Chemdependency
	MAPK14	Mitogen-activated protein kinase 14	Chemdependency
	CREB1	cAMP responsive element-binding protein 1	Histiocytoma
	SOX2	SRY-box 2	Optic nerve hypoplasia and abnormalities of the central nervous system
	KLF4	Kruppel like factor 4	Cardiovascular and metabolic diseases
2	MTOR	Mechanistic target of rapamycin	Aging, cancer, chemdependency
	PRKACA	Protein kinase cAMP-activated catalytic subunit alpha	Cushing syndrome
	MAPK10	Mitogen-activated protein kinase 10	Metabolic diseases
	STAT1	Signal transducer and activator of transcription 1	Mycobacterial and viral infections
	ACTB	Actin beta	Baraitser-winter syndrome 1 and dystonia
	BCL2L11	BCL2 like 11	Cancer, cardiovascular diseases
	SIRT1	Sirtuin 1	Aging, cancer, cardiovascular diseases
	TUBB3	Tubulin beta 3 class III	Fibrosis, cortical dysplasia, brain malformations
	MYC	v-myc avian myelocytomatosis viral oncogene homolog	Burkitt lymphoma
3	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Aging
	CASP8	Caspase-8	Breast cancer, lung cancer
	AKT1	AKT serine/threonine kinase 1	Breast cancer, somatic, colorectal cancer, ovarian cancer, schizophrenia
	BCL2	BCL2, apoptosis regulator	Leukemia/lymphoma
	CYCS	Cytochrome c, somatic	Thrombocytopenia
	MAPK11	Mitogen-activated protein kinase 11	Unknown
	MAPK13	Mitogen-activated protein kinase 13	Unknown
	CTNNB1	Catenin beta 1	Colorectal cancer, hepatocellular carcinoma, ovarian cancer and mental retardation

verification of predicted genes that are expressed in neurological disorders and express at the developmental stage. More research is required in the field of neurodevelopmental biology to identify neurological abnormalities at its budding stage. This paper also highlights the importance of microarray experiment in understanding the neurological diseases and methodology to study various outcomes of gene expression data, like coexpression analysis, pathway and process identification, and protein-protein interaction network study.

Data Availability

The microarray data used to support the findings of this study are included within the supplementary information file.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Supplementary Materials

Gene expression matrix microarray raw files (CEL and CDF files) were used to make gene expression matrix using Affy package and RMA (robust multiarray average) method. This matrix file was used for further microarray analysis like clustering, pathway, and protein-protein interaction analysis. Pathway and protein-protein interaction result as generated from the Metascape tool is given. This file includes information about clustered formed within genes and network details with scores. Annotation and enrichment annotation file and pathway enrichment result as generated from the Metascape tool is given. Annotation file includes information about genes like gene symbol, gene description, GO biological process, protein function, and so on. (*Supplementary Materials*)

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Research Article

Comparison of Antimicrobial Activity of *Allium sativum* Cloves from China and Taşköprü, Turkey

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In this study, antimicrobial activities of two different samples of *Allium sativum* L. from Turkey (TR) (Taşköprü, Kastamonu, Turkey) and China (CN) were determined. A broad spectrum of Gram-negative and Gram-positive bacteria (17 bacteria) including species of *Bacillus*, *Enterobacter*, *Enterococcus*, *Escherichia*, *Klebsiella*, *Listeria*, *Pseudomonas*, *Salmonella*, and *Staphylococcus* were used for testing antibacterial activity. In addition, antifungal activity against *Candida albicans* was also investigated. Antimicrobial activity was tested by using 3 different processes (chopping, freezing, and slicing by the disk diffusion method). The results showed that TR garlic presented more antimicrobial activity than CN garlic. Mechanism of activity of CN garlic could be proposed to be different from that of TR garlic.

1. Introduction

Prevention of food infection, related to pathogens and spoilers, is a significant topic at the last decades [1]. In order to inhibit food pathogens, medicinal plant research supply abundant source as natural preservatives [2]. Garlic is one of the oldest agricultural harvests, which has historical records dating back to BC 800 and today being used worldwide as food and medicine [3]. Garlic has several different uses, such as garlic volatile oil, garlic powder, and garlic juice for its antimicrobial activity. Main garlic species is *A. sativum*, which is not only accepted as an ethnopharmaceutical drug but also proved to have therapeutic effects by several scientific research studies. It has been used as food and medicine starting from ancient times in India, Egypt, Greece, and Rome [4]. Several research studies about antibacterial, antiprotozoal, anticancer, antifungal, and antiviral activity of garlic can be found as the current literature is concerned [5].

The most significant component of garlic is allicin (diallyl thiosulfinate), and its activity is investigated against a broad range of Gram-positive and Gram-negative bacteria. Allicin is not present in fresh clove of garlic, but it is released after crushing and chopping with the alliinase enzyme activity. Alliums, component of garlic, include largely cysteine sulfoxides. Conversion of alliinase to allicin by cysteine sulfoxides transforms to thiosulfates, which are volatile and lachrymatory [6]. Allicin, an organosulfur compound, which prevents lipid biosynthesis, was proved to damage *Candida albicans* cell wall [7] and cause inhibition of RNA synthesis in bacteria [8]. The antimicrobial activities of allicin and garlic extract investigated a large spectrum against *Mycobacterium*, *Photobacterium*, *Proteus*, *Pseudomonas*, *Salmonella*, *Staphylococcus*, *Escherichia*, *Helicobacter*, *Clostridium*, *Cryptocaryon*, *Klebsiella*, and *Bacillus* species [9].

In this study, the antimicrobial activity of *A. sativum* was analyzed after 3 different processes, namely, chopping, freezing, and slicing, by using the disk diffusion method. In

addition, the activities of two garlic samples, one from Taşköprü, Kastamonu, Turkey (TR), and the other from China (CN), are also compared.

2. Materials and Methods

2.1. Garlic. Two kinds of *A. sativum*, from Turkey (Taşköprü, Kastamonu) and China, were obtained for this study from a local company. Garlic, cultivated in Taşköprü region, is free of any chemical treatment; however, garlic cultivated in China region could possibly be treated by chemicals due to its industrial production.

2.2. Microbial Strains. Seventeen bacteria and 1 fungus species were used, and these microorganisms were sustained on nutrient agar (BD Difco, USA). There are 11 standard bacteria and 1 standard fungus. Five of them are standard Gram-positive bacteria, which are *Bacillus subtilis* DSMZ 1971, *Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 25923, and *Staphylococcus epidermidis* DSMZ 20044. The others are standard Gram-negative bacteria, which are *Enterobacter aerogenes* ATCC 13048, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* DSMZ 50071, *Pseudomonas fluorescens* P1, *Salmonella enteritidis* ATCC 13075, and *Salmonella typhimurium* SL1344. There is 1 standard fungus, which is *Candida albicans* DSMZ 1386. Besides, there are 6 nonstandard bacteria, which are isolated from food at Ankara University microbiology laboratory. Three of them are Gram-positive bacteria, which are *Enterococcus durans*, *Enterecoccus faecium*, and *Listeria innocua*. The others are Gram-negative bacteria, which are *Klebsiella pneumonia*, *Salmonella infantis*, and *Salmonella Kentucky*.

2.3. Garlic Ethanol Extracts. Garlic samples were prepared by 3 different processes: chopping, freezing, and slicing. Garlic is chopped in small pieces using a grinder. Garlic is sliced just into two pieces using a knife. In the freezing process, using an ultra freezer, garlic was frozen, and then it was ground immediately using a cold grinder. In all processes, 50 g of garlic was used, and these samples were shaken in ethanol (Sigma-Aldrich) at 125 rpm for 2 days at room temperature [10]. After that, all of them were filtrated using Whatman No. 1 filter paper into evaporation flasks. Filtrates were evaporated by a rotary evaporator (Buchi R3) at 45°C [11]. Finally, remnants were collected, and the quantities used for each process are as given in Table 1. In order to compare the results, the first and second quantities were adjusted to the same values in μg, where the third was set to the same value as μL.

2.4. Preparation of Inocula. All bacterial strains were incubated at 37°C for 24 hours; however, *Candida albicans* DSMZ 1386 was incubated at 27°C for 48 hours [12]. Each bacteria and yeast were inoculated into 0.9% sterile saline solution and adjusted to 0.5 McFarland standard, in order to standardize the inocula to contain about 10^8 cfu·mL^{-1} for bacteria and 10^7 cfu·mL^{-1} for *Candida albicans* [13].

Table 1: Amount of garlic samples which were loaded on disks in μg and μL.

Garlic	1.	2.	3.
TC	303.75 μg (112.5 μL)	607.5 μg (225 μL)	656.25 μg (262.5 μL)
TF	303.75 μg (87.5 μL)	607.5 μg (175 μL)	708.75 μg (262.5 μL)
TS	151.9 μg (66 μL)	303.75 μg (131.2 μL)	607.5 μg (262.5 μL)
CC	303.75 μg (51 μL)	607.5 μg (112.5 μL)	1155 μg (262.5 μL)
CF	303.75 μg (51 μL)	607.5 μg (112.5 μL)	1155 μg (262.5 μL)
CS	303.75 μg (87.5 μL)	607.5 μg (175 μL)	708.75 μg (262.5 μL)

2.5. Antimicrobial Activity Test. The antimicrobial activity of garlic ethanol extract was performed by he disk diffusion test, as described by Andrews [14]. First, Mueller-Hinton Agar (BD Difco, USA) was poured into 90 mm sterile Petri dishes until reaching a mean depth of 4.0 mm ± 0.5 mm. Extracts were loaded on 6 mm Oxoid Antimicrobial Susceptibility Test Disk as given in Table 1. Disks were left to dry overnight at 30°C under sterile conditions in order to prevent any remaining solvent, which may interfere with the result. After that, prepared microorganisms, which were inoculated into the saline solution, were streaked on the surface of Petri dishes. The plates were left to dry for 5 minutes at room temperature under aseptic conditions [15]. Next, disks were tightly applied to the surface of plates. Finally, the plates were incubated, and inhibition zone diameters were observed [16, 17].

2.6. Controls. Empty sterile disks and extraction solvent (ethanol) were used as negative controls.

2.7. Statistics. The statistical analysis was executed by a nonparametric method, Kruskal–Wallis, which is one-way analysis of variance with $p < 0.05$.

3. Results

Antimicrobial activity of *A. sativum* cloves (from Turkey and China) ethanol extracts were analyzed in our study. In order to load extract, empty sterile disks were used, and then these disks were applied on a culture medium (Mueller-Hinton Agar), which is inoculated with microorganisms. Inhibition zones were observed, when the extracts had activity against these microorganisms. The diameter of these zones was measured in millimetres as given in Tables 2 and 3. No activity for empty sterile disks and ethanol loaded on disks and evaporated before application, which are negative controls, was observed.

Table 2: Disk diffusion test result for *A. sativum* from Kastamonu (inhibition zones in mm).

	TC			TF			TS		
	1.	2.	3.	1.	2.	3.	1.	2.	3.
B. subtilis DSMZ 1971	17	23	24	10	13	15	-	8	10
C. albicans DSMZ 1386	25	30	30	11	16	19	-	9	16
E. aerogenes ATCC 13048	7	7	7	-	-	-	-	-	7
E. durans	-	8	8	-	-	-	-	-	-
E. faecalis ATCC 29212	-	8	9	-	-	7	-	-	-
E. faecium	11	17	17	-	10	10	-	-	8
E. coli ATCC 25922	-	7	7	-	-	-	-	-	-
K. pneumonia	7	7	7	7	7	7	7	8	7
L. innocua	7	8	9	-	-	-	-	-	-
L. monocytogenes ATCC 7644	18	20	18	-	-	-	-	8	8
P. aeruginosa DSMZ 50071	7	7	8	-	-	7	-	-	-
P. fluorescens P1	-	-	7	-	-	-	-	-	-
S. enteritidis ATCC 13075	-	7	8	-	-	7	-	-	-
S. infantis	-	7	7	-	-	-	-	-	-
S. kentucky	7	7	7	-	-	-	-	-	7
S. typhimurium SL1344	-	-	7	-	-	7	-	-	-
S. aureus ATCC 25923	9	11	13	-	7	8	-	-	-
S. epidermidis DSMZ 20044	-	9	10	-	7	8	-	-	-

"-": no activity observed.

Table 3: Disk diffusion test result for *A. sativum* from China (inhibition zones in mm).

	CC			CF			CS		
	1.	2.	3.	1.	2.	3.	1.	2.	3.
B. subtilis DSMZ 1971	12	21	24	17	19	28	14	20	23
C. albicans DSMZ 1386	16	24	30	17	20	28	18	25	30
E. aerogenes ATCC 13048	-	7	7	-	-	7	-	-	-
E. durans	-	-	8	-	-	8	7	7	7
E. *faecalis* ATCC 29212	-	-	8	-	-	8	-	-	8
E. faecium	9	10	17	8	9	18	9	11	14
E. coli ATCC 25922	-	7	7	-	7	7	-	7	7
K. pneumonia	7	7	7	7	7	7	7	7	7
L. innocua	-	-	9	-	-	8	-	-	8
L. monocytogenes ATCC 7644	-	8	10	-	9	12	8	9	10
P. aeruginosa DSMZ 50071	-	7	7	-	7	7	-	7	7
P. fluorescens P1	-	-	9	-	-	8	-	-	-
S. enteritidis ATCC 13075	-	-	7	-	-	7	-	-	7
S. infantis	-	7	7	-	7	7	-	-	-
S. kentucky	-	-	-	-	-	-	-	-	-
S. typhimurium SL1344	-	-	-	-	-	-	-	-	-
S. aureus ATCC 25923	-	8	10	-	8	11	8	10	11
S. epidermidis DSMZ 20044	-	8	9	-	7	8	-	7	9

"-": no activity observed.

The diameter of inhibition zones for *A. sativum* from Turkey is given in Table 2. Turkey-chopped garlic (TC) has antimicrobial activity against 17 bacteria; however, Turkey-frozen garlic (TF) has antimicrobial activity against 9 bacteria, whereas Turkey-sliced garlic (TS) has antimicrobial activity against 6 bacteria. According to Table 2, TC has high antimicrobial activity against *B. subtilis* DSMZ 1971 (24 mm), *E. faecium* (17 mm), and *L. monocytogenes* ATCC 7644 (18 mm) at 656.25 mg sample. TF has only high antimicrobial activity against *B. subtilis* DSMZ 1971 (15 mm). Furthermore, TC, TF, and TS have high antifungal activity against *Candida albicans* DSMZ 1385 (30, 19, and 16 mm, respectively); however, the antifungal activity dramatically decreased in TF and TS. These results demonstrate that freezing and slicing negatively affected the antimicrobial activity of *A. sativum* from Turkey.

The diameter of inhibition zones for *A. sativum* from China is given in Table 3. China-chopped garlic (CC) has antimicrobial activity against 15 bacteria, China-frozen garlic (CF) has antimicrobial activity against 15 bacteria, and China-sliced garlic (CS) has antimicrobial activity against 12 bacteria. According to Table 3, CC, CF, and CS have high antimicrobial activity against *B. subtilis* DSMZ 1971 (24, 28, and 23 mm, respectively) and *E. faecium* (17, 18, 14 mm respectively). Furthermore, CC, CF, and CS have high antifungal activity against *Candida albicans* DSMZ 1385 (30, 28, and 30 mm, respectively). These results demonstrate that freezing and slicing did not negatively affect the antimicrobial activity of *A. sativum* from China.

4. Discussion

In this research, ethanol is used as an extraction solvent because it has best solvability of active ingredients when compared to other solvents, such as methanol, ethyl acetate, and chloroform [18]. Gram-positive bacteria are more sensitive to antimicrobials, and they have no powerful wall because of the existence of only thick peptidoglycan layers on the outer surface [19]. However, Gram-negative bacteria are less susceptible due to phospholipidic membrane, which prevents the permeability of lipophilic solutes. These hydrophilic solutes can pass with porines, which are selective barriers.

L. monocytogenes has critical food-borne pathogen activity, and it causes listeriosis with serious illness [20]. Since the production of ready-to-eat food is increasing, discovering some alternative compounds has become critical against listeriosis for the food industry [21]. For listeriosis treatment, natural garlic product can be used significantly rather than industrial product.

According to Kallel et al. [22], *A. sativum* ethanol extract had moderate antibacterial activity against *B. subtilis* and *S. aureus*, which were 10–15 mm and low-level activity against *B. thuringiensis* and *P. aeruginosa*, which were <10 mm, however, presented no activity against *K. pneumoniae*, *E. coli*, and *S. typhimurium*. Also, Karuppiah and Rajaram [23] reported that *A. sativum* ethanolic extract had antibacterial activity against all tested multiple antibiotic-resistant (MAR) bacteria such as *P. aeruginosa* (19.45 mm), *E. coli* (18.50 mm), *Bacillus* sp. (16.5 mm), *Proteus* sp. (13.50 mm), *Enterobacter* sp. (13.50 mm), and *S. aureus* (13.50 mm).

Our results are also important since a broad range of strains were tested, and as a result, we can propose that TR garlic is more effective than CN garlic. These activity differences are not related to garlic clove amount, because their first and second volumes were equaled at sort of μg, and third volumes were equaled at sort of μL. For this reason, their differences are related to their ingredients.

5. Conclusion

According to our results, TR *A. sativum* (Turkey) has more antimicrobial activities than CN *A. sativum* (China). In addition, freezing and slicing negatively affected the antimicrobial activity of *A. sativum* from Turkey, and in contrary, no reverse effect was observed for freezing and slicing against *A. sativum* which is from China. By using freezing and slicing, motor force was prevented in order to inhibit the transformation of alliin to allicin. The process of industrial production could lead to change in antimicrobial activity and composition and concentration of active components. However, further research studies are required in order to analyse these active substances and their mechanism of activity in detail. Besides, these results should be supported by further large-scale studies; however, by keeping in mind that geographical differences can cause active compound differences, these differences must also be taken into account in further studies.

Abbreviations

CC: China-chopped garlic
CF: China-frozen garlic
CS: China-sliced garlic
CN: Garlic cloves from China
TR: Garlic cloves from Turkey
TC: Turkey-chopped garlic
TF: Turkey-frozen garlic
TS: Turkey-sliced garlic.

Disclosure

An earlier version of this study was presented as an abstract in "The Second Japan—Turkey International Symposium on Pharmaceutical and Biomedical Sciences, 2017".

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

HPTLC Analysis of *Solanum xanthocarpum* Schrad. and Wendl., a *Siddha* Medicinal Herb

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In the present study, HPTLC is used to detect the presence and amount of triterpenoids and phytosterols in different plant parts (fruit, stem, leaf, and root) of *Solanum xanthocarpum* Schrad. and Wendl.; such analysis is done for the first time. Each plant part has its own medicinal value and is used as *Siddha* medicinal herb. The employed statistical analysis ensures that the developed method is reproducible and selective. The results show that the fruit samples contain highest amount of tested phytochemicals. This method can be used as an important tool to ensure the therapeutic dose in herbal formulations, standardization, and quality control of bulk drugs.

1. Introduction

Solanum xanthocarpum Schrad. and Wendl., commonly known as *Kantkari*, belongs to family Solanaceae. It comprises 90 genera and 2000–3000 species. It is distributed to plains and lower hills of India. It is an herbaceous spiny perennial herb with prominent nodes and internodes. Roots are almost cylindrical and tapering. Flowers are purple colored and few flower axillary cymes with glabrous, globular berry are green when young and turn yellow at maturity. The seeds are smooth, compressed, and reniform with bitter taste. It is known for its traditional medical value, and recent scientific studies have emphasized the possible use of this plant in the modern medicine system. India is rich with its biodiversity and knowledge of rich ancient traditional systems of medicine like Ayurveda, Siddha, Unani, Amchi, and local health traditions [1]. Wild plants serve as an indispensable constituent of the human diet. They supply with minerals, vitamins, protein, and certain hormone precursors [2–4]. Roots, stem, leaves, flowers, and fruits are useful parts of this herb as *siddha* medicinal herb [5]. However, there is need to study the inexpensive nutritive value of these wild plants so that these can be exploited for their pharmaceutical preparations.

2. Experimental

At present triterpenoids and phytosterols are separated from methanolic extracts in different plant parts (fruit, stem, leaf, and root) in *S. xanthocarpum* by using high-performance thin layer chromatography (HPTLC). Details of qualification and quantification of different mobile phases used are mentioned in Table 1. Part-based separation of bioactive compounds from the wild samples is done first.

2.1. Plant Material. Plant samples were collected from different localities of Indian Thar desert, Rajasthan. The specimens were collected and deposited in the herbarium of Punjabi University, Patiala, with accession numbers 59194 and 59931. The plants parts, i.e., fruit, leaf, stem, and root samples, were separated washed and dried at room temperature (25°C–30°C).

2.2. Stock Solution. All the organic solvents as well standards of analytical grade used in the present study were purchased from Merck (Darmstadt, Germany). Solutions of standards were prepared by using 1 : 1 methanol.

Table 1: Data showing bioactive markers, composition of solvent system, derivatizing reagent used and wavelength of all the marker compounds used in the present study for HPTLC analysis.

S. no.	Bioactive compound	Solvent system	Composition (v/v/v/v)	Derivatizing reagent	Wavelength (nm)
Triterpenoids					
1.	Lupeol	Toluene:methanol:formic acid	9 : 4: 0.2	*p*-Anisaldehyde sulphuric acid	530
2.	Oleanolic acid	Toluene: ethylacetate:formic acid	7 : 3 : 0.3	*p*-Anisaldehyde sulphuric acid	540
3.	Ursolic acid	Toluene: ethylacetate:formic acid	8 : 2 : 0.1	*p*-Anisaldehyde sulphuric acid	510
Phytosterols					
4.	β-Sitosterol	Toluene: ethylacetate	9 : 4	*p*-Anisaldehyde sulphuric acid	530
5.	Campesterol	Toluene:methanol:formic acid	9 : 4 : 0.2	*p*-Anisaldehyde sulphuric acid	530
6.	Ergosterol	Toluene:methanol:formic acid	9 : 4 : 0.2	*p*-Anisaldehyde sulphuric acid	530
7.	Withanolide B	Toluene:methanol:formic acid	9 : 4 : 0.2	*p*-Anisaldehyde sulphuric acid	530

2.3. Apparatus. For the acquisition, a Camag HPTLC system comprising a Linomat-V automatic sample applicator and Camag TLC scanner III with win CATS 4 software for interpretation of data was used. A Camag 100 μL precision syringe from Hamilton, Bonaduz, Switzerland, was used for sample application under gentle stream of nitrogen. Camag aluminum precoated silica gel 60-$_{F254}$ plates with 200 μm thickness × 5 μm particle size from Merck (Darmstadt, Germany) was used. For the plate development, a Camag twin-trough chamber 20 cm W × 10 cm H was used.

2.4. Chromatography. Chromatographic studies were performed using the following conditions: HPTLC was carried out using aluminum plates precoated with silica gel 60F_{254}. Combinations of different mobile phases were used to quantify different standards (Table 1); volume of mobile phase was kept up to 20 mL; chamber saturation time: 30 min; temperature: 25 + 18°C; relative humidity: 35%–40%; migration distance: 80 mm; migration time: 30 min; wavelength of detection (Table 1); scanning speed: 20 mm/s; data resolution: 100 mm/step; and band width: 4mm. A Camag video documentation system was used for imaging and archiving the thin layer chromatograms. The object was captured by means of a highly sensitive digital camera. Image acquisition processing and archiving were controlled via Win CATS software.

2.5. Preparation of Derivative Reagent. Anisaldehyde sulphuric acid was prepared by dissolving 5 mL of *p*-anisaldehyde solution in 1 ml of 98% sulphuric acid and 50 ml of acetic acid. After development and derivatization of the plate, measurements were made by winCATS software. Concentration of the target analytes in the separated bands was determined from the intensity of the reflected light indicated and the peak areas produced were correlated to the analyte concentrations using six-level linear calibration curves.

2.6. Chromatographic Separation. Each extract of 5 μL *S. xanthocarpum* solution was spotted on the HPTLC silica gel plate, 4 mm band length, using a Camag ATS4 automatic TLC sampler spotting device. The TLC plate was developed in the ascending mode in a twin-trough chamber presaturated for 30 mins with particular mobile phase. Linear ascending plate development was performed until a migration of distance 8 cm from the origin was reached. The plate was removed from the chamber, air dried, derivation with *p*-ansaldehyde sulphuric acid, heated, and scanned in the absorbance/reflectance mode of a Camag TLC scanner 3 (Figures 1–7). Peak area data were recorded using Camag Win CATS software.

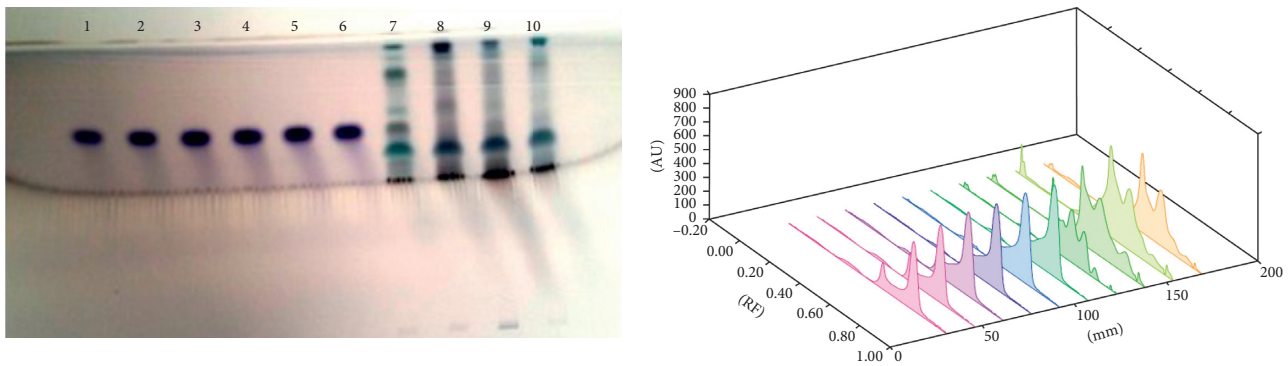
2.7. Calibration Curve. A standard solution volume of 2–10 μL of all the analyzed sugars was used. Concentration of the target analysts in the separated bands were determined from the intensity of the reflected light indicated, and the produced peak areas were correlated to the analyst concentrations using six-level linear calibration curves. The employed statistical analysis ensures that the developed method is reproducible and selective. This method can be used as an important tool to ensure the therapeutic dose in herbal formulations, standardization, and quality control of bulk drugs.

3. Validation of HPTLC Densitometry Method Specificity

3.1. Specificity. The specificity of the method was ascertained by analyzing standard compounds and samples. The spots for standards in samples were confirmed by comparing the R*f* and spectra of the spots with that of the standards. The peak purity of all standards were assessed by comparing the spectra at three different levels, i.e., peak start, peak apex, and peak end positions of the spot.

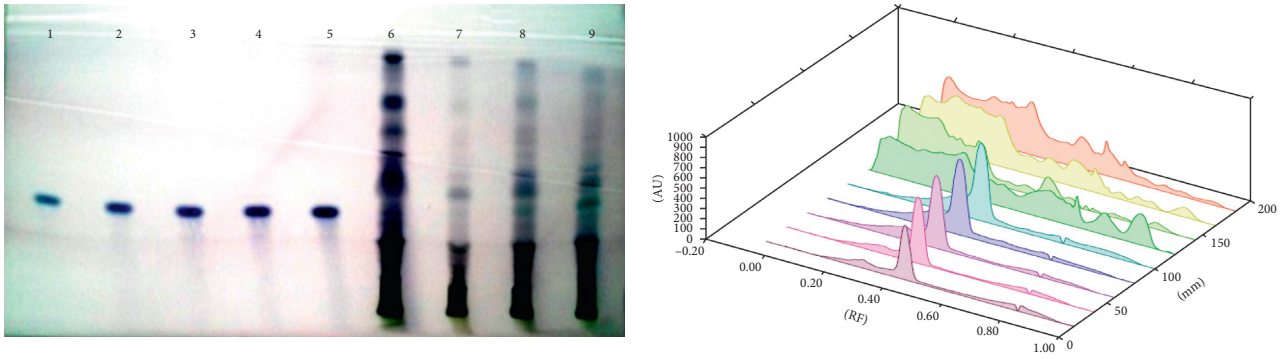
3.2. Precision. To define deviations due to the instrument, six different samples of the same were spotted on HPTLC silica gel plates and analyzed to determine variations arising due to method itself (Table 1).

3.3. Recovery. The recovery of the method was determined at two levels, i.e., 50% and 100%, by adding a known amount of particular standard to the extracts of plant part, and the mixtures were analyzed by the proposed method.



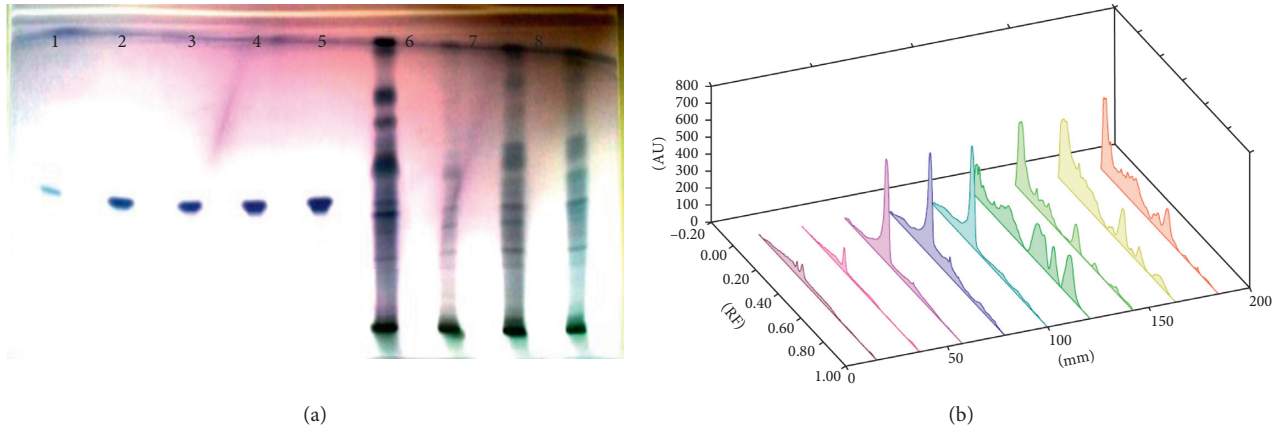
(a) (b)

Figure 1: (a) HPTLC fingerprint profile of lupeol (tracks 1–6) in fruit (track 7); leaf (track 8); stem (track 9); and root (track 10) of *S. xanthocarpum*; (b) 3D view of densitogram at 530 nm.



(a) (b)

Figure 2: (a) HPTLC fingerprint profile of oleanolic acid (tracks 1–5) in fruit (track 6); leaf (track 7); stem (track 8); and root (track 9) of *S. xanthocarpum*; (b) 3D view of densitogram at 530 nm.

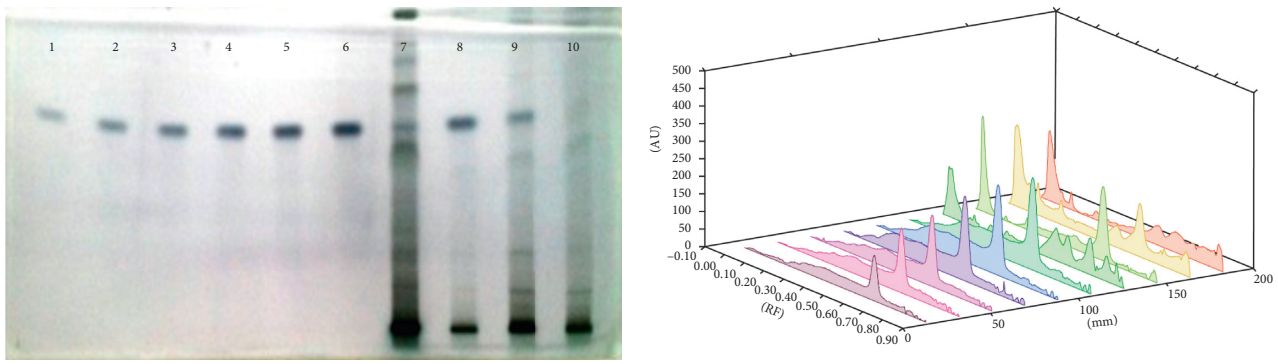


(a) (b)

Figure 3: (a) HPTLC fingerprint profile of ursolic acid (tracks 1–5) in fruit (track 6); leaf (track 7); stem (track 8); and root (track 9) of *S. xanthocarpum*; (b) 3D view of densitogram at 510 nm.

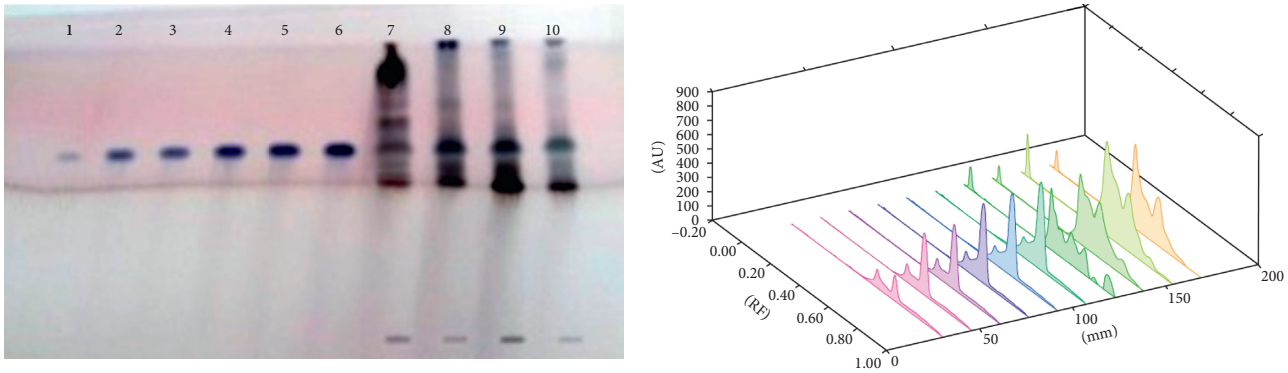
3.4. Ruggedness. The ruggedness of the proposed method was studied using reagents from different lots and different manufacturers.

3.5. Limit of Detection and Limit of Quantitation. The limit of detection (LOD) and limit of quantitation (LOQ) were determined, and data pertaining to LOD, LOQ, interday,



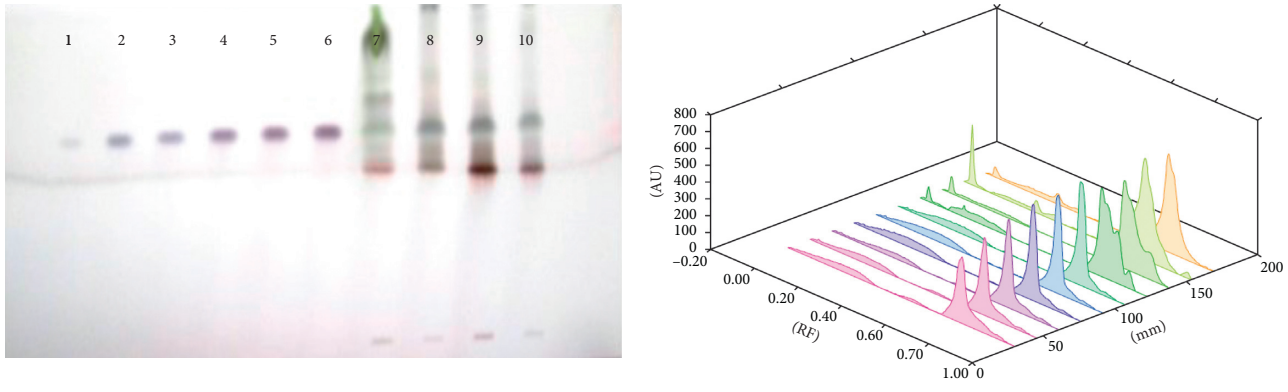
(a) (b)

Figure 4: (a) HPTLC fingerprint profile of β-sitosterol (tracks 1–6) in fruit (track 7); leaf (track 8); stem (track 9); and root (track 10) of *S. xanthocarpum*; (b) 3D view of densitogram at 530 nm.



(a) (b)

Figure 5: (a) HPTLC fingerprint profile of campesterol (tracks 1–6) in fruit (track 7); leaf (track 8); stem (track 9); and root (track 10) of *S. xanthocarpum*; (b) 3D view of densitogram at 530 nm.

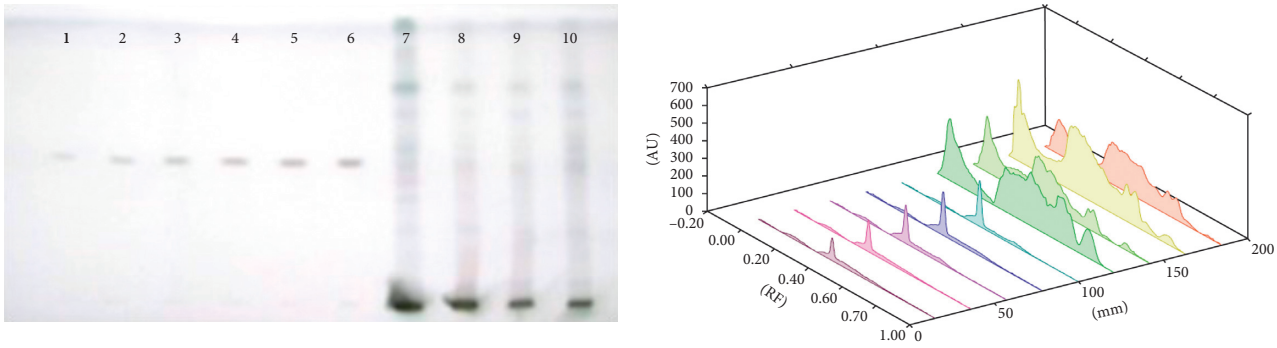


(a) (b)

Figure 6: (a) HPTLC fingerprint profile of ergosterol (tracks 1–6) in fruit (track 7); leaf (track 8); stem (track 9); and root (track 10) of *S. xanthocarpum*; (b) 3D view of densitogram at 530 nm.

and intraday precision are given in Table 1. The significant difference between the amounts of particular compound in each plant is also mentioned in Table 3.

3.6. Sample Preparation. Plant parts like fruit, leaf, stem, and root of the plants were extracted with methanol by using the Soxhlet apparatus. The plant material was shade-dried and



(a) (b)

Figure 7: (a) HPTLC fingerprint profile of withanolide B (tracks 1–6) in fruit (track 7); leaf (track 8); stem (track 9); and root (track 10) of *S. xanthocarpum*; (b) 3D view of densitogram at 530 nm.

Table 2: Data showing different parameters analyzed for the reference compounds during the present study by using HPTLC.

Reference compound	Lupeol	Oleanolic acid	Ursolic acid	β-Sitosterol	Campesterol	Ergosterol	Withanolide B
Working concentration (μg/band)	2–10	2–10	2–10	2–10	2–10	2–10	2–10
Rf value	0.84	0.47	0.36	0.64	0.74	0.90	0.64
Regression equation	$Y = 2653 * X + 1548$	$Y = 3621 * X - 969.3$	$Y = 924.3 * X - 155.1$	$Y = 1308 * X + 609.7$	$Y = 2554 * X + 214.4$	$Y = 1652 * X + 348.7$	$Y = 470 * X + 319.4$
Correlation coefficient (r^2)	0.998	0.997	0.997	0.995	0.993	0.995	0.995
LOD (ng)	407	304	578	419	928	527	488
LOQ (ng)	1234	921	987	1272	2813	1598	1479

coarsely powdered before Soxhlet apparatus application. 10 g of each dried and powdered aerial plant parts was applied to the methanolic extraction independently in the Soxhlet apparatus. The extracts were concentrated using a rota-evaporator and then lyophilized. Powdered extracts was weighed, and 5 mg of each was dissolved in 5 mL of methanol to obtain 1 mg/1 mL concentration.

4. Results

Plant samples were collected from different localities of Indian Thar desert, Rajasthan. Present studies reveal unequal concentration of bioactive compounds in different plant parts. This phenomenon is very common in many secondary metabolities [6, 7].

Different solvents are examined for the separation of these bioactive compounds, and the best combinations for separation are listed in Tables 1 and 2. Triterpenoids detected are lupeol, oleanolic acid, and ursolic acid. The fruit (6.81 ± 0.23 μg/mg DWE) and stem (6.179 ± 0.61 μg/mg DWE) samples of the plant are rich in lupeol content. It is completely absent in leaf samples and present in very less amount in root samples (Figure 1; Table 3). Roots are found to be quite rich in oleanolic acid (24.67 ± 0.582 μg/mg DWE) and ursolic acid (8.48 ± 0.31 μg/mg DWE) followed by stem samples (6.39 ± 0.97 μg/mg DWE; 1.07 ± 0.19 μg/mg DWE), (Figures 2 and 3; Table 3). Among the triterpenoids, earlier oleanolic acid was isolated using paper, thin layer, and column chromatography [8]. There is no earlier report of part-based isolation or separation of these triterpenoids by using HPTLC in wild samples of the plant.

Amount of β-sitosterol is reported to be high in stem samples of the plant (20.85 ± 0.96 μg/mg DWE) followed by the leaf samples (19.89 ± 1.53 μg/mg DWE) and root samples (8.04 ± 0.055 μg/mg DWE). The least amount of β-sitosterol was reported in fruits (6.42 ± 0.91 μg/mg DWE) of the plant (Figure 4). The root samples are quite rich in phytosterodial composition (28.19 ± 0.01 μg/mg DW) of campesterol and ergosterol (24.27 ± 0.28 μg/mg DW; Figures 5 and 6). Withaferin A and withanolide A and B were also tested for their presence. But only withanolide B was present. Withanolide B is also separated along with other bioactive compounds (Tables 1 and 2). It is present only in the fruit (17.59 ± 0.12 μg/mg DWE) and the root samples (10.09 ± 0.14 μg/mg DWE) of the plant (Figure 7). Phytosterols are the most important constituents which increases the medicinal value of the plant. Earlier, there was no report of determination of different bioactive compounds from the plant by using HPTLC. At present, the methanolic extract of the plant are used to separate different bioactive compounds (Tables 1 and 2). The plant is reported to be very rich in phytosteroidal content (Table 3).

Among triterpinoids, earliar oleanolic acid was isolated using paper, thin layer, and column chromatography [9], and lupeol was reported in fruits in tissue cultures of *solanum xanthocarpum* [10]. Phytosterols are the most important constituents which increases its medicinal value of

Table 3: Phytochemical studies in different plant parts of selected marker compounds in *S. xanthocarpum* by using HPTLC.

Bioactive compound	Fruit (μg/mg)	Stem (μg/mg)	Leaf (μg/mg)	Root (μg/mg)
Chlorogenic acid	20.86 ± 0.25	7.75 ± 0.47	0.37 ± 0.07	0.90 ± 0.06
Apigenin	2.95 ± 0.36	6.57 ± 0.32	6.61 ± 0.76	10.12 ± 0.65
Lupeol	6.81 ± 0.23	0.17 ± 0.61	Nd	1.73 ± 0.14
Oleanolic acid	16.43 ± 0.66	6.39 ± 0.97	17.98 ± 0.67	24.67 ± 0.58
Ursolic acid	5.45 ± 0.24	11.07 ± 0.19	8.64 ± 0.16	8.48 ± 0.31
β-Sitosterol	6.42 ± 0.91	20.85 ± 0.96	19.89 ± 1.53	8.049 ± 1.05
Campesterol	26.73 ± 0.004	14.34 ± 0.95	13.86 ± 0.43	28.19 ± 0.018
Ergosterol	12.09 ± 0.40	9.35 ± 0.32	12.09 ± 0.48	24.27 ± 0.28
Withanolide B	1.95 ± 0.068	8.29 ± 0.37	3.43 ± 0.072	34.09 ± 0.53
Emodin	Nd	0.82 ± 0.40	1.01 ± 0.74	0.61 ± 0.04

the plant. Heble et al. [11] reported the presence of β-sitosterol through tissue culture techniques. The plant was estimated for its fatty acids content in [12].

5. Discussion

A simple, rapid, reliable method is developed and validated for the qualitative and quantitative determination of different phytochemicals in plant matrices. A significant difference was obtained among the different plant parts. The highest amount of most of the compounds was noted in fruit samples of the plant. The results clearly show that the fruits are the very supplier of different phytochemicals mainly phytosterols and should be explored more in the production of medicinal drugs in pharmaceutical companies.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Investigation of Antioxidant/Oxidant Status and Antimicrobial Activities of *Lentinus tigrinus*

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In the present study, antioxidant and antimicrobial potential of the *Lentinus tigrinus* (Bull.) Fr. mushroom was determined. Total antioxidant status (TAS), total oxidant status (TOS), and oxidative stress index (OSI) of the mushroom were measured with Rel Assay kits. Antimicrobial activities were tested on 9 standard bacterial and fungal strains (*Staphylococcus aureus*, *Staphylococcus aureus* MRSA, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Candida albicans*, *Candida krusei*, and *Candida glabrata*) with a modified agar dilution method. It was determined that the TAS value of *L. tigrinus* was 1.748 ± 0.071, TOS value was 19.294 ± 0.237, and OSI was 1.106 ± 0.031. It was also found that mushroom extracts generally exhibited higher activity on *Candida albicans*, *C. krusei*, and *C. glabrata*. In conclusion, it was suggested that *L. tigrinus* can be used as a natural source due to its antioxidant and antimicrobial activities.

1. Introduction

Since historical times, several mushroom species have been consumed as nutrients and medicine of natural origin by humans [1]. Mushrooms can be considered as functional nutrients due to their health benefits and nutritional properties. In recent years, functional nutrients were again the center of focus for the consumers whose interest in human health, nutrition, and prevention from diseases has increased [2, 3]. Previous studies on mushrooms reported that mushrooms possessed several medical properties such as antioxidant, antimicrobial, DNA-protective, analgesic, anti-inflammatory, cytotoxic, antiviral, anticancer, anti-parasitic, immunomodulation effects, and hepatoprotective activity [4–18]. The identification of medical potential of the mushrooms is significant for identification of new natural resources for fighting the diseases.

Lentinus tigrinus is a wood-rotting basidiomycete with leathery flesh, strong aroma, and taste that makes it applicable in gourmet preparations [19]. This basidiomycetous mushroom is often seen growing on fallen logs in the forest from May to September [20]. Previous studies reported that this mushroom contains high amounts of carbohydrates, proteins, fibers, and minerals [19].

The present study aimed at determining the total antioxidant status, total oxidant status, and oxidative stress index of *L. tigrinus* (Bull.) Fr. mushroom and the antimicrobial activities of the ethanol (EtOH), methanol (MeOH), and dichloromethane (DCM) extracts of the mushroom. This study will evaluate the availability of *L. tigrinus* mushroom for pharmacological designs.

2. Materials and Methods

2.1. Laboratory Studies. Lentinus tigrinus (Bull.) Fr. study samples were collected in Gaziantep province, Turkey. Morphological (shape, color, and size) and ecological characteristics of the samples were recorded in the field conditions. The microscopic characteristics of the specimens transported to the laboratory under appropriate conditions were determined by light microscopy using a 3% KOH solution (Leica DM750). The specimen was identified morphologically using the references of Käärik [21], Knudsen [22], Bresadola [23], Dähncke [24], Roux [25], and

Boccardo et al. [26]. After the collected mushroom samples were identified, they were dried at 40°C in an incubator. Then, they were pulverized in a mechanical grinder. Then, pulverized 30 g mushroom samples were placed in cartridges, and the extracts were obtained with ethanol (EtOH) (Merck), methanol (MeOH) (Merck), and dichloromethane (DCM) (Merck) in a soxhlet extractor (Gerhardt EV) at 50°C for approximately 6 hours. The extracts were then concentrated under pressure at 40°C in a rotary evaporator (Heidolph Laborota 4000 Rotary Evaporator) to conduct the tests at +4°C [1].

2.2. Determination of Total Antioxidant Status (TAS), Total Oxidant Status (TOS), and Oxidative Stress Index (OSI). The mushroom total antioxidant status (TAS), total oxidant status (TOS) levels, and oxidative stress index (OSI) were determined with Rel assay brand commercial kits (Rel Assay Kit Diagnostics, Turkey). Trolox was used as the calibrator in the TAS tests and hydrogen peroxide in the TOS tests [27, 28]. To determine the OSI, the mmol unit of TAS and the μmol unit of the TOS were cross-converted and the index value was expressed as percentage [28]. The TAS and TOS tests were conducted on 5 mushroom samples in 5 replicates.

2.3. Antimicrobial Activity Tests. Antimicrobial activity tests were conducted with the agar dilution method recommended by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) on mushroom EtOH, MeOH, and DCM extracts. Minimal inhibitor concentration (MIC) for each extract was determined against standard bacterial and fungal strains. *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* MRSA ATCC 43300, and *Enterococcus faecalis* ATCC 29212 were used as Gram-positive bacteria. *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Acinetobacter baumannii* ATCC 19606 were used as Gram-negative bacteria. *Candida albicans* ATCC 10231, *Candida krusei* ATCC 34135 ATCC 13803, and *Candida glabrata* ATCC 90030 were used as fungi. Bacterial strains were precultured in Muller-Hinton Broth medium, and fungal strains were precultured in the RPMI 1640 broth medium. To obtain a standard inoculum, the turbidity of the bacteria and fungi was designed based on the McFarland 0.5 scale. All extracts were tested at concentrations of 800-12.5 μg/mL, and all dilutions were prepared with distilled water. Solvents used for the extraction were also tested for antimicrobial activity. Fluconazole and amphotericin B were used as reference drugs for the fungi and amikacin, and ampicillin and ciprofloxacin were used as reference drugs for the bacteria. The minimal dilution that inhibited the growth of bacteria and fungi was identified as the minimum inhibitory concentration (MIC) [29–34].

3. Results and Discussion

3.1. Total Antioxidant Status (TAS), Total Oxidant Status (TOS), and Oxidative Stress Index (OSI). The mushroom TAS, TOS, and OSI values were determined with the Rel Assay kits. The findings demonstrated that the *L. tigrinus* TAS value was 1.748 ± 0.071, TOS value was 19.294 ± 0.237, and OSI was 1.106 ± 0.031. Mushrooms have the potential to contain several antioxidant enzymes and reduced coenzymes and reduced molecules such as phenolic compounds that include electron sources with the antioxidant effect. The analysis and evaluation of TAS as a marker of the system that reflects the whole of the enzymatic and nonenzymatic molecules that the fungi potentially produce and maintain are significant in identification and determination of new natural antioxidant sources. There are no previous studies that aimed at determining TAS, TOS, and OSI of *L. tigrinus*. In previous studies conducted with mushrooms on oxidative stress, it was determined that TAS values of *Omphalotus olearius* and *Paxillus involutus* were 2.827 and 1.230, TOS values were 14.210 and 7.533, and OSI values were 0.503 and 0.613, respectively [35, 36]. It was also reported that the TAS values of *Helvella leucomelaena* and *Sarcosphaera coronaria* were 2.367 and 1.066, TOS values were 55.346 and 41.662, and OSI values were 2.338 and 3.909, respectively [37]. In other studies, it was determined that the TAS value of *Pleurotus eryngii* was 1.93, and the TAS value of *Auricularia polytricha* was 0.93 [38, 39]. In the present study, it was observed that the TAS value of *L. tigrinus* used in this study was higher when compared to *P. involutus*, *S. coronaria*, and *A. polytricha* mushroom and lower when compared to *O. olearius*, *H. leucomelaena*, and *P. eryngii*. It was also observed that *L. tigrinus* TOS and OSI values were higher when compared to *P. involutus* and *O. olearius* and lower when compared to *H. leucomelaena* and *S. coronaria*. It was considered that the difference among the mushrooms was due to the variation in their capacity for reactive oxygen species production as a result of the environmental factors in fungal habitats. Thus, it is suggested that *L. tigrinus* had antioxidant potential; however, due to its high oxidant compound production capacity, the samples collected in Gaziantep province should not be consumed in excess. Furthermore, it was determined that samples collected in regions with adequate mushroom oxidative stress levels can be consumed as a natural antioxidant source.

3.2. Antimicrobial Activity. It was reported that mushrooms produce a variety of biologically active compounds, often associated with the cellular wall, and it was determined that several such compounds have biological activities. Indigenous communities considered mushrooms as potential sources of antibacterial drugs, and antibiotic research were initially started and succeeded with mushrooms [4, 40]. Thus, identification of fungal antimicrobial activities is very important for identification of the new antibacterial and antifungal agents. In the present study, EtOH, MeOH, and DCM extracts of *L. tigrinus* were evaluated against *S. aureus*, *S. aureus* MRSA, *E. faecalis*, *E. coli*, *P. aeruginosa*, *A. baumannii*, *C. albicans*, *C. krusei*, and *C. glabrata*. *L. tigrinus* extracts were compared with ampicillin, amikacin, ciprofloxacin, fluconazole, and amphotericin B which were used to treat general bacterial and fungal infections. In particular, *L. tigrinus* extracts showed antibacterial activity to different widths depending on the type of the infectious agent. The findings are presented in Table 1.

Table 1: Minimum inhibitory concentrations of different extracts of *L. tigrinus* and standard antibiotics against test microorganisms.

	S. aureus	*S. aureus* MRSA	*E. faecalis*	*E. coli*	*P. aeruginosa*	*A. baumannii*	*C. albicans*	*C. glabrata*	*C. krusei*
EtOH	200	200	200	800	800	None	400	100	200
MeOH	200	400	200	None	None	None	800	200	200
DCM	800	800	800	None	None	None	800	400	400
Ampicillin	1.56	3.12	1.56	3.12	3.12	—	—	—	—
Amikacin	—	—	—	1.56	3.12	3.12	—	—	—
Ciprofloxacin	1.56	3.12	1.56	1.56	3.12	3.12	—	—	—
Fluconazole	—	—	—	—	—	—	3.12	3.12	—
Amphotericin B	—	—	—	—	—	—	3.12	3.12	3.12

The MIC values are presented in units of μg/mL.

Antimicrobial activity test findings demonstrated that EtOH extracts generally exhibited higher levels of activity on test microorganisms. Table 1 shows that the mushroom extracts were not effective on *A. baumannii*. Furthermore, mushroom EtOH extract exhibited activity against *E. coli* and *P. aeruginosa*, while MeOH and DCM extracts did not exhibit any activity in tested concentrations. It was found that the mushroom extracts were generally more active on fungal strains. Previous studies that were conducted to determine the antimicrobial activities of *L. tigrinus* reported that the acetonitrile extract was active against *E. coli* and *S. aureus* [20]. In a separate study, it was determined that water and *n*-hexane extracts of *L. tigrinus* were active against *E. coli*, *Bacillus subtilis*, *B. licheniformis*, *S. aureus*, and *Agrobacterium tumefaciens* in various concentrations [41]. Furthermore, it was determined that mushroom extracts were active on *S. aureus*, *S. aureus MRSA*, *E. faecalis*, *C. albicans*, *C. glabrata*, and *C. krusei* in concentrations of 100–800 μg/mL. In conclusion, it was determined that *L. tigrinus* can be consumed as a natural antimicrobial source against the microorganism that demonstrated the abovementioned activities. Mushrooms contain many compounds that show antimicrobial and antioxidant effects. In future, GC-MS studies can identify compounds in *L. tigrinus*. These compounds can be isolated and identified as compounds that cause antimicrobial and antioxidant effects. Crude extracts of *L. tigrinus* were used in our study. Antioxidant and antimicrobial potential of *L. tigrinus* was determined.

4. Conclusions

In the present study, total antioxidant status, total oxidant status, oxidative stress index, and antimicrobial potential of *L. tigrinus* were determined. It was determined that the mushroom possessed antioxidant potential as a result of the conducted analyses. However, it was recommended to limit the consumption of this mushroom due to high oxidant values. It was determined that *L. tigrinus* mushroom collected in regions with adequate oxidative stress levels may be consumed as a natural antioxidant source. Furthermore, the present study demonstrated that *L. tigrinus* may serve as a natural antimicrobial source against the microorganisms that exhibited activity in the tests conducted in the study.

Data Availability

No data were used to support this study.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Simultaneous Determination of Phenolic Compounds in *Leptadenia pyrotechnica* (Forssk.) Decne. by Using High-Performance Liquid Chromatography (HPLC-DAD-UV)

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During the present study, an endeavor has been made to produce a simple, rapid, and simultaneous method for determination of phenolic compounds by using high-performance liquid chromatography in aerial parts of *Leptadenia pyrotechnica* (Forssk.) Decne. collected from the Indian Thar Desert. The optimized process was used for the quantification of ten phenolic compounds. The chromatographic separation was accomplished on an Atlantis T3 column at 25°C with isocratic elution. A mixture of acetonitrile and water was used as the mobile phase at a flow rate of 0.8 mL/min. The linear regression examination data for the calibration plots displayed a good linear relationship with $r^2 > 0.999$ in the concentration range of 2–20 μL. In the methanolic extracts of the whole plant of *L. pyrotechnica*, the content of caffeic acid (3.3%) was reported to be the highest concentration.

1. Introduction

L. pyrotechnica is a characteristic desert plant [1] commonly known as Khimp, Kip, or Kheep [2]. It is an erect ascending, leafless, sappy, evergreen shrub which grows from 1.5 m to 3 m high. The stem is glabrous, pale yellow to light green in color and excretes watery fluid or sap. Flowers are bisexual, pentamarous, actinomorphic, and yellowish green in color. Flowering and fruiting of the plant occurs during the months of August to January. It is native to semiarid deserts of African and Asian countries. In India, it is found in dry sandy soil area, and in western India, especially in Rajasthan, it is found with less frequency in Gujarat and Punjab.

It is a wonderful desert plant, of which almost every plant part is employed in the traditional medicinal system to treat several ailments [3]. This species embraces diversity of bioactive ingredients that generate therapeutic properties; it has also been reported to possess the antitumor and anticancer activity. This plant species holds antifungal, antibacterial, anticancer, antioxidant, anthelmintic, antiatherosclerotic, antidiabetic, wound-healing, and hepatoprotective activities [4, 5]. Nearly every plant parts are used in the therapeutic system for the treatment of various disorders. It is distributed throughout the desert habitats of the state [2]. This plant species is an important component of an arid ecosystem and a good source of medicines, forage, and fiber. It is one of the botanical sources of the Ayurvedic drug *Jivanti*. *Jivanti* is one of the important *rasayana* drugs in Ayurveda. It is used as an ingredient in formulations like *Jivantadya taila*, *J. rasa*, *J. ghrita*, *Ashwagandhadi ghritha*, *Anuthaila*, and *Chandanadi thaila*. These formulations are effective in the treatment of diseases like hemorrhage, tuberculosis, emaciation, fever, and cardiac ailments [6, 7]. Earlier, HPLC studies also showed the high concentration of β-sitosterol, lupeol, and oleanolic acid in *L. pyrotechnica* [8]. The plant is one of the ingredients of many formulations which have been used to recover from physiological, bacterial diseases, or even from cancer. The goal of this paper is to analyze

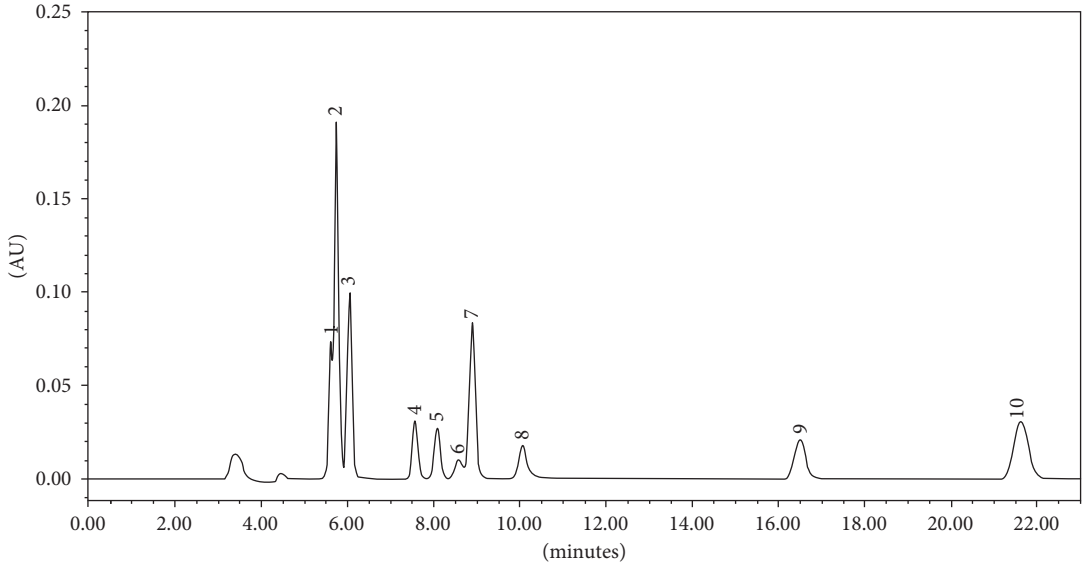

Figure 1: A typical HPLC chromatograph showing the standard mixture of phenolic compounds, i.e., [1] caffeic acid, [2] resorcinol, [6] vanillic acid, [7] *p*-coumaric acid, [9] ferulic acid, [10] vanillin, [11] veratric acid, [4] myristicin, [5] coumarin, and cinnamic acid.

Table 1: Data showing the amount of ten phenols in aerial parts of *L. pyrotechnica*.

S. no.	Phenolic compound/flavonoid	Amount (%)
1	Caffeic acid [1]	3.305
2	Resorcinol [2]	0.008
3	Vanillic acid [6]	0.063
4	p-Coumaric acid [7]	0.034
5	Ferulic acid [9]	0.054
6	Vanillin [10]	0.181
7	Veratric acid [11]	0.015
8	Myristicin [4]	0.013
9	Coumarin [5]	0.004
10	Cinnamic acid	0.042

qualitatively and quantitatively the phenolic compounds in *L. pyrotechnica*, a highly important medicinal plant of the Indian Thar Desert.

2. Experimentation

2.1. Sample Collection and Preparation. Fresh healthy aerial plant parts were collected from Rajasthan. Plants were shade-dried, crushed into fine powder, accurately weighed, and exhaustively extracted using methanol. Methanolic extractions were carried out by using Soxhlet apparatus. Samples were clarified by nylon 6, 6 ultipore film filter papers through a pore size of 0.22 μm prior to giving the injection.

2.2. Chemicals and Reagents. HPLC-grade methanol, acetic acid, water, and acetonitrile were obtained from MERCK, Mumbai. Ten phenolic standards were purchased from Himedia, India. The stock solution was made by using methanol. The standard calibration curves have high degrees of linearity ($r^2 > 0.999$). The plant extracts were recognized from the source of the retention of the standard, and quantification was carried out by equating the peak areas with the standards.

2.3. Instrumentation and Apparatus. Compounds were investigated by using high-performance liquid chromatography, and simultaneous determination of the phenolic compounds was performed. The separation process was performed on an Atlantis T3 column (5 μm pore size; 4.6 mm × 250 mm) with a diode-array detector held at 25°C. Determination of analytes was carried out on the peak area. Spectra were obtained at 200–600 nm. The developed method has been applied to the plant extracts.

2.4. Chromatographic Conditions. The mobile phase was purified using acetonitrile and water with 0.1% acetic acid. The mobile phase was filtered with 5 μm pore filters and then, sonicated in PCI analytics for degassing. Prior to running the baseline of the HPLC-DAD system, the mobile phase was monitored until it became stable before sample analysis.

2.5. Method Development. The method has been effectively applied to the determination of phenolic compounds. HPLC-DAD separation of phenolic acids was accomplished according to previous methods with little alterations [9]. The present study is carried out to study the diversity of phenolic acids in methanolic extracts of this plant species.

Acetonitrile is preferred in method development as it provides lesser viscosity, lower pressure, and superior peak regularity of phenolic compounds. Initially, a gradient run from 2% to 92% was achieved to determine whether isocratic or gradient elution was appropriate [9]. In most of the cases, gradient elution is preferred but during the present study, satisfactory separation was achieved by isocratic elution, to decrease analysis time and enhance the separation

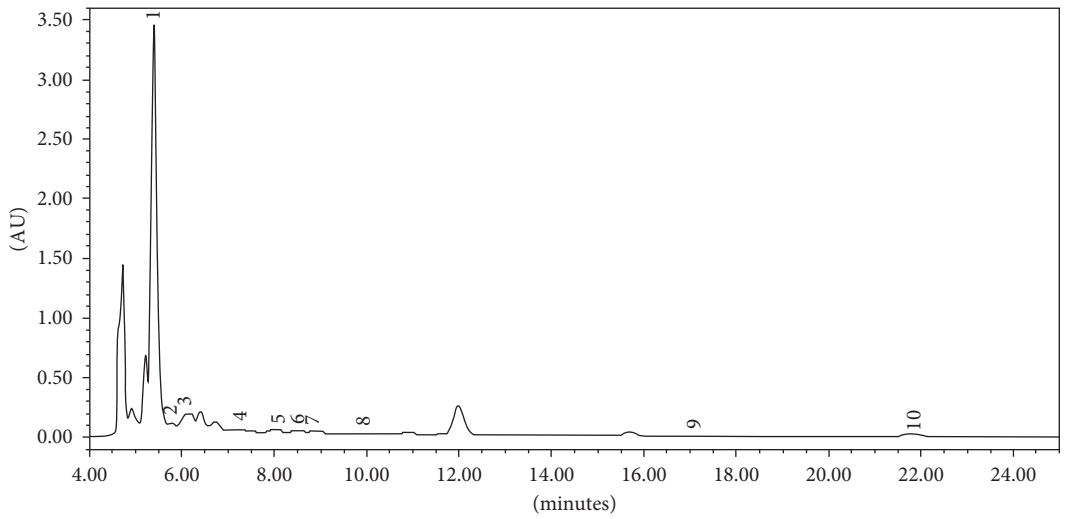

Figure 2: A typical HPLC chromatograph showing phenolic compounds in aerial parts of *Leptadenia pyrotechnica*.

Table 2: Validation of the liquid chromatographic method.

S. no.	Analyte	Retention time	Concentration range (μg/mL)	Calibration equation	Correlation coefficient	Limit of detection (μg/mL)	Limit of quantification (μg/mL)
1	Caffeic acid [1]	5.616	1–20	$Y = 8.20e + 004X - 4.64e + 004$	0.999836	0.22	0.63
2	Resorcinol [2]	5.745	1–20	$Y = 3.66e + 004X - 1.03e + 004$	1.000000	0.28	0.66
3	Vanillic acid [6]	6.050	1–20	$Y = 1.10e + 005X - 5.72e + 004$	0.999948	0.22	0.70
4	*p*-Coumaric acid [7]	7.563	1–20	$Y = 1.88e + 004X - 8.97e + 003$	0.999995	0.21	0.66
5	Ferulic acid [9]	8.084	1–20	$Y = 1.97e + 004X - 2.17e + 004$	0.999669	0.28	0.75
6	Vanillin [10]	8.575	1–20	$Y = 1.59e + 004X - 8.61e + 003$	0.999997	0.21	0.65
7	Veratric acid [11]	8.899	1–20	$Y = 6.32e + 004X - 3.56e + 004$	0.999940	0.22	0.71
8	Myristicin [4]	10.061	1–20	$Y = 1.14e+ 0\ 04X - 2.36e + 004$	0.997837	0.25	0.72
9	Coumarin [5]	16.501	1–20	$Y = 1.67e + 005X + 2.60e + 004$	0.999182	0.22	0.69
10	Cinnamic acid	21.630	1–20	$Y = 7.73e + 004X - 4.34e + 004$	0.999998	0.23	0.67

selectively. Subsequently, the separation was performed on isocratic elution.

The phenolic compounds have the ionizable carboxyl group in their structures; hence, pH has critical significance in separation of these compounds. A mobile phase of lower pH using acetic acid (2-3) was used, and it was buffered to constrain the ionization of analytes and increase column efficiency. The separation of overlapping peaks and restriction of peak tailing of caffeic acid and resorcinol of phenolic compounds was achieved at pH 3. The peak tailing might occur due to the interface between partially ionized analyte and surface column. The separation of all the peaks with higher resolution was found at 23°C. The consequence of the flow rate on separation was discovered by altering the flow rate from 0.5 to 1.5 mL/min. By reducing the flow rate, a proportional increase in the analysis time was obtained in order to sustain peak spacing. The analysis flow rate of the mobile phase was preserved at 0.8 mL/min, and adequate separation was achieved.

These optimal chromatographic conditions were employed to resolve the phenolic compounds in 22 min. All the peaks were baseline-separated (>1.5) with the resolution in 22 min. Figure 1 shows a characteristic chromatogram of 10 μg/mL standard blend of phenolic acids using the optimized conditions. Among the ten phenolic compounds, caffeic acid (3.30%) was reported in maximum amount (Table 1).

2.6. Validation. These studies resembling linearity range, precision, accuracy, limit of detection, and limit of

qualification have also accomplished to recognize that the formed method was appropriate for practical examination in agreement with the guidelines of the International Conference on Harmonization [10]. The method linearity has been assessed by plotting the peak area versus the concentration of the analytes from 2.0 to 20 μg/mL. Calibration curves were obtained by three sequential quantities of the five concentrations. All the correlation coefficients achieved indicate that the method displayed excellent linearity within the studied range.

The limits of detection and limits of quantification were calculated using the following equations: LOD = $3.3s/m$ and LOQ = $10s/m$, employing the standard deviation of y-intercept (s) and the slope (m) of the corresponding calibration curve [11]. The consequences demonstrated that the mentioned method delivers suitable linearity, sensitivity, accuracy, and precision for the synchronized analysis of phenolic compounds in any plant metrics.

3. Results and Discussion

L. pyrotechnica is considered an important medicinal plant and is widely used by local/tribal people of the Indian Thar Desert. The objective of this study was to produce a technique for simultaneous separation of phenolic compounds from a crude methanolic extract by means of HPLC. To obtain a more comprehensive profile of these phenolic compounds, a simple, rapid, analytical HPLC method was developed, using acetonitrile and water as the mobile phase (Figure 1). As a result, it was found that it contains the highest concentration of caffeic acid (3.3%) followed by vanillin (0.018%) (Figure 2). Table 1 demonstrates the concentration of entirely studied phenolic compounds, and Table 2 shows validation parameters of the liquid chromatographic method. Previously, there were many reports of composition of various phytochemicals in this plant species, but the present study is the first attempt to develop a simultaneous method of determination of phenols in crude plant matrices.

4. Conclusion

An efficient, rapid, simple method for simultaneous separation of phenolic compounds is developed. The method is suitable for obtaining a comprehensive HPLC profile. The respective compounds were identified by peak areas and R_f values. The content/concentration was calculated by calibration curves. The presence of caffeic acid in the highest concentration followed by other compounds in aerial parts of this desert plant indicates its rich phytochemical composition and its potential to be used in drug and pharmaceutical companies and hence proves its wide usage by local/tribal people for various alignments.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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