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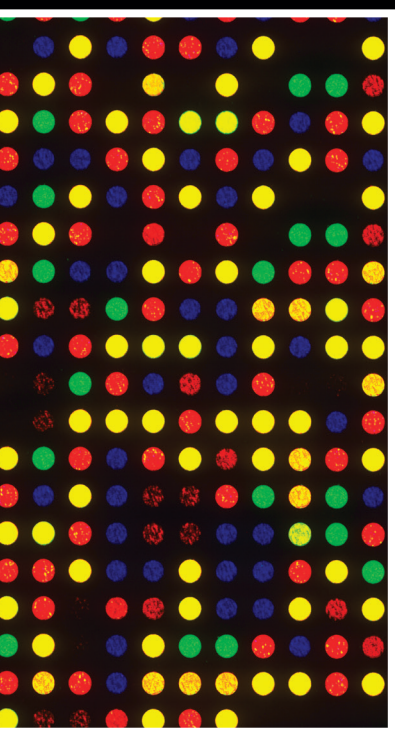
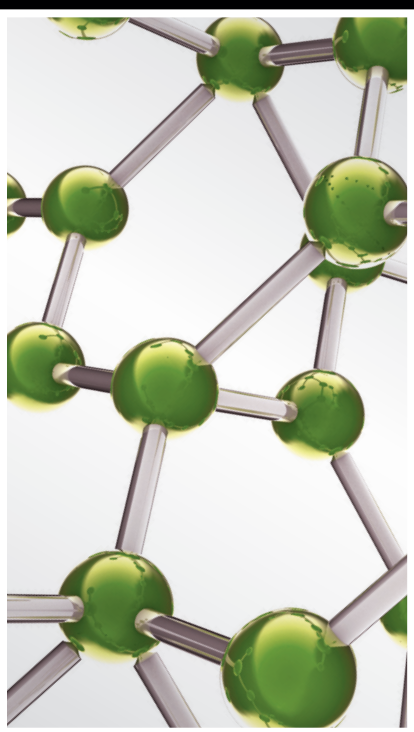
Natural Foods from Plant Sources in Preventing Nontransmissible Diseases

Special Issue Editor in Chief: Almir Gonçalves Wanderley
Guest Editors: Randhir Singh Dahiya and Sérgio Faloni de Andrade



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Editorial

Natural Foods from Plant Sources in Preventing Nontransmissible Diseases

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The nontransmissible diseases also known as noncommunicable diseases (NCDs) or chronic diseases are noninfectious health condition that cannot spread from person to person. Generally, these diseases have slow progression and long duration. In accordance with World Health Organization, there are four main types of NCDs: (1) cardiovascular diseases (like heart attacks and stroke), (2) cancer, (3) chronic respiratory diseases (such as chronic obstructed pulmonary disease and asthma), and (4) diabetes. These diseases are responsible for 63% of all annual deaths provoking the death of more than 36 million people. Currently, these diseases kill more than all communicable diseases, such as HIV, malaria, and tuberculosis diarrhea.

There is growing evidence that positively correlates the consumption of natural foods with the reduction/prevention of diseases, mainly noncommunicable diseases. Within this criteria, consumption of plants and their derivatives represents important options in prevention of these diseases. Considering these view points, special issue in ECAM has been published, in order to report contributions of several researchers in this area.

In the present issue, seven articles have been published which are briefly described below.

In one of the articles, B. B. N'guessan et al. investigated the effect of CellGevity®, on rat liver microsomal cytochrome P450 (CYP) enzymes. This preparation is a dietary supplement contained riboceine (D-ribose-L-cysteine) a GSH-precursor molecule, which is reported to effectively deliver cysteine into the cell and enhance GSH level. Besides, CellGevity® contains other constituents such as turmeric root extract (curcumin), resveratrol, aloe extract, milk thistle, quercetin, broccoli seed extract, alpha lipoic acid, grape seed extract, vitamin C, selenomethionine, cordyceps, and piperine. Moreover, antioxidant potential of this dietary supplement *in vitro* was also estimated. The results showed that CellGevity® dietary supplement possesses moderate antioxidant activity *in vitro* and possesses inhibitory effect on selected rat liver CYP enzymes, suggesting its potential interaction with drugs metabolized by CYP enzymes.

In another study, S. Rampogu et al. undertook the chemical analyses from fenugreek (*Trigonella foenum-graecum*) seeds and also evaluated the potential of their main compounds against type 2 diabetes and breast cancer using molecular docking and molecular dynamics simulation-based computational drug discovery methods. The main compounds identified have been galactomannan and 4-hydroxyisoleucine and computational analysis displayed that galactomannan is an interesting compound from fenugreek seeds with a docking score compared to established drugs, such as canagliflozin and anastrozole in binding simulations of therapeutics against type 2 diabetes and breast cancer, respectively. Of this mode, the authors concluded that galactomannan, derived from fenugreek seeds, is a candidate for further experiments considering its value as a possible drug to treat type 2 diabetes and breast cancer.

In some countries fenugreek is commonly recommended as a galactagogue to breastfeeding women in case of hypogalactia. Thus, R. Shawahna et al. have analyzed the use of fenugreek among lactating women, in order to achieve formal consensus among breastfeeding women and healthcare providers on which potential harms and benefits of using fenugreek need to be communicated and discussed during the clinical consultations. The study involved breastfeeding women and healthcare providers to achieve formal consensus on a list of 24 and 16 items related to potential harms and benefits of fenugreek consumption during lactation. It achieved consensus about 21 potential harms and 14 potential benefits of using fenugreek to enhance human milk supply that needs to be discussed with breastfeeding women during consultations. Moreover, the authors pointed out that further observational studies are needed to assess what is being discussed in daily consultations when herbal remedies are recommended as galactagogue agents.

J. A. Pereira-Freire et al. investigated the phytochemistry profile and antioxidant potential of *Mauritia flexuosa* (Arecaceae) fruits and determined the bioaccessibility of its phenolic compounds. *M. flexuosa* is a palm tree widely distributed in South America, especially in the Amazon region and Brazilian Cerrado. In the Brazilian food industry, the peel and endocarp are commonly discarded or underutilized. The results have shown higher values for phenols, flavonoids, carotenoids, tannins, and ascorbic acid in peels when compared to the pulps and endocarps. Moreover, phenolic compounds identified by HPLC have shown reduced bioaccessibility after *in vitro* simulated gastrointestinal digestion. All samples showed capacity to scavenger free radicals but peels presented higher scavenger action in all methods explored and also protected rat blood cells against lysis induced by peroxyl radicals. Based on results, authors highlighted the nutritional characteristics of these by-products for human or livestock which otherwise are commonly discarded or are used as feed for ruminant animals only.

Another contribution of this special issue was the work of the H. Hong et al., which assessed the effects of *Glehnia littoralis* (GLE) root hot water extract and its underlying mechanism on 3T3-L1 cell adipogenesis and in high fat diet-induced obese mice. The GLE dose-dependently inhibited 3T3-L1 adipocyte differentiation and intracellular lipid accumulation in differentiated adipocytes. Further, body weight gain and fat accumulation were significantly lower in the GLE-treated HFD mice than in the untreated HFD mice and treatment suppressed the expression of adipogenic genes such as peroxisome proliferator-activated receptor (PPAR) γ, CCAAT/enhancer-binding protein (C/EBP) α, fatty acid synthase (aP2), and fatty acid synthase (FAS). These results suggested that GLE inhibits adipocyte differentiation and intracellular lipid accumulation by downregulating the adipogenic gene expression both *in vitro* and *in vivo*.

A Nationwide Cohort study was carried out by W.-C. Chen et al. to evaluate the effect of *Salvia miltiorrhiza* (Danshen) in the treatment of urolithiasis. The authors described that usage of *S. miltiorrhiza* decreased the ratio of subsequent stone treatment after the first treatment in the study population; there was no increased bleeding risk due to long-term use of it. Therefore, they suggested this is a safe herb having a potential for calculus prevention.

Finally, the effect of resveratrol suspension on the immune function of piglets has been evaluated by Q. Fu et al. showing that the treatment with it provoked significant effects on the development, maturation, proliferation, and transformation of T lymphocytes. The activity appears related to regulation of the humoral immune responses by upregulating the release of IFN-γ and downregulating the release of TNF-α. Moreover, there was significant increase in antibody titers of the piglets after immunization using swine fever vaccine (CSFV) and foot-and-mouth disease vaccine (FMDV). These positive effects indicate that resveratrol could be considered as an adjuvant to enhance the body's immune response to vaccines, as well as dietary additive for animals in order to enhance humoral and cellular immunity.

Thus, with contributions from research groups from different countries, this special issue exhibited that natural products have great potential to be explored in the prevention and treatment of not transmissible diseases. The editors of the special issue would like to thank all authors which submitted their works and allowed the success of this special issue.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Almir Gonçalves Wanderley
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Research Article

Exploring the Therapeutic Ability of Fenugreek against Type 2 Diabetes and Breast Cancer Employing Molecular Docking and Molecular Dynamics Simulations

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Fenugreek (Trigonella foenum-graecum) is used as a spice throughout the world. It is known for its medicinal properties such as antidiabetic, anticarcinogenic, and immunological activities. The present study shows the properties and the nutritional quality of fenugreek seed extract and focuses on screening of active compounds in drug designing for type 2 diabetes and breast cancer. Quantitative analysis was used to calculate the percentages of protein, carbohydrates moisture, fatty acid, galactomannan, oil, and amino acid. Phytochemical analysis revealed the presence of flavonoids, terpenoids, phenols, proteins, saponins, and tannins in fenugreek seed extracts. Molecular docking and molecular dynamics simulation-based computational drug discovery methods were employed to address the role of fenugreek seed constituents against type 2 diabetes and breast cancer. The computational results reveal that the compound galactomannan can be ascribed as potential drug candidate against breast cancer and type 2 diabetes rendered by higher molecular dock scores, stable molecular dynamics (MD) simulations results, and lower binding energy calculations.

1. Introduction

The legume fenugreek *(Trigonella foenum-graecum)* is a short annual plant from the Fabaceae family [1, 2]. The name Trigonella foenum-graecum is a Latin-Greek name as it bears a typical triangular shaped flowers and is employed as a common fodder for animals in Greece [1]. It is found in various parts of the globe and is often used as spice, condiment, and medication [3, 4]. Largely, fenugreek leaves and seeds have been used as spices in different parts of the globe. In Africa, fenugreek is used as supplement during bread preparation and the seed components of fenugreek are known to enhance the nutritional quality of the bread. In India, the leaves and seeds are utilized as favouring and seasoning agents [1]. In China, it is used as cure edema, while the ancient Egyptians employed fenugreek to incense the mummies [1, 5, 6]. Additionally, fenugreek is used as a medicine to treat several diseases besides being used as antioxidant [7], against inflammation [8, 9], as anticancer [10], as hepatoprotective agent [11, 12], as antibacterial [13–15], and as antifungal [16]. Additionally, fenugreek is also used as off-season fodder and animal food supplement [17].

Fenugreek seeds are widely studied part of the plant. The powdered fenugreek is used as condiment and the seed endosperm serves to secure fenugreek gum [1]. The seeds have a strong aroma with bitter taste [18]. The major chemical constituents found in fenugreek seed are galactomannan (fibre), diosgenin (sapogenin), trigonelline (alkaloid), and 4-hydroxyisoleucine that have the antidiabetic properties and are also employed to treat breast cancer [19].

Diabetes mellitus is a common and chronic disease concern globally associated with a ten-year shorter life expectancy [20]. According to WHO, type 2 diabetes occurs because either body does not produce enough insulin or body resists the effects of insulin [21, 22]. Type 2 diabetes is dominant in developing countries and accounts to around 85%–90% worldwide [20, 21]. Fenugreek is another promising antidiabetic drug [23]. It was also confirmed that consuming fenugreek as a dietary supplement in the prediabetic patients could efficiently reduce the outbreak of type 2 diabetes [24]. Additionally, it was further reported that the socked fenugreek seeds can act as adjuvant in mitigating the type 2 diabetes and also in noninsulin dependent diabetes [25, 26] and serum lipids in type I diabetes [27]. Additionally, it is well evidenced that the fenugreek seeds are antidiabetic in nature [24, 28, 29].

Fenugreek also possesses anticancer properties and chemical constituents of fenugreek are known to induce apoptosis [30, 31]. Furthermore, it induces dose-dependent effect on human breast cancer cell line [32]. Breast cancer is the most common cause of death in female worldwide [33, 34]. The discovery of BRCA1 and BRCA2 genes helped to understand that hereditary factor is the main cause of most cancers [35]. Chloroform seed extract studies by Khoja *et al.* proved the effective killing of MCF-7 human immortalized breast cells [30]. Amin *et al.* (2005) studies suggest that fenugreek seed chemical constituents have preventive effect against breast cancer which inhibit MDA 231-induced mammary hyperplasia [36]. However, it is not yet delineated on the most effective compound that can act on both the morbidities. Therefore, in the current investigation, we employed the computational technique such as molecular docking and molecular dynamics simulations to identify candidate compound as compared with the reference compounds.

Molecular docking is one of the widely adapted methods to predict the binding affinities between the ligand and the target protein and further the lead optimization [37]. Additionally, the molecular docking imparts knowledge on the interactions at the atomic level [37] and predicts the ideal binding mode [33, 38]. Molecular docking mechanism generally evaluates the binding conformations, its orientation, and the accommodation of the small molecule at the active site of the proteins binding site and are read as scores [39]. The molecular dynamics simulation imparts knowledge on the nature of the small molecules at the proteins binding pocket thereby affirming the appropriate binding modes [38]. The identified Hits that have demonstrated a higher dock score than the reference compounds or the known drugs, exhibiting the interactions with the key residues complemented by stable molecular dynamics simulation results, are considered the most promising candidate compounds.

In the current investigation, the quantitative analysis of fenugreek seeds was conducted to gain information on the components and further the computational analysis was performed to discover the potential compound against breast cancer and type 2 diabetes. The *in silico* results have illuminated galactomannan as the prospective compound against both diseases.

2. Materials and Methods

Fenugreek seeds were used as a sample to test the medicinal properties. Fenugreek seeds were sourced from a local market (Hyderabad, India) and were of high quality grade. They were shade dried, cleaned, and finely powdered and used for chemical analysis.

2.1. Biochemical Analysis. The biochemical studies were carried out to identify the protein content, total soluble carbohydrates, oil content and fatty acid values, free amino acids, and soluble fibres from the collected seed samples.

2.1.1. Estimation of Total Protein. Percentage of proteinaceous nitrogen and proteins was estimated by the Micro-kjeldahl method [40]. Proteinaceous nitrogen was calculated by the following formula.

$$\%\ \text{of Nitrogen} = (T - B) \times N \times 10 \times \frac{1.4}{28} \times S \quad (1)$$

T is titration reading of the sample,

B is blank reading of the sample,

S is the amount of sample taken in grams,

N is normality of hydrochloric acid (N/28).

To calculate the percentage of protein, the nitrogen value was multiplied by the factor 6.25.

2.1.2. Estimation of Total Carbohydrate. Total carbohydrate content of the seed samples was estimated by the procedure suggested by Loewis (1952) [41]. Anthrone reagent was used and the developed colour was read at 620nm in a colorimeter against blank.

2.1.3. Estimation of Oil Content. Total oil content of the said spices was estimated as suggested by Meara (1955) [42].

Percentage of oil was calculated by following formula:

$$\%\ \text{of oil} = \frac{Wo}{Ws} \times 100 \quad (2)$$

Wo is the weight of oil extracted,

Ws is the weight of seed taken.

2.1.4. Estimation of Fatty Acid Value. Method used to estimate the fatty acid value is suggested by Meara (1955) [42].

Fatty acid value was calculated using the formula

$$\text{Fatty acid value} = U \times \frac{56.1}{W} \quad (3)$$

U is the volume of titration of 0.1 n KOH,

W is the grams of oil taken.

2.1.5. Isolation of Amino Acids. Column chromatography was used to isolate free amino acids from fenugreek seeds [43].

To find the concentration of 4-hydroxyisoleucine, first the total amino acid content was determined by using spectrophotometric method. Then the relative concentration of 4-hydroxyisoleucine in the mixture of amino acid was determined by high performance thin layer chromatography (HPTLC).

2.1.6. Isolation of Galactomannans. Extraction and isolation of the water-soluble polysaccharides (galactomannans) from endosperm of fenugreek seeds were done using the procedure of Kooiman (1971) [44].

2.1.7. Estimation of Moisture Percentage. Moisture content of seeds was estimated by "Dry air oven" method association of official analytical chemists (AOAC) (anonymous, 1947)[45] and the percentage was calculated from the following formula:

$$\%\ \text{moisture} = \text{fresh weight of the seed} - \frac{\text{dry wt. of the seed}}{\text{dry wt. of the seed}} \times 100 \quad (4)$$

2.2. Molecular Docking, Simulations, and Free Energy Calculations. To further assess the suitability of the compounds as antidiabetic and potential breast cancer agents, the investigation proceeds employing the computational methods such as molecular docking recruiting CDOCKER available on Discovery Studio (DS) v4.5, molecular dynamics (MD) simulations conducted using GROningen MAchine for Chemical Simulations (Gromacs) v5.0, which was followed by MM/PBSA calculations.

2.2.1. Molecular Docking. For the execution of the docking protocol, the proteins for both the diseases were imported from protein data bank (PDB) of high resolution. The protein with the PDB id 3EQM (2.9Å) was chosen for breast cancer and 1GFY (2.1Å) was elected for type 2 diabetes, respectively. These proteins were prepared on DS by initiating the *clean protein* module embedded with the DS and subsequently heteroatoms together with the water molecules were dislodged and the addition of hydrogens was performed adapting the CHARMm force field accessible on the DS. The active sites were selected in accordance with the co-crystal geometry, thereby, considering the residues around 10 Å radius [46, 47].

Phytochemicals along with the type 2 diabetic and breast cancer drugs, canagliflozin [48] and anastrozole [49], were used to comparatively evaluate the effect of the prospective drug molecules on the diseases labelling the latter as reference drug. These compounds were imported onto the DS to obtain their 3D structures and were subsequently minimized. The prepared proteins and the ligands were subjected to molecular docking studies employing the CDOCKER protocol.

CDOCKER available on the DS happens to be the most reliable method as it employs the CHARMm-based dynamics methods [50]. Subsequently, 30 conformations were allowed to be generated for each ligand, while the other parameters were set at default. The results were evaluated based upon the higher –CDOCKER interaction energy and higher –CDOCKER energy that significantly correspond to the favourable binding. The most appropriate binding mode was judged by the maximum clusters formed and was therefore subjected to MD simulations to understand its dynamic behaviour.

2.2.2. MD Simulations. Molecular dynamics (MD) simulations were performed for the favourable systems obtained after docking using GROMACS 5.0 with CHARMm27 force field. Ligand topologies were generated adapting the SwissParam [51]. All the parameters were attributed as described earlier [52–56]. Dodecahedron water box was generated and the systems were solvated comprising three-site transferrable intermolecular potential (TIP3P) water model, to which the counter ions were added. The system was energy minimized with steepest descent algorithm with 10000 steps which was then subjected to equilibration using constant number N, volume V, and temperature T (NVT) [57] and constant number N, pressure P, and temperature T (NPT) [58]. During this process, the protein backbone was restrained and the periodic boundary conditions were fostered to avoid bad effects. Thereafter, the MD run was conducted for 10 ns, saving the data for every one picosecond (ps). Visual molecular dynamics (VMD)[59] and DS were utilized to analyse the MD results.

2.2.3. Binding Free Energy Calculations. Molecular Mechanics/Poisson Boltzmann Surface Area (MM/PBSA) was recruited to compute the binding free energy calculations [60, 61]. 10 snapshots were evenly extracted from the MD trajectories of the protein ligand complex. A variety of energetic values were calculated using

$$\begin{aligned}
\Delta G_{\text{binding}} &= G_{\text{complex}} - \left(G_{\text{protein}} + G_{\text{ligand}}\right) \\
G_X &= E_{MM} + G_{\text{solvation}} \\
E_{MM} &= E_{\text{bonded}} + E_{\text{non-bonded}} \\
&= E_{\text{bonded}} + \left(E_{\text{vdw}} + E_{\text{elec}}\right) \\
G_{\text{solvation}} &= G_{\text{polar}} + G_{\text{non-polar}} \\
G_{\text{non_polar}} &= \gamma \text{SASA} + b
\end{aligned} \quad (5)$$

3. Results

3.1. Biochemical Analysis. The total seed percentage revealed that galactomannan and 4-hydroxyisoleucine were present in 26.4 and 13 percentages, respectively, as in Table 1.

Further phytochemical screening of acetone seed extract of fenugreek was carried out to test the presence of tannins, phenols, terpenoids, flavonoids, saponins, and alkaloids [62] and are tabulated in Table 2.

Test for flavonoids: 1 ml of extract in a test tube and 5ml of diluted ammonium solution were added followed by few drops of concentrated sulphuric acid. Formation of yellow colour indicated the presence of flavonoids [62].

Table 1: Percentage of the seed contents.

Contents of fenugreek seed extract	Average percentage of the seed extracts (%)
protein	28.5
carbohydrate	16.2
oils	5.3
fatty acid	3.8
galactomannan	26.4
moisture	6.8
4-hydroxyisoleucine	13

Table 2: Summary of Phytochemicals in Acetone Extract of Fenugreek Seed.

Tests	Results
Flavanoid	+ve
Tannin	+ve
Terpenoids	+ve
Alkaloids	+ve
Saponins	+ve

Test for tannins: Formation of reddish-brown colour indicated the presence of tannins (ferric chloride test) when 1% ferric chloride solution was added to 1 ml of extract of fenugreek seeds [62].

Test for terpenoids: To find out the presence of terpenoids, Salkowski test was conducted. 1 ml of extract was taken and dissolved in chloroform and then a few drops of concentrated sulphuric acid were added to it. On the inner face, a reddish-brown colour was formed that indicated the presence of terpenoids [62].

Test for alkaloids: Dragendorffa's test results indicated the presence of alkaloids by giving orange-red precipitate, when 1 ml of Dragendroffa's reagent was added (potassium bismuth iodide solution) to 1 ml of extract [62].

Test for saponins: Frothing test was conducted to test for saponins in the seed extract. 1ml of extract was vigorously shaken with distilled water and was allowed to stand for 10 min. Stable froth indicated the presence of saponins [62].

3.2. Molecular Docking, Simulations, and Free Energy Calculations

3.2.1. Molecular Docking Studies. Molecular docking was executed independently for diabetes and breast cancer. The ligands along with their respective proteins were docked to assess their binding affinities. It was interesting to note that 4-hydroxyisoleucine has generated a relatively lower dock score while galactomannan produced higher dock score as compared to their respective reference compounds, as in Table 3. Therefore, 4-hydroxyisoleucine was refrained from further calculations and the other systems were proceeded forward.

3.2.2. Molecular Dynamics Simulations. To secure the results obtained from the docking, the MD simulations were performed to establish the most reliable ligand-receptor complex

Table 3: Molecular dock scores between the drug targets and the compounds.

S. no.	Name of the compound	-CDOCKER interaction energy
Dock scores of diabetes mellitus		
1	canagliflozin	36.55
2	galactomannan	43.19
3	4-hydroxyisoleucine	28.27
Dock scores of breast cancer		
1	anastrozole	34.05
2	galactomannan	58.15
3	4-hydroxyisoleucine	23.88

and additionally to understand their behaviour at proteins active site [52, 53]. The MD for 10 ns was initiated and the behaviour of each system was monitored. Accordingly, root mean square deviation (RMSD), root mean square fluctuation (RMSF), and potential energies were calculated for each system. The RMSD for the breast cancer systems were observed to be stable after 4000 ps with no significant variation, thereafter, implying that the system is well converged, as in Figure 1. Moreover, the RMSD values were demonstrated to be less than 0.25 nm. Similar results were noted with RMSF values as well, as in Figure 2. The potential energy further states that there were no abnormal behaviours of the systems which were stable throughout the simulations, as in Figure 3. The last 5ns trajectories were retrieved to study the binding mode analysis. Upon superimposition, it was conceived that the binding mode pattern of the reference and the galactomannan were similar, as in Figure 4. The interactions of the ligand with the protein were evaluated with the key residues located at the active site. The reference compound anastrozole was seen to form a hydrogen bond with the NH atom of Met374 residue, joined by N5 atom with a bond length of 2.9 Å. Phe134 was found to form the π – π with the ligand molecule. Galactomannan was found to interact with the protein by forming 7 hydrogen bonds. The O13 atom of the ligand has interacted with the HH22 atom of Arg115 with a bond length of 2.8 Å. The HH21 atom of Arg115 has interacted with O15 atom of the ligand with a bond length of 2.5 Å. The O atom of Ile132 has joined with H62 of the ligand displaying a bond length of 2.6 Å. Another hydrogen bond was observed between the HH11 atom of Arg145 and the O14 atom of the ligand with a length of 2.0 Å. The OD2 atom of the residue Asp309 has interacted with the H57 of the ligand with a bond distance of 2.8 Å. The O atom of the key residue Met374 has interacted with the H53 atom of the ligand with a bond length of 2.5 Å. The SG atom of the Cys437 residue has interacted with the H63 atom of the ligand with a distance of 2.5 Å. The details of the interaction are represented in Figure 5 and Table 4. Furthermore, the intermolecular hydrogen bond interactions were recorded during the simulations to elucidate deposition of the ligand within the active site. It was observed that the reference molecule displayed an average of 0.3 hydrogen bonds, while those within 0.35 nm were observed to be 0.7, as in Figure 6,

Table 4: The molecular interactions between the compounds and the protein.

S. no.	Compound	Ligand Atom	Amino acid	Amino acid atom	Bond length (Å)	Hydrophobic interactions
1	anastrozole	N5	Met374	HN	2.9	Ile133,Asp309,Val370, Leu372, Val373,Pro429,Phe430,Cys437,Leu 477
2	galactomannan	O13	Arg115	HH22	2.8	Ala306, Asp309, Phe430.
		O15	Arg115	HH21	2.5	
		H62	Ile132	O	2.6	
		O14	Arg145	HH11	2.0	
		H57	Asp309	OD2	2.8	
		H53	Met374	O	2.5	
		H63	Cys437	SG	2.5	

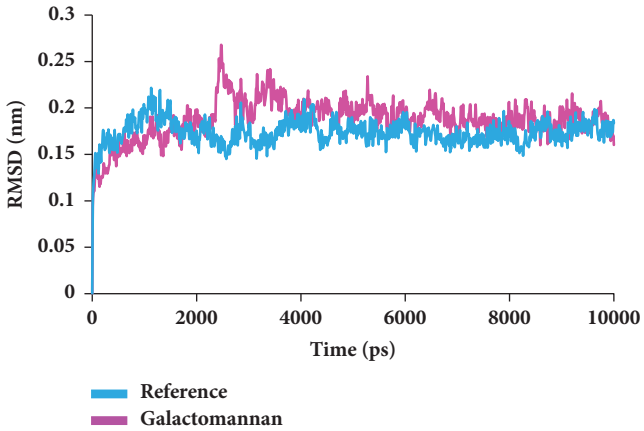

Figure 1: RMSD plots for backbone atoms.

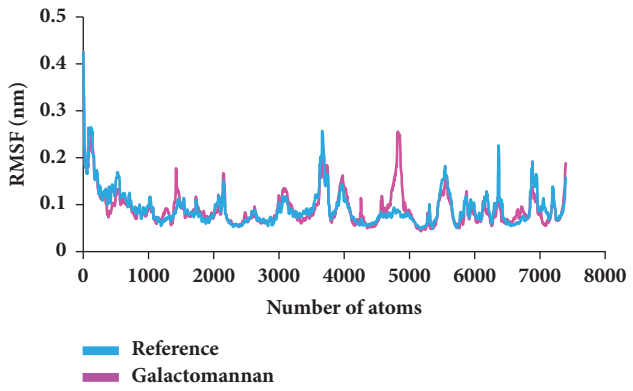

Figure 2: RMSF profiles for backbone atoms.

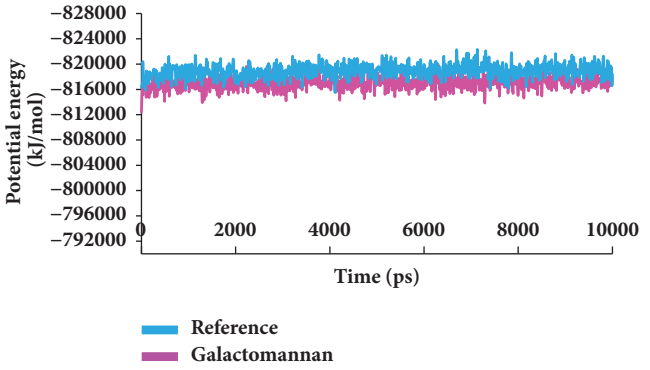

Figure 3: Potential energy graphs of the systems.

while the candidate molecule demonstrated an average of 1.3 hydrogen bonds and the bonds within 0.35 nm were 4.4, as in Figure 7.

Similar types of calculations were determined for the type 2 diabetes disease target and its respective ligands. The RMSD was recorded to be stable after 7000 ps for both the reference and galactomannan. Further, it was noted that the RMSD of the reference was established to be within 0.2 nm while the drug-like molecule demonstrated a RMSD within 0.15 nm, as in Figure 8. However, no major fluctuations were noticed during the simulations referring to the stability of the systems. The same results were depicted through the RMSF, as in Figure 9, and the potential energy calculations, as in Figure 10. Therefore, to examine the binding mode of the ligand molecules, the last 5 ns trajectories were extracted and were superimposed. The results represented a similar binding mode between the reference and the galactomannan, as in Figure 11. Furthermore, intermolecular interactions were inspected with the key residues residing at the active site. It revealed that the reference molecule has formed three hydrogen bonds with the active site residues. The F2 of the ligand has interacted with the HG atom of Cys215 with bond length of 2.6 Å. The other two hydrogen bonds are formed with HN and HE atoms of Arg221 and 2.1 Å each. Tyr46 and Phe182 have been involved with the $\pi - \pi$ interactions. On the contrary, Galactomannan on the other hand generated eight hydrogen bonds, two hydrogen bonds with Lys120 and Asp181 amino acid residues and one hydrogen bond with Arg221, Ser216, Gln262, and Gln266, respectively. The details of the interactions are tabulated in Figure 12 and Table 5. Furthermore, the intermolecular hydrogen bonds were evaluated throughout the simulations. The average hydrogen bonds were computed to be 0.09 and those within 0.35 nm were

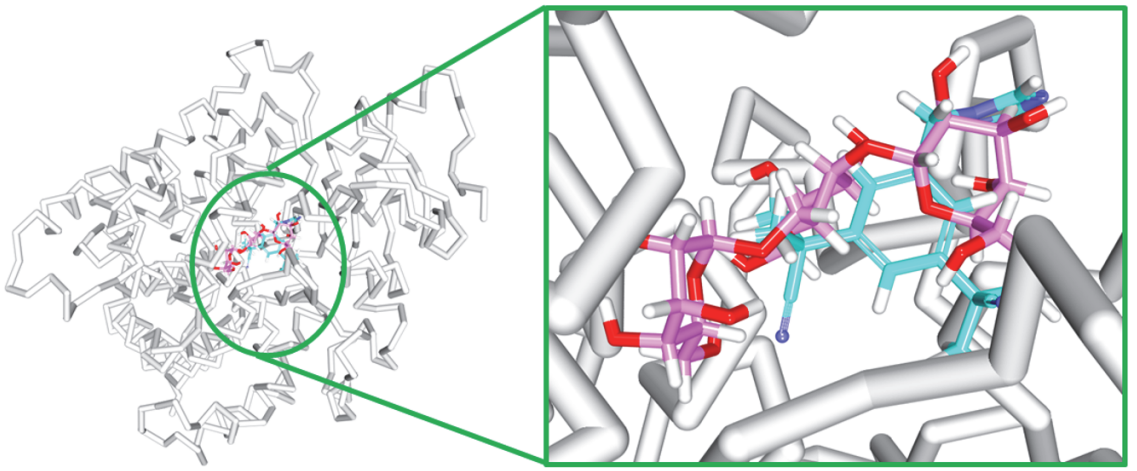

Figure 4: Binding mode assessment of the reference (cyan) and galactomannan (pink). Superimposition of the representative structures (left) and zoomed (right).

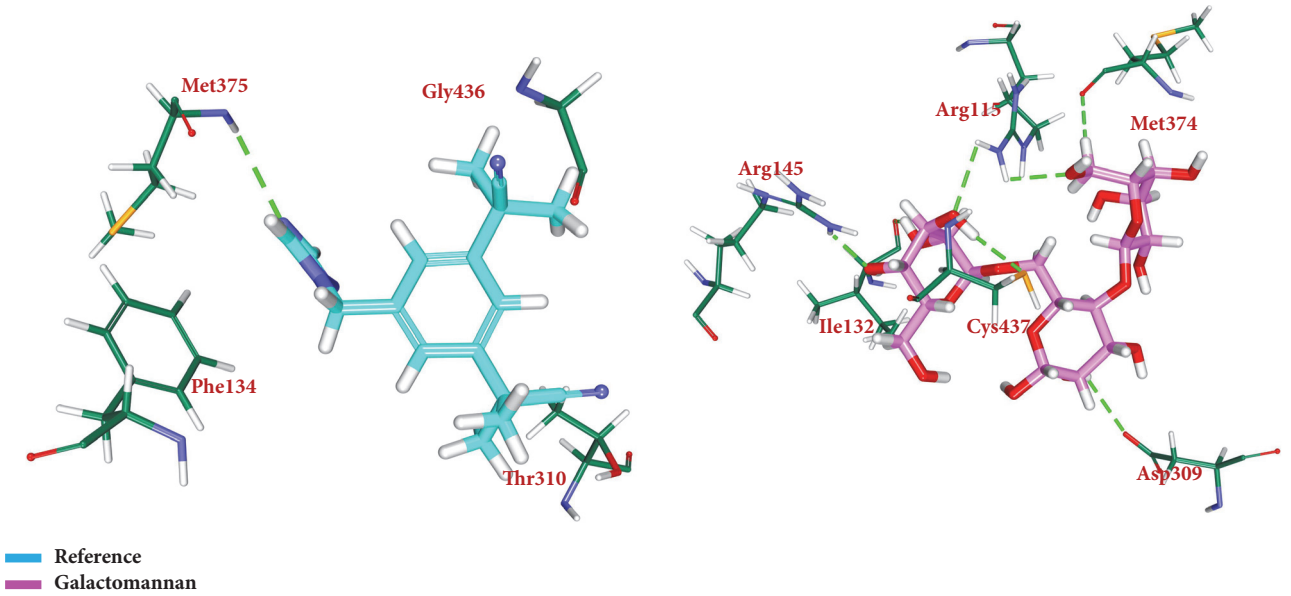

Figure 5: Depiction of hydrogen bond interactions and binding conformations. Only polar atoms are displayed for clarity.

found to be 0.7, as in Figure 13. The prospective drug molecule however has represented average hydrogen bonds of 3.9 while the bonds within 0.35 nm were enumerated to be 4.4 projecting the superiority of galactomannan, as in Figure 14.

3.3. Binding Free Energy Analysis. Binding free energies are computed after the MD simulations that inspect protein fluctuations and ligand conformations thereby ensuring a suitable positioning of the ligand within the binding site. The MM/PBSA calculations have produced a favourable ΔG that ranged between -10 to 100 kJ/mol for breast cancer target, as in Figure 15. Furthermore, the average binding energy produced by reference was -42.45 kJ/mol while that generated by galactomannan was -47.95 kJ/mol, respectively, as in Table 6.

The binding free energies were subsequently calculated for canagliflozin-protein and galactomannan-protein systems for type 2 diabetes. 10 snapshots were evenly extracted and the binding energies were computed accordingly. The binding energies ranged between -15 kJ/mol and -100 kJ/mol, as in Figure 16. Additionally, it was observed that the average binding energy was calculated as -51.75 kJ/mol for the reference and -68.11 kJ/mol for galactomannan, as in Table 7.

From the results, it is evident that galactomannan has represented higher –CDOCKER interaction energy values and lower binding free energies than their respective reference compounds. These results demonstrate that galactomannan has stronger binding affinities than the reference inhibitors.

Table 5: The molecular interactions between the compounds and the protein.

S.no	Compound	Ligand Atom	Amino acid	Amino acid atom	Bond length Å	Hydrophobic interactions
1	canagliflozin	F2	Cys215	HG	2.6	Lys120,Lys116, Ser216,Gly218, Ile219,Gly220, Ala217,Gln262
		F2	Arg221	HN	2.1	
		F2	Arg221	HE	2.1	
2	galactomannan	O9	Lys120	HZ2	1.7	
		O3	Lys120	HZ1	2.0	Tyr46,Lys116, Phe182,Gly183, Cys215,Ser216, Gly218,Ile219, Gly220
		H66	Asp181	OD1	2.3	
		H64	Asp181	OD1	1.9	
		O14	Arg221	HN	2.4	
		O16	Ser216	HN	2.4	
		H62	Gln262	OE1	2.1	
		O13	Gln266	HE22	2.4	

Table 6: Comparative assessment between dock scores and the binding energies of breast cancer systems.

S. no.	Name of the compound	-CDOCKER interaction energy	Average binding energy (kJ/mol)
1	anastrozole	34.05	-42.45
2	galactomannan	58.15	-47.95

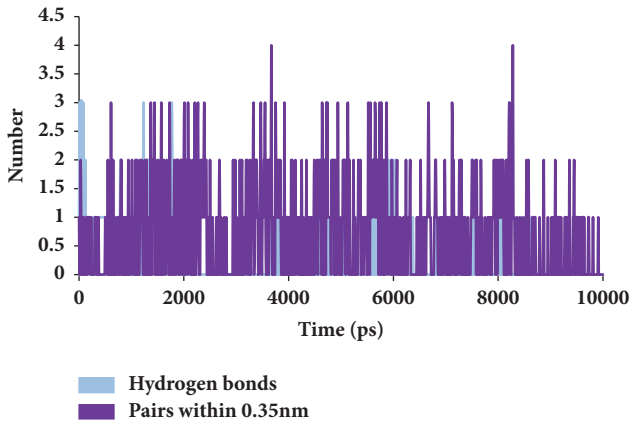

Figure 6: Graphical depiction of number of intermolecular hydrogen bond interactions between the protein and the reference compound.

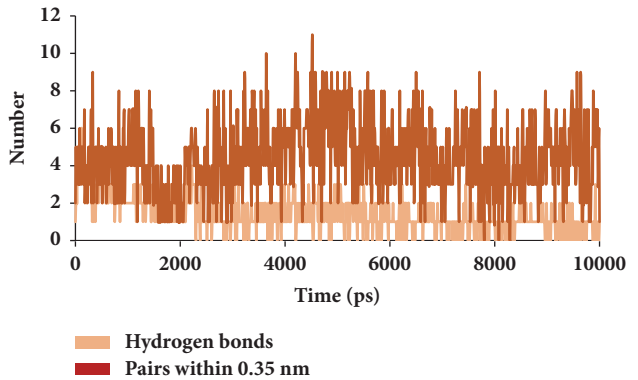

Figure 7: Graphical depiction of number of intermolecular hydrogen bond interactions between the protein and the candidate compound.

4. Discussion

In the present study, the seed extract showed the presence of proteins, carbohydrates, fatty acids, oils, saponin, flavonoids, tannins, terpenoids, alkaloids, soluble fibre galactomannan, and amino acid 4 hydroxyisoleusine (Tables 1 and 2). Some chemicals screened are similar to the work done by Yadav R. et al. 2014 [63].

Out of these chemicals, the special interest in this investigation is on the percentages of soluble fibre galactomannan 26.4 % and free amino acids 4 hydroxyleucine 13% and the presence saponins, as these are linked to human health benefits mainly in the reduction of plasma glucose levels and anticancer activities [64].

In order to further evaluate molecular inhibitory effect of the selected phytochemicals, the investigation proceeds *in silico*. Computational results have revealed that the phytochemical 4 hydroxyisoleucine could not induce the inhibitory activity against both the diseases. Although reports exist to explain its antidiabetic and antibreast cancer activity, the present finding foretells its inability as an inhibitor [31, 65]. Therefore, this amino acid was not forwarded for further studies. The other compound galactomannan has proved to be potential against both the diseases. This was represented by the RMSD, RMSF, and the potential energy values. The results were found to be unaltered as compared with the reference throughout the simulations. Moreover, the binding energies of the prospective drug molecules are found to be less, while rendering the highest –CDOCKER interaction energies. It is documented from the previous reports regarding the role of breast cancer inhibitors on diabetes mellitus as there exists a linkage between them [66, 67]. All the above results conclude that galactomannan could be considered as a potential drug for both the diseases.

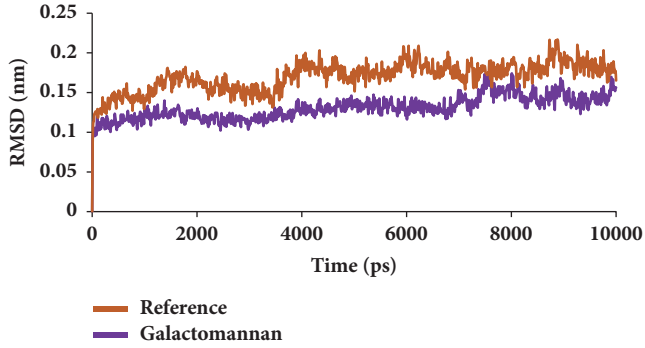

Figure 8: RMSD plots for backbone atoms.

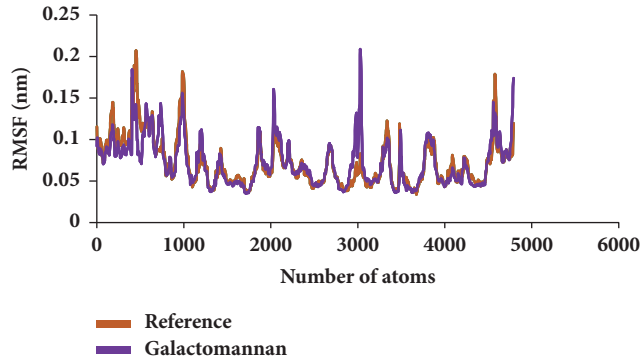

Figure 9: RMSF profiles for backbone atoms.

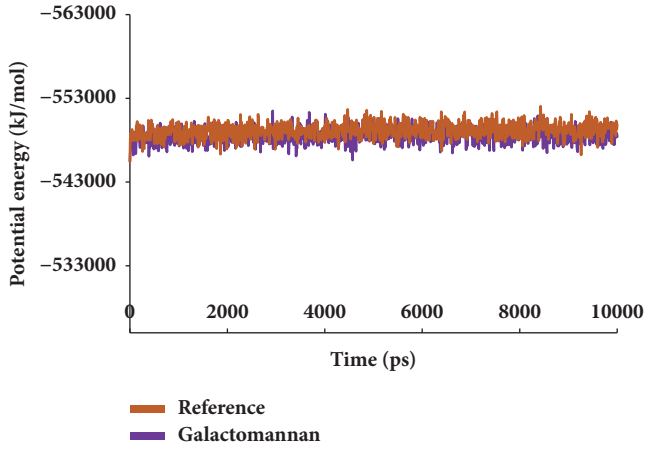

Figure 10: Potential energy graphs of the systems.

Chemically, galactomannan is a polysaccharide molecule comprising a mannose backbone and the galactose side groups, hence, the name. More precisely they exist with 1-6 alpha-D-galactopyranose linkage. However, in fenugreek, mannose and galactose are linked by 1:1 linkage. Upon observing the docking conformations, it can be elucidated that the galactose side groups have involved in forming the hydrogen bond interaction with the active side residues, with the ring structures of the mannose involved in the formation of the π bond interactions.

In conclusion, the present study has examined the active components of fenugreek seeds against two common but different diseases, *viz-a-viz*: type-2 diabetes and breast cancer, using a well-established computational drug discovery method. The chemical composition of fenugreek seeds was assessed, and galactomannan and 4-hydroxyisoleucine were identified as major components and are similar to previous studies [68]. The therapeutic potential of these two identified active components was further assessed using molecular docking and molecular dynamics simulations. Our results

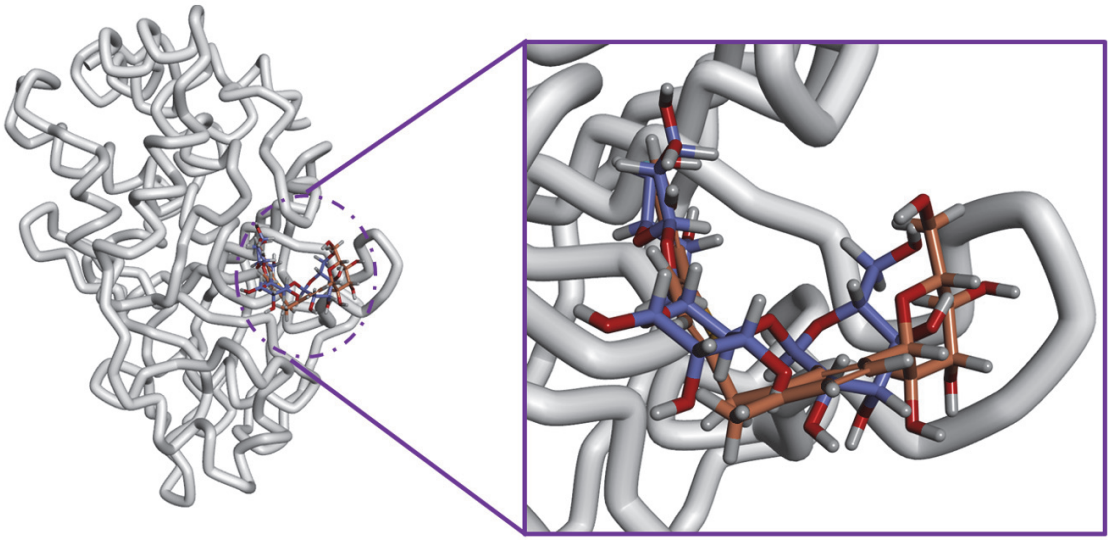

Figure 11: Binding mode assessment of the reference (purple) and galactomannan (orange). Superimposition of the representative structures (left) and zoomed (right).

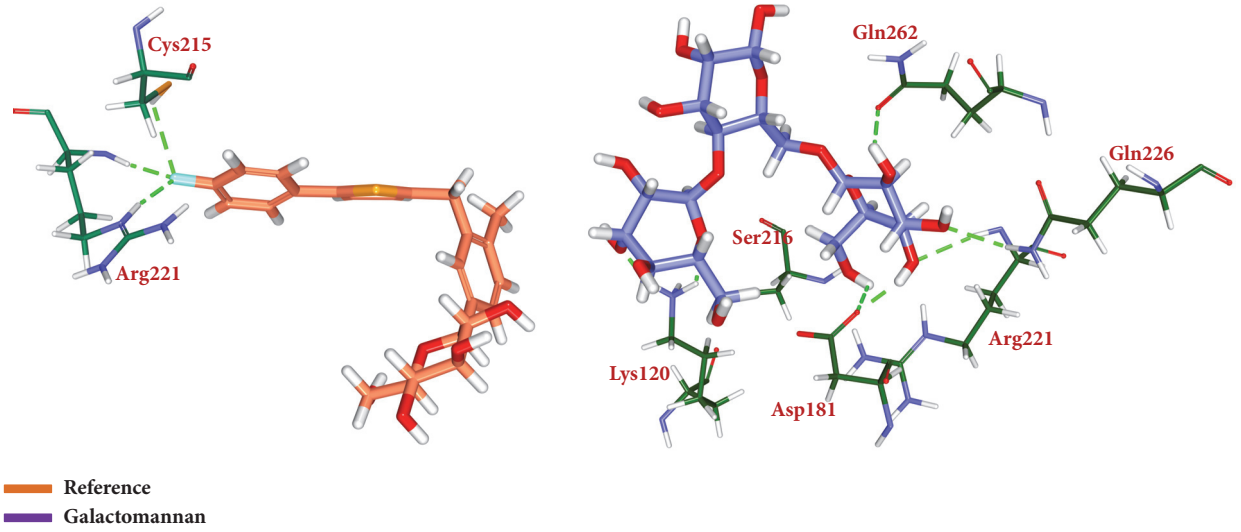

Figure 12: Depiction of hydrogen bond interactions and binding conformations. Only polar atoms are displayed for clarity.

identify galactomannan as a potential active component of fenugreek seeds, with a docking score compared to established drugs such as canagliflozin and anastrozole in binding simulations of therapeutics against type-2 diabetes and breast cancer, respectively. These results establish galactomannan, derived from fenugreek seeds, as a potential candidate for further drug discovery experiments in establishing their value as therapeutics against type-2 diabetes and breast cancer.

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

Shailima Rampogu and Saravanan Parameswaran contributed equally to this work.

Acknowledgments

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Table 7: Comparative assessment between dock scores and the binding energies type 2 diabetes systems.

S. no.	Name of the compound	-CDOCKER interaction energy	Average binding energy (kJ/mol)
1	canagliflozin	36.55	-51.75
2	galactomannan	43.19	-68.11

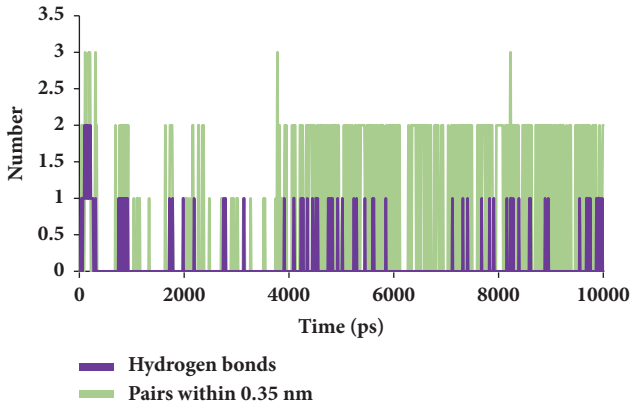

Figure 13: Graphical depiction of number of intermolecular hydrogen bond interactions between the protein and the reference compound.

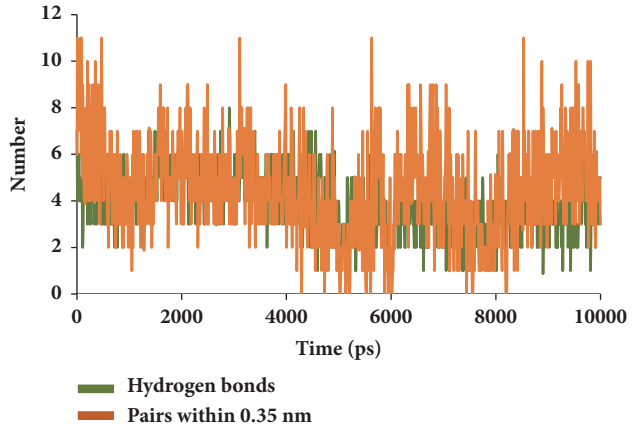

Figure 14: Graphical depiction of number of intermolecular hydrogen bond interactions between the protein and the candidate compound.

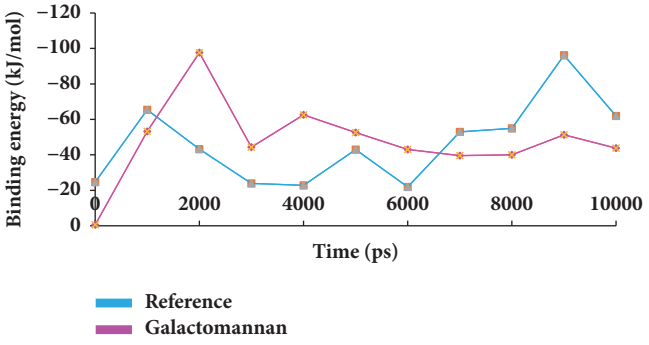

Figure 15: MM/PBSA binding energy representation of the reference and the candidate compound.

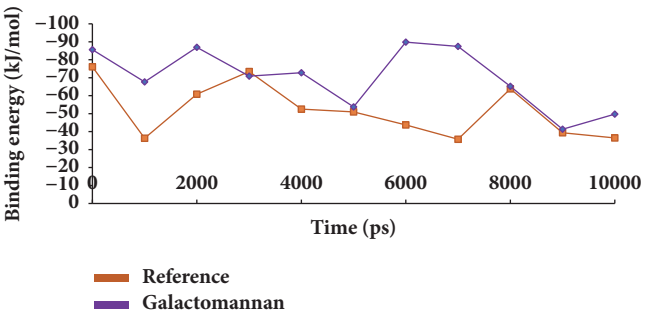

Figure 16: MM/PBSA binding energy representation of the reference and the candidate compound.

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Research Article

In Vitro and *Ex Vivo* Chemopreventive Action of *Mauritia flexuosa* Products

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Mauritia flexuosa (Arecaceae), known as "Buriti," is a Brazilian palm tree with high economic potential for local communities. Herein, we investigated the phytochemistry profile and antioxidant potential of *M. flexuosa* fruits and determined the bioaccessibility of phenolic compounds. Peels revealed upper values for phenols, flavonoids, carotenoids, tannins, and ascorbic acid when compared to the pulps and endocarps. All samples showed capacity to scavenger free radicals (0.5, 1.0, 2.0, 4.0, and 8.0 mg/mL) but peels presented higher scavenger action in all methods explored. Phenolic compounds identified by HPLC displayed reduced bioaccessibility after *in vitro* simulated gastrointestinal digestion for pulp (38.7%), peel (18.7%), and endocarp (22.3%) extracts ($P < 0.05$). Buriti fruits also protected rat blood cells against lysis induced by peroxyl radicals. We demonstrated the promising chemopreventive potentialities of *M. flexuosa* fruits and their by-products and peels with higher quantities of bioactive compounds and phenolic substances before and after *in vitro* bioaccessibility investigation. In Brazil, these parts are discarded or underused, mainly as feed for ruminant animals. Consequently, it is extremely important to explore nutritional characteristics of these by-products for human/livestock foods and to install biofriendly techniques and sustainable biotechnology handling of natural resources.

1. Introduction

Bioactive compounds have natural functions in plants such as sensory properties (color, aroma, flavor, and astringency) and defense against microorganisms and predators [1]. On the other hand, intake of vegetal nutrients has functional benefits for consumers and enables increasing supply for healthy foods. A diet rich in antioxidant compounds associated with endogenous enzymatic mechanisms can help to minimize the development of oxidative damage caused

by free radicals (free electrons), mainly reactive oxygen (ROS)/nitrogen (RNS)/sulfur (RSS)/and chlorine species, since these unstable molecules are consequence of normal and/or unbalanced metabolic activities and studies have demonstrated epidemiological and biological correlations with chronic or nonchronic diseases such as hypercholesterolemia, atherosclerosis, hypertension, ischemia-reperfusion injury, inflammation, cystic fibrosis, diabetes, Parkinson's disease, Alzheimer, cancer, and aging process itself or premature aging [2–8].

In this context, plant species produce secondary metabolites belonging to different chemical groups such as alkaloids and cyanogenic glycosides and nonnitrogenous compounds, such as tannins, flavonoids, terpenes, and anthocyanins, which present antioxidant activity [9–12].

"Buriti," *Mauritia flexuosa* L. f., belongs to the family Arecaceae, a palm tree widely distributed in South America, especially in the Amazon region and Brazilian Cerrado, where it has demonstrated high economic potential for the biotechnology development based on the sustainability of natural resources. In the Brazilian food industry, the peel and endocarp are commonly discarded or underutilized for the preparation of candies, ice creams, juices, jams, porridges, and/or oils [13]. Additionally, some studies have emphasized pharmacological potentialities of the *M. flexuosa* parts, such as antimicrobial [14–16], antitumor [16], hypolipemiant [17], hypoglycemiant [18], and healing activities [19].

For exotic and underexploited plants, in particular, there is little and shallow knowledge about key interfering factors in the biological significance of foods on human health, intake of nutrients, and their bioaccessibility/bioavailability throughout the gastrointestinal tract [20, 21]. In this perspective, the development of studies on the use of regional and tropical fruits should be encouraged, advancing about the knowledge and exploring the use of fresh fruits for Research and Development (R&D) of novel products [22, 23]. Herein, we investigated the phytochemistry profile and antioxidant potential of *M. flexuosa* fruits and determined the bioaccessibility of phenolic compounds using *in vitro* simulated gastrointestinal digestion.

2. Materials and Methods

2.1. Chemical Reagents. Chemical reagents 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$), 2,20-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$), thiobarbituric acid, trichloroacetic acid, ferric chloride, potassium ferricyanide, dihydrochloride 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), sodium nitroprusside (SNP), Triton X-100, Folin-Ciocalteu, sodium carbonate, gallic acid, aluminum chloride, quercetin, β-carotene, potassium iodide, and potassium persulfate were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2. Plant Material: Origin and Preparation. A sample of *Mauritia flexuosa* was deposited in the Graziela Barroso Herbarium at Federal University of Piauí (UFPI) (voucher specimen #30567). About 300 fruits were collected in Água Branca, Piauí, Brazil, in December 2014 (latitude: 5°54′50.5″S; longitude: 42°38′03.4″W) and taken to the Federal Institute of Piauí, Teresina, Brazil. Fruits were selected regarding sanity and same maturation stage and cleaned in water containing 25 ppm of commercial sodium hypochlorite. These fruits presented an elongated oval shape surrounded by the epicarp (peel) of reddish brown color, mesocarp (pulp), orange, and endocarp with a white or yellowish spongy tissue [24]. Subsequently, fruits were separated in pulp, peel, and endocarp. These parts were frozen separately at −70°C. For the lyophilization process, stainless steel tray of lyophilizer model L101 (Liotop, São Carlos, Brazil) was used. Lyophilization conditions (temperature: 40°C; vacuum pressure: <500 mmHg; lyophilization rate: 1 m/h) were well controlled during 72 h [25]. After such process, fruits were packaged in plastic bags under refrigeration at 4°C before process for preparation of powder samples using rotor mill (0.08 mm) (Figure 1).

2.3. Content of Phenols, Flavonoids, Carotenoids, Tannins, and Ascorbic Acid. Pulverized pulp, peel, and endocarp samples were submitted to extraction of bioactive compounds with methanol. Samples were mixed with mortar and pestle for 10 min (1 : 10; sample/solvent) until reaching uniform consistency. Methanol extracts were stored at 4°C for 2 days up to quantification of bioactive compounds (phenols, flavonoids, carotenoids, and tannins) and antioxidant activity, respectively. All analyses of bioactive compounds were carried out in quintuplicate.

2.3.1. Total Phenolics. The total phenolic content was determined with Folin-Ciocalteu reagent according to [3], with some modifications. For 1 mL of pulp, peel, and endocarp methanol solution (10 mg/mL), 1 mL of Folin-Ciocalteu reagent (1 : 4) and 1 mL of 15% sodium carbonate (Na_2CO_3) were added and the final volume was filled with distilled water to 10 mL. The mixture was maintained for 2 h and centrifuged at 4000 rpm during 4 min. The supernatant was measured at 750 nm. Stock solution without fruit parts was used as negative control. Results were expressed as mg of gallic acid equivalents per 100 g of sample (mg GAE/100 g sample) and a gallic acid calibration curve was determined ($0.9497x\ y = -0.0527$; $r^2 = 0.999$).

2.3.2. Total Flavonoids. The content of total flavonoids was determined based on the formation of the flavonoid-aluminum complex, according to [3] with some modifications. For 1 mL of pulp, peel, and endocarp methanol solution (10 mg/mL), 1 mL of 20% aluminum chloride and 100 μL of 50% acetic acid were added. The mixture was maintained for 30 min and centrifuged at 4000 rpm during 4 min. The supernatant was measured at 420 nm. Results were expressed as mg of quercetin equivalent per 100 g of sample (mg EQE/100 g sample) and a quercetin calibration curve was prepared ($y = 0.0136x - 0.0422$; $r^2 = 0.999$).

2.3.3. Total Carotenoids. Total carotenoids were determined according to [26] with some modifications. A total of 0.1 g of pulp, peel, and endocarp diluted in 10 mL of acetone : hexane solution (4 : 6) was stirred for 10 min at room temperature

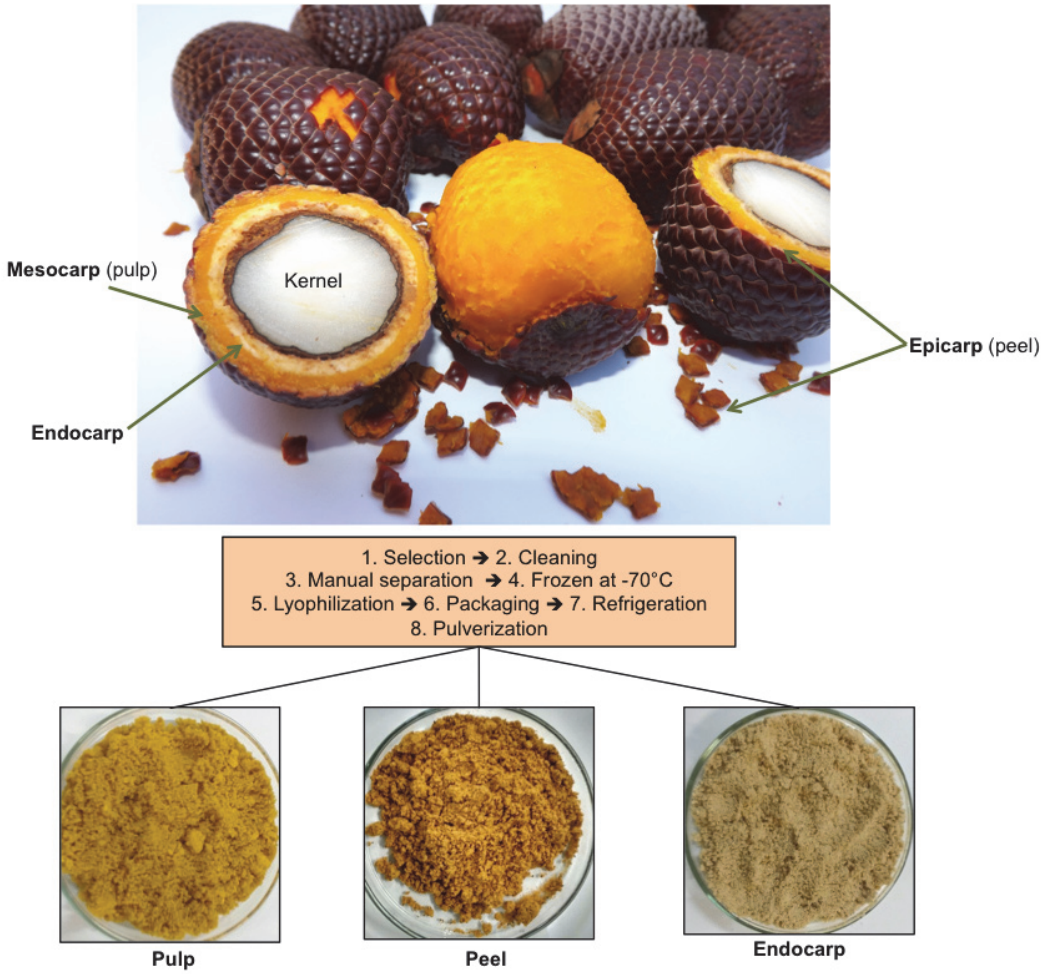


FIGURE 1: Preparation of *Mauritia flexuosa* fruits: lyophilization, pulverization, and stocking preceded phytochemical and biological analysis.

(400 rpm) and centrifuged for 4 min at 4000 rpm. Reading was performed at 450 nm and the results were expressed as mg of β-carotene equivalent per 100 g of sample (mg βCTE/100 g sample). A β-carotene calibration curve was prepared ($y = 0.3099x - 0.341$; $r^2 = 0.991$).

2.3.4. Condensed Tannins. The content of condensed tannins was determined using the methodology of vanillin [27]. To the methanol solution containing 1 mL of pulp, peel, and endocarp (10 mg/mL), 3 mL of 2% vanillin prepared with sulfuric acid (70%) was added. Subsequently, the reaction mixture was performed in water bath at 20°C for 15 min. Samples were centrifuged for 4 min at 4000 rpm and reading was carried out in digital spectrophotometer at 500 nm. Results were expressed as milligrams of catechin equivalents per gram of sample (mg CTQ/100 g sample). A catechin calibration curve was performed ($y = 0.008x + 0.096$; $r^2 = 0.999$).

2.3.5. Hydrolysable Tannins. The hydrolysable tannin concentration was determined using potassium iodide according to [28]. One milliliter of saturated potassium iodide solution was added to the methanol solution containing 3 mL of pulp, peel, and endocarp (10 mg/mL) and allowed to rest at room temperature for 40 min and centrifuged for 4 minutes at 4000 rpm and the absorbance was measured at 550 nm. Results were expressed as mg of tannic acid equivalents per gram of sample (mg ACT/100 g sample) and a tannic acid calibration curve ($0.0122x + y = 0.26$; $r^2 = 0.981$) was performed.

2.3.6. Ascorbic Acid . Ascorbic acid content was determined using the titrimetric *Tillmans*' method. We used a solution of oxalic acid as a solvent to substitute metaphosphoric acid. Twenty milliliters was mixed with 80 mL of 1% oxalic acid and 10 mL of such solution was titrated with Tillmans reagent, using 2,6-dichlorophenolindophenol. Results were calculated based on a standard solution of ascorbic acid and expressed in mg/100 mL.

2.4. In Vitro Quantification of Total Phenolics after Simulated Gastrointestinal Digestion. The digestion was performed using simulated gastric (pepsin solubilized with 0.1 mol/L HCl) and intestinal fluids (pancreatin-bile salts solubilized with 0.1 mol/L $NaHCO_3$), which were prepared according to [29]. We added 1 mL of pulp, peel, and endocarp methanol

solution (10 mg/mL) to 100 mL of 0.01 mol/L HCl, and pH was adjusted to 2 with 2 mol/L HCl solution. Equal quantity of phenols was used as positive control (10 mg/mL). Afterwards, 3.2 mL of pepsin was added, maintaining samples under stirring at 37°C for 2 h to simulate food digestion in the stomach. Then, to simulate the pH found in human intestines, titration was carried out with 0.5 mol/L NaOH to obtain pH 7.5. Subsequently, a dialysis process was performed for 2 h (dialysis membrane with 33 × 21 mm, molecular weight of 12.000 to 16.000, and porosity of 25 angstroms, Inlab, Brazil) with 0.1 mol/L $NaHCO_3$ equivalent to titratable acidity. After pH adjustment, dialysis membranes were added and the solution was stirred in water bath at 37°C/30 min. Then, 5 mL of pancreatin-bile salts solution was added and the mixture was stirred again for additional 2 h to mimic food digestion in the intestine. Finally, the membrane content (dialysate) was removed and samples were stored at 20°C until analysis.

Finally, dialyzed material was analyzed to determine total phenolics [3]. Results were expressed as mg gallic acid/100 g sample. The bioaccessible percentage was calculated according to [20]: % bioaccessible = 100 × (DPC/CPC), where F is the content of dialyzable phenolic compounds (mg gallic acid/100 g sample) and G corresponds to the content of phenolic compounds in the sample (mg gallic acid/100 g sample).

2.5. In Vitro Antioxidant Capacity. For *in vitro* antioxidant evaluation, the antioxidant capacity of the samples was assayed against 1,1-diphenyl-2-picrylhydrazyl [$DPPH^{\bullet}$] [30], 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid [$ABTS^{\bullet+}$] [31], reducing potential [Fe^{3+}/Fe^{2+}] [32], lipid peroxidation [thiobarbituric acid reactive substances (TBARS) removal [33, 34], and nitrite content [nitrite production induced by sodium nitroprusside [35, 36]. Aqueous stock solutions of the samples (pulp, peel, and endocarp: 0.5, 1.0, 2.0, 4.0, and 8.0 mg/mL), $DPPH^{\bullet}$ (40 μg/mL), $ABTS^{\bullet+}$ (7 mM), 1% potassium ferricyanide, sodium nitroprusside (10 mM), and 0.67% thiobarbituric acid, were prepared. Trolox (0.5 mg/mL) was used as positive standard.

Values of 50% effective concentration (EC_{50}) for Buriti extracts were spectrophotometrically quantified (T80+ UV/VIS Spectrometer, PG Instruments Ltd.®, Leicestershire, UK) at 515 nm for $DPPH^{\bullet}$, 734 nm for $ABTS^{\bullet+}$, 700 nm for potassium ferricyanide, 532 nm for TBARS (thiobarbituric acid reactive substances), and 540 nm for nitrite radicals 30 min after the reaction started. Antioxidant evaluation was performed in triplicate from two independent experiments and absorbance values were converted to the inhibition percentage (I) of radicals using the equation of [37]: (%) = [(absorbance of negative control − absorbance of sample) × 100]/absorbance of negative control, where absorbance of negative control is, for example, the initial absorbance for $DPPH^{\bullet}$ solution and absorbance of sample is the absorbance for reaction mixture ($DPPH^{\bullet}$ and sample).

2.6. Ex Vivo Analysis on Murine Erythrocytes. All procedures were approved by the Committee on Animal Research at UFC (#054/2014) and they are in accordance with Brazilian (COBEA, *Colégio Brasileiro de Experimentação Animal*) and international guidelines on the care and use of experimental animals (Directive 2010/63/EU of the European Parliament and of the Council).

Blood was collected from retroorbital plexus of anesthetized female *Wistar* rats (180–220 g) with ketamine (90 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). Blood was mixed with 0.85% NaCl solution containing 10 mM $CaCl_2$ and submitted to three centrifugations (2000 rpm/5 min). Erythrocytes were suspended in NaCl to obtain a cell suspension (10%). Hemolytic investigations were performed in 96-well plates following the method described by [38].

2.6.1. Hemolytic Capacity Determination. Each well received 50 μL of 0.85% NaCl. The first well was the negative control that contained only the vehicle (PBS), and in the second well 50 μL of test substance that was diluted in half was added. The extracts were tested at concentrations ranging from 0.5 to 8 g/mL. The last well received 50 μL of 0.2% Triton X-100 (in 0.85% saline) to obtain 100% hemolysis. Then, each well received 50 μL of a 10% suspension of mice erythrocytes in 0.85% saline containing 10 mM $CaCl_2$. After incubation at room temperature for 2 h, cells were centrifuged, the supernatant was removed, and the liberated hemoglobin was measured spectroscopically as absorbance at 540 nm. For comparison, a solution of 0.5 mg/mL Triton X-100 was used as positive control.

2.6.2. Antioxidant Capacity against Oxidative Hemolysis. The antioxidant capacity against oxidative hemolysis was performed by inhibition of oxidative hemolysis induced by peroxyl radicals generated following thermal decomposition of 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) in method described by [39] with some modifications. Briefly, aliquots of pulp, peel, and endocarp aqueous extracts (0.5 to 8 mg/mL) were mixed with 30 μL of 10% erythrocyte suspension and 50 μL of AAPH (200 mM in PBS, pH 7.4) in 96-well plates. The reaction mixture was incubated for 120 minutes at 37°C. Afterwards, the reaction mixture was diluted with 240 μL of PBS and centrifuged at 2000 rpm for 5 min and the liberated hemoglobin was measured spectroscopically as absorbance at 540 nm. Results were expressed as percentage inhibition of hemolysis compared to the complete hemolysis of erythrocyte suspensions induced by AAPH. Liberated hemoglobin was measured spectroscopically as absorbance at 540 nm. The inhibition of erythrocyte hemolysis was calculated as $(1 - A_{\text{sample}}/A_{\text{control}}) \times 100\%$. Trolox (0.5 mg/mL) was used as positive standard.

2.7. Chromatographic Analysis. For chromatographic analysis, methanol extracts of pulp, peel, and endocarp were used. Mobile phases were represented by solvents A–C using three pumps associated with the chromatograph (Shimadzu® liquid chromatograph with a diode array detector, Japan: solvent A, 0.1% trifluoroacetic acid in acetonitrile; solvent B, 0.1% trifluoroacetic acid in HPLC grade water; solvent C, 100% methanol). A TSK-GEL Super-ODS (Supelco) column was used. The effluent was monitored at 250 and 330 nm. Flow rate was fixed at 1.0 mL/min, and column temperature was maintained at 37°C throughout the test. Initially, the solvent

Table 1: Quantification of phenols, flavonoids, carotenoids, condensed tannins, and hydrolysable tannins in the lyophilized methanolic extracts of pulp, peel, and endocarp from *Mauritia flexuosa* fruits.

Class of compounds	Pulp	Peel	Endocarp
Total phenols (mg GAE/100 g)	553.5 ± 7.7^{b}	$1288.0 \pm 10.4^{a,c}$	597.1 ± 6.5^{b}
Total flavonoids (mg EQE/100 g)	$264.4 \pm 2.1^{b,c}$	$339.4 \pm 3.9^{a,c}$	$145.4 \pm 10.2^{a,b}$
Total carotenoids (mg βCTE/100 g)	$58.9 \pm 0.1^{b,c}$	$88.3 \pm 0.3^{a,c}$	$19.1 \pm 0.2^{a,b}$
Hydrolysable tannins (mg ACT/100 g)	$47.4 \pm 0.3^{b,c}$	$56.2 \pm 0.4^{a,c}$	$0.1 \pm 0.02^{a,b}$
Condensed tannins (mg CTQ/100 g)	$69.6 \pm 1.8^{b,c}$	$118.3 \pm 2.1^{a,c}$	$36.5 \pm 1.4^{a,b}$
Ascorbic acid (mg/100 mL)	4.3 ± 1.3^{c}	5.9 ± 0.2^{c}	$2.5 \pm 0.3^{a,b}$

Data were presented as mean ± standard error of the mean (SEM). $^{a}P < 0.05$ compared to pulp; $^{b}P < 0.05$ compared to peel; $^{c}P < 0.05$ compared to endocarp by ANOVA followed by Student-Newman-Keuls test.

was represented by 100% solvent B, but a linear gradient was used to increase solvent A from 0 to 10% within 7 min. Its composition was maintained at an isocratic flow for 3 min. Then, solvent A increased from 10 to 40% during 20 min. Such composition was maintained for additional 2 min and returned to the initial condition in 3 min. A volume of 20 μL for the standards substances and samples was injected for each HPLC analysis.

2.8. Statistical Analysis. Data were presented as mean ± standard error of the mean (SEM) and compared by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test using GraphPad Prism® software 5.0 (San Diego, CA, USA). EC_{50} values were calculated by nonlinear regression (95%). Statistical correlation among experimental data was performed using the Pearson coefficient (r) and results were statistically significant when $P < 0.05$.

3. Results

3.1. Bioactive Compounds and Bioaccessibility. The screening of bioactive compounds in *M. flexuosa* fruit is described in Table 1. Peel revealed the highest values for phenols (1288.0 ± 10.4 mg GAE/100 g), flavonoids (339.4 ± 3.9 mg EQE/100 g), carotenoids (88.3 ± 0.3 mg βCTE/100 g), tannins (hydrolysable: 56.2 ± 0.4 mg ACT/100 g; condensed: 118.3 ± 2.1 mg CTQ/100 g), and ascorbic acid (5.9 ± 0.2 mg/100 mL) when compared to the pulp and endocarp ($P < 0.05$).

The correlation of chromatographic peaks was achieved by comparison of experimental retention times (t_R) with reference standards (Table 2). All chromatographic analyses were carried out in triplicate and revealed phenolic compounds (protocatechuic acid, quercetin, apigenin, catechin, and epicatechin) with the following t_R: 16.3, 33.6, 41.7, 53.6, and 49.3 minutes, respectively.

Subsequently, we analyzed the quantity of phenolic compounds before and after *in vitro* simulated gastrointestinal digestion for pulp, peel, and endocarp (Table 3). All samples (pulp, peel, and endocarp) displayed reduction in bioaccessibility after *in vitro* digestion of 38.7, 18.7, and 22.3%, respectively ($P < 0.05$).

3.2. In Vitro Antioxidant Capacity. In this step, we carried out quantification of the antioxidant capacity of Buriti samples (pulp, peel, and endocarp) at concentrations of 0.5, 1, 2, 4, and 8 mg/mL. This capacity is described as free radical inhibition (Figure 2).

For all parameters and samples, we determined EC_{50} values: 1.6 ± 0.1, 0.1 ± 0.1, and 1.5 ± 0.1 mg/mL ($DPPH^{\bullet}$); 2.3 ± 0.1, 0.1 ± 0.1, and 1.9 ± 0.1 mg/mL ($ABTS^{\bullet+}$); 2.1 ± 0.3, 1.2 ± 0.1, and 1.9 ± 0.4 mg/mL (potassium ferricyanide); 1.6 ± 0.2, 0.7 ± 0.1, and 2.3 ± 0.2 mg/mL (TBARS); and 2.6 ± 0.1, 1.1 ± 0.1, and 6.4 ± 0.14 mg/mL (nitrite content) for pulp, peel, and endocarp, respectively. Trolox (0.5 mg/mL), the positive standard, showed free radical inhibition capacity upper to 90% for the antioxidant assessments (Figure 2). Then, all samples showed growing capacity in a concentration-dependent manner to scavenger free radicals, but it is important to note that peels' samples presented a higher scavenger capacity in all methods explored ($P < 0.05$).

3.3. Antioxidant Capacity against Oxidative Hemolysis. Firstly, we analyzed the capacity of the samples to cause hemolysis. None of the extracts induced lysis of rat erythrocytes even 8.0 mg/mL. On the other hand, Triton X-100, used as positive control, caused 100% hemolysis.

Based on these promising findings (scavenger of free radicals and absence for cellular lysis), we evaluated the antioxidant capacity against oxidative hemolysis induced by AAPH (100% hemolysis). Once again, all concentrations

Table 2: Identification of compounds by high-performance liquid chromatography (HPLC) in *Mauritia flexuosa* samples.

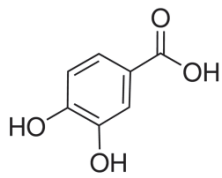
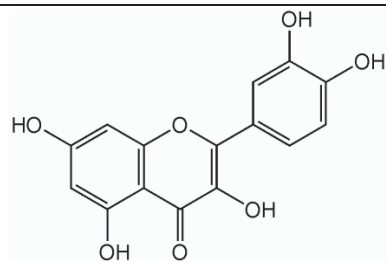
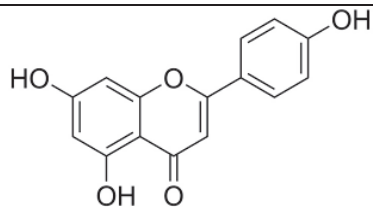
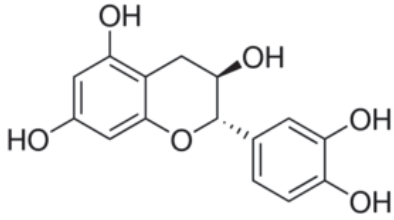
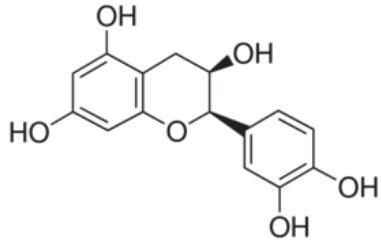
IUPAC Name Chemical Name	Chemical structures	Class	Retention time (min)	Sample
3,4-Dihydroxybenzoic acid (protocatechuic acid)		Phenol	16.3	Pulp
2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one *(quercetin)*		Flavonoid	33.6	Pulp
4′,5,7-Trihydroxyflavone *(apigenin)*		Flavonoid	41.7	Pulp Endocarp
(−)-*trans*-3,3′,4′,5,7-pentahydroxyflavane, (2S,3R)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-1(2H)-benzopyran-3,5,7-triol (catechin)		Condensed tannin	53.6	Endocarp Peel Pulp
(−)-*cis*-3,3′,4′,5,7-pentahydroxyflavane, (2*R*,3*R*)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-1(2*H*)-benzopyran-3,5,7-triol (epicatechin)		Condensed tannin	48.3	Peel

Table 3: Contents of phenolic compounds in the lyophilized methanolic extracts of pulp, peel, and endocarp from *Mauritia flexuosa* fruits before and after simulated gastrointestinal digestion.

Sample	Bioaccessibility before *in vitro* digestion (mg/L)	Bioaccessibility after *in vitro* digestion (mg/L)	Reduction (%)
Pulp	553.5 ± 7.7	102.2 ± 0.4*	18.7
Peel	1288.0 ± 10.4	498.5 ± 13.9*	38.7
Endocarp	597.1 ± 6.5	133.4 ± 7.8*	22.3

*$P < 0.05$ compared to bioaccessibility before *in vitro* digestionby ANOVA followed by Student-Newman-Keuls test.

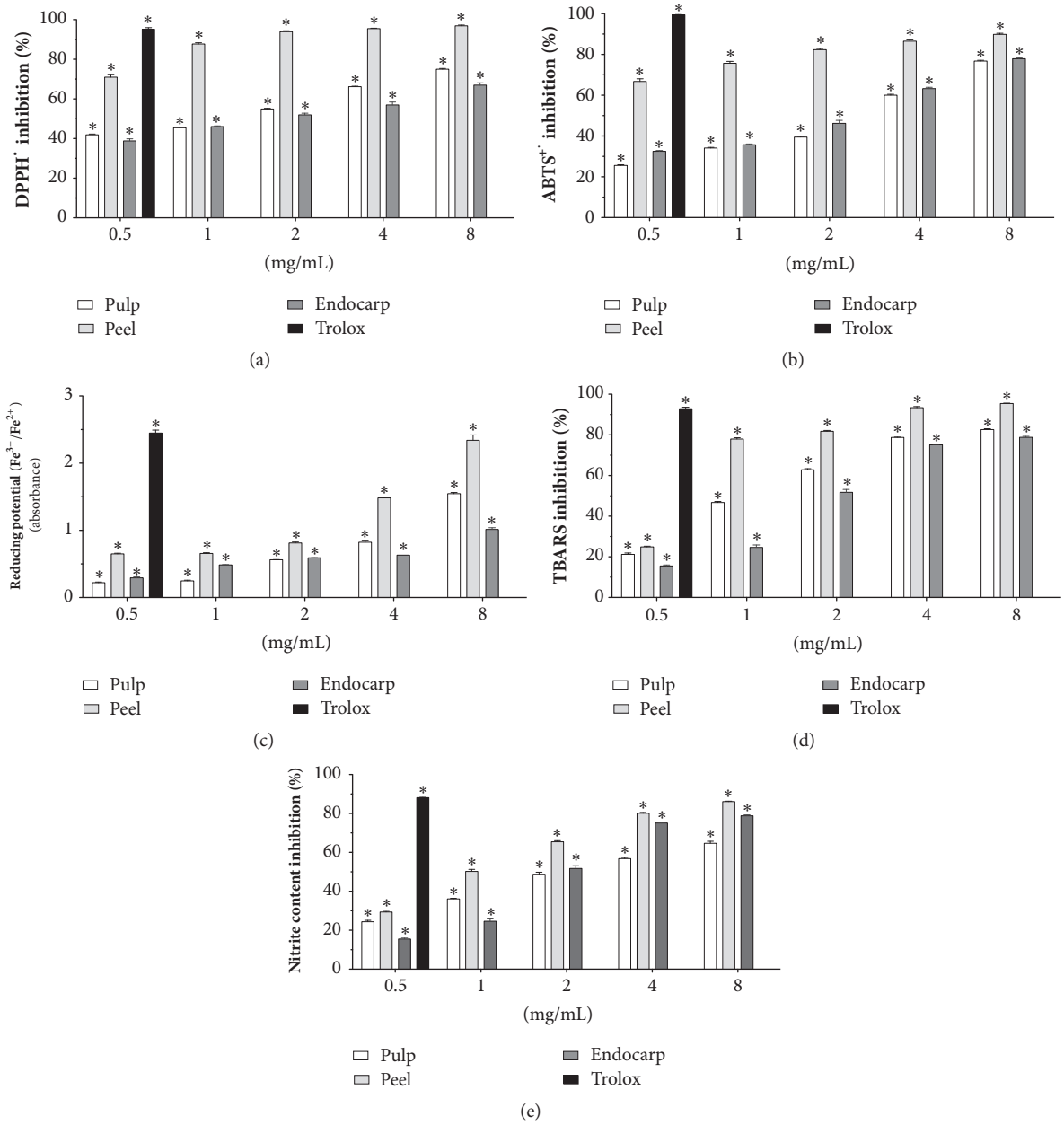


Figure 2: Effects of lyophilized fruits (pulp, peel, and endocarp) from *Mauritia flexuosa* (0.5, 1, 2, 4, and 8 mg/mL) on the removal of (a) 1,1-diphenyl-2-picrylhydrazyl (DPPH•), (b) 2,20-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$), (c) reducing potential (Fe^{3+}/Fe^{2}), (d) reactive substances to thiobarbituric acid [TBARS levels induced by 2,2′-azo-bis (2-methylpropionamidine]) dihydrochloride, AAPH), and (e) nitrite content (induced by sodium nitroprusside). Trolox (0.5 mg/mL) was used as positive standard. Results are expressed as mean ± standard error of measurement (SEM) from two independent experiments in triplicate. Negative control was treated with the solution used for diluting the tested substance. With exception of reducing potential, absorbance values were converted to inhibition (I) percentage of radicals: I (%) = [(absorbance of negative control − absorbance of sample) × 100]/absorbance of negative control. $^{*}P < 0.05$ compared to negative control by ANOVA followed by Student-Newman-Keuls test.

used (0.5, 1.0, 2.0, 4.0, and 8.0 mg/mL) were able to protect blood cells when compared to positive control exposed to peroxyl radicals generated following thermal decomposition of AAPH as follows: pulp (15.0 ± 1.1, 26.9 ± 0.7, 27.6 ± 0.4, 36.8±0.1, and 49.3±2.7%), peel (26.9±0.6, 46.9±1.2, 51.2±0.3, 60.1±0.8, and 74.3±0.5%), and endocarp (19.6±1.7, 25.7±0.9, 28.5 ± 0.3, 31.8 ± 0.5, and 40.2 ± 0.7%), respectively (Figure 3). Trolox showed an antioxidant perceptual protection of 73.2 ± 0.5%. EC_{50} values were 7.7±0.4, 1.8±0.1, and 11.4±0.5 mg/mL for pulp, peel, and endocarp, respectively.

Pearson's correlation, a measure of the strength of linear relationship between two variables, revealed a positive relationship between bioactive compounds (total phenol, total flavonoids, total carotenoids, and condensed and

Table 4: Analysis of Pearson's correlation among bioactive compounds and antioxidant capacity in samples of pulp, peel, and endocarp from *Mauritia flexuosa*.

Class of compounds	DPPH$^{\bullet}$	ABTS$^{\bullet+}$	Reducing potential	TBARS	Nitrite content	Oxidative hemolysis
			Pulp			
Total phenols	0.956*	0.978*	0.978*	0.867	0.931*	0.954*
Total flavonoids	0.957*	0.979*	0.978*	0.869	0.933*	0.956*
Total carotenoids	0.951*	0.974*	0.975*	0.859	0.926*	0.951*
Condensed tannins	0.955*	0.977*	0.978*	0.866	0.930*	0.954*
Hydrolysable tannins	0.923*	0.953*	0.956*	0.822	0.898*	0.935*
			Peel			
Total phenols	0.681	0.847	0.928*	0.749	0.854	0.907*
Total flavonoids	0.956*	0.978*	0.978*	0.867	0.931*	0.954*
Total carotenoids	0.966*	0.984*	0.983*	0.881*	0.941*	0.959*
Condensed tannins	0.963*	0.982*	0.982*	0.876	0.937*	0.957*
Hydrolysable tannins	0.972*	0.988*	0.987*	0.890*	0.947*	0.961*
			Endocarp			
Total phenols	0.682	0.848	0.930*	0.751	0.854	0.907*
Total flavonoids	0.951*	0.974*	0.975*	0.860	0.926*	0.952*
Total carotenoids	0.949*	0.973*	0.974*	0.857	0.924*	0.950*
Condensed tannins	0.952*	0.975*	0.976 *	0.861	0.927*	0.952*
Hydrolysable tannins	0.948*	0.972*	0.973 *	0.855	0.923*	0.950*

*$P < 0.05$. Pearson's correlation coefficient was calculated using Student's t-test for all variables at 5% significance levels. 1,1-Diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS$^{\bullet+}$), reducing potential (Fe^{3+}/Fe^{2}), reactive substances to thiobarbituric acid [TBARS levels induced by 2,2′-azo-bis (2-methylpropionamidine]) dihydrochloride, AAPH), and nitrite content (induced by sodium nitroprusside).

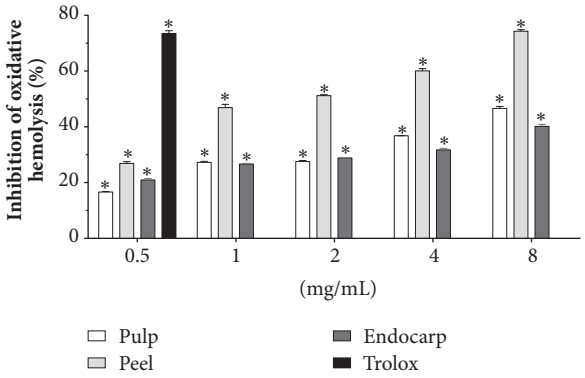


Figure 3: Protection against oxidative hemolysis induced by peroxyl radicals generated following thermal decomposition of 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) by lyophilized fruits (pulp, peel, and endocarp) from *Mauritia flexuosa* (0.5, 1, 2, 4, and 8 mg/mL). Trolox (0.5 mg/mL) was used as positive standard. Results are expressed as mean ± standard error of measurement (SEM) from two independent experiments in triplicate. Negative control was treated with the solution used for diluting the tested substance. *$P < 0.05$ compared to control by ANOVA followed by Student-Newman-Keuls test.

hydrolysable tannins) and antioxidant capacity ($r > 0.881$; $P < 0.05$) and bioactive compounds and protection against oxidative hemolysis ($r > 0.907$; $P < 0.05$) (Table 4). On the other hand, Pearson's correlation did not show association between antioxidant activity against TBARS and presence of bioactive compounds for most correlations analyzed ($P > 0.05$).

4. Discussion

Since oxidative damage contributes significantly to pathologies, herein, we performed different biochemical methods to support the antioxidant and functional action of *M. flexuosa* fruits.

Peels from *M. flexuosa* fruits presented highest values of bioactive compounds when compared to the pulp and endocarps. Previously, studies demonstrated that pulp extracts from Amazon Buriti have mainly quinic acid, caffeic acid, chlorogenic acid, ferulic acid, p-Coumaric acid, protocatechuic acid, catechin, epicatechin, luteolin, apigenin, myricetin, kaempferol, and quercetin, some of them also found in lower concentrations [18]. Moreover, as confirmed here, Buriti seems to be an excellent source of carotenoids (44600 μg/100 g), especially α- and β-carotene and *cis*- and *trans*-áṭđ-carotene [40–43], which are normally found in carrots and are considered the most known and accepted source by consumers, justifying its use to treat hypovitaminosis A.

Our results presented differences per 100 g of dry material, since Buriti samples were collected under natural conditions from Cerrado Brazilian (a type of savanna) and most studies presented outcomes with fruits from Amazon region. These findings are explained by differences in biome conditions. Amazon is hot and humid, while Cerrado presents a dryer climate. Besides, the Cerrado soil is more acid and rich in aluminum salts, which will probably generate

higher oxidative stress for the plants. They react, producing antioxidant agents [41].

Polyphenol substances with high *in vitro* antioxidant activity do not necessarily have similar actions after gastrointestinal process and absorption [20, 44]. Therefore, we verified the bioaccessibility of phenolic compounds from pulp, peel, and endocarp methanol extracts. For this, we used an *in vitro* method that has recently gained much attention because it simulates the process of gastrointestinal digestion, enabling studying changes that occur in the diet components during gastric and intestinal digestion. Moreover, *in vitro* techniques have the advantage to substitute animals and are time-efficient and cost-effective and require less manpower [21, 23, 44, 45]. Interestingly, *M. flexuosa* methanol extracts showed reduction of bioaccessible polyphenols after digestion simulation ranging from 18.7 (pulp) to 38.7% (peel).

It is important to note that only solubilized nutrients from the food matrix which are not destroyed during gastrointestinal digestion are bioaccessible and potentially bioavailable [22, 23]. Since dietary fiber components are not absorbed, they achieve the large intestine and provide the substrate for intestinal digestion. Soluble fibers are usually fermented quickly, while insoluble fibers are slowly or only partially fermented. The fermentation is carried out by anaerobic bacteria of the colon (e.g., *Lactobacillus* and *Bifidobacterium* genera), leading to the production of lactic acid, short-chain fatty acids, and gas, events that can alter food components and their bioavailability [46]. Furthermore, the consumption of high quantities of phytates and oxalates can cause chelation of metal ions (e.g., calcium and zinc) and induce cholelithiasis [24].

Although *M. flexuosa* fruits have been associated with multiple nutritional properties that can be favorable to the human health, their fibers and polyphenols may link to macromolecular compounds that are not dialyzable or generate mineral complexes, further decreasing solubility and bioaccessibility of phenols [47, 48]. Furthermore, because dialysis process during *in vitro* gastrointestinal digestion separates bioactive substances, this can interfere with biological activity and quantity of phenolic compounds, which may work more efficiently together rather than individually as synergists to reduce free radicals [49].

Investigators working with cashew fruits from *Anacardium occidentale* L., another typical natural delight from Brazilian Northeast known as "caju," "acajuíba," and "açajaíba," but more popular, accepted, studied, and economically exploited than *M. flexuosa*, also showed a considerable loss of phenolic compounds in cashew apple juice and cashew apple fiber after bioaccessibility tests, mainly due to the type of food matrix elements, and this often alters absorption of phenolic compounds [44].

In vitro antioxidant activity is mainly based on chemical assays that assess the ability of a substance to reduce the concentration of free radicals in a specific reaction medium [50, 51]. Then, we performed methods to determine the *in vitro* scavenging actions.

Firstly, we used the DPPH method, since it is a rapid, simple, accurate, and inexpensive assay for measuring the ability of different compounds to act as free radical scavengers or hydrogen donors and to evaluate the antioxidant activity of foods and beverages independent of sample polarity [11, 52]. In the ABTS test, 2,2′-azino-bis (3-ethylbenzthiazoline-6-acid) (ABTS) is converted into its radical ($ABTS^{\bullet+}$) by addition of sodium persulphate and is reactive towards most antioxidants. Since it is not affected by ionic strength, it can be used to determine both hydrophilic and hydrophobic antioxidant capacities [10]. The total antioxidant activity was also measured by the ferric reducing antioxidant power assay. Flavonoids and phenolic acids presented in the medicinal plants exhibit strong antioxidant activity, which is depending on their potential to form the complex with metal atoms, particularly iron and copper. This method is based on the principle of increase in the absorbance of the reaction mixtures [32]. Subsequently, lipid peroxidation was determined by TBARS removal. Since polyunsaturated fatty acids are easy targets for oxidants, and the process of lipid peroxidation is, once initiated, a self-sustaining free radical chain process, the accumulation of lipid peroxidation products provides the most common biochemical marker of oxidative stress [33, 34]. Finally, nitrite ion technique was carried out based on the decomposition of sodium nitroprusside in nitric oxide at physiological pH, under aerobic conditions, which produces nitrites [35, 36]. It was important to perform the evaluation of samples against RNS, since these radicals may cause damage to biological components such as the aromatic amino acid tyrosine and DNA bases, particularly in guanines, by nitration or hydroxylation [51].

Buriti samples presented antioxidant capacity, and peel extracts were more active scavengers. References [14] also demonstrated antioxidant potential in leaves (iron reduction test) and fruit pulps (DPPH method) from *Mauritia flexuosa*. Differences in the antioxidant action found are probably associated with distinctive concentrations of the chemical constituents in each part of the plant, mainly flavonoids and anthocyanins [53]. So, there is a huge possibility that this effect repeats in Buriti fruits in different Brazilian regions, once *M. flexuosa* in the "Cerrado" biome is exposed to a higher incidence of sunlight in a soil of dry climate [54]. It is supposed that climate conditions interfere even in the constitution of the general parts, with average values of 22.1–25.1, 11–24.2, 21.0, and 32.6–63.9% for peel, pulp, endocarp, and seed, respectively [25, 55].

Typically, phenols and carotenoids are found in higher concentrations in peels due to their pigmentation, regulation of enzymatic activity, and protection against sunlight and pathogenic microorganisms [1, 56]. So, we noted superior presence of phenolic compounds (57.0 and 53.6%), flavonoids (22.1 and 57.2%), tannins (hydrolysable: 15.7 and 99.8%; condensed: 41.1 and 69.1%), and ascorbic acid levels in peels when compared to pulp and endocarp, respectively, which improved antioxidant activity in peels, respectively. Taking into consideration the fact that the Dietary Reference Intake (DRI) of ascorbic acid for adults is 45 mg/day [57], one cup with 200 mL of peel extract from *M. flexuosa* fruits (11.7 mg/mL) would correspond to 26% of the RDI, while consumption of pulp would reach 19.1%. Anyway, it is important to note that vitamin C is converted to oxalate when it is present in higher concentrations [24].

For *M. flexuosa* fruit, protection by antioxidant compounds is required and could be a reason for the higher concentration of bioactive compounds found in the peel than in pulp and endocarp. Using Pearson's correlation, we found a good correlation index among bioactive compounds and antioxidant capacity for pulp, peel, and endocarp from *Mauritia flexuosa*, which supports the suggestion that protection against oxidative hemolysis is directly associated with levels of bioactive substances.

Since vegetal extracts are rich in different classes of compounds that can attack or interact with cellular membranes, hemolysis assay is frequently used to test materials, compounds, or mixture of compounds at defined pHs that mimic extracellular environments. So, the evaluation of membrane stability during exposure to phytotherapeutic products must be routinely considered in their evaluation, since the consumption of these products is increasing globally and may constitute a serious public health problem. So, membrane stability represents the capacity of this biological complex to maintain its structure under chaotropic conditions such as hypotonicity, pH extremes, heat, and the presence of solutes (such as ethanol, urea, and guanidine) and oxidative stress [38, 58–60]. When submitted to the cell assays, none of the Buriti samples caused lysis of erythrocytes and reversed hemolysis induced by peroxyl radicals and, once again, better results were found with peel extracts.

The antihemolytic action described for fruit extracts from *M. flexuosa* may be associated with an osmotic stabilization of erythrocytes. It is possible that the exacerbation of Van der Waals contacts inside the lipid bilayer could be a source of membrane stabilization, though such membrane protection is normally related to the prevention of lipoperoxidation triggered by secondary metabolites such as flavonoids and phenols that can be incorporated into erythrocyte membranes [39, 58, 61]. Indeed, there is a strong correlation between thiobarbituric acid-reactive substances (TBARS) as a marker of lipid peroxidation and products that protect cells against oxidative damage [50]. Such protection can explain, at least in part, some folk uses and pharmacological properties of these fruits, such as protective effects against cognitive impairment [24, 62], antiplatelet, antithrombotic [63], lowering cholesterol [43, 64], and healing [19, 41] activities.

5. Conclusions

In summary, the antioxidant analysis of *M. flexuosa* fruits and their by-products showed promising chemopreventive potentialities, and peels demonstrated higher quantities of bioactive compounds and phenolic substances before and after *in vitro* bioaccessibility investigation. Since the processing of *M. flexuosa* fruits generates a large quantity of agricultural residues such as peels, endocarps, and seeds, most of them are commonly discarded or are used as feed for ruminant animals only, especially after production of sweets and oil extraction. Consequently, it is extremely important to explore the nutritional characteristics of these by-products for human/livestock foods and to install biofriendly techniques and sustainable biotechnology handling of natural resources. For Brazilian local communities, it is really important to reuse such residues, especially for people from poor regions, as a way to give better opportunities and improve quality of life.

Data Availability

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

Conflicts of Interest

All authors declare that there are no conflicts of interest.

Acknowledgments

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Research Article

In Vitro Antioxidant Potential and Effect of a Glutathione-Enhancer Dietary Supplement on Selected Rat Liver Cytochrome P450 Enzyme Activity

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Background. There is considerable evidence that many people take dietary supplements including those of herbal origin as an alternative therapy to improve their health. One such supplement, with an amalgam of constituents, is CellGevity®. However, the effect of this dietary supplement on drug-metabolizing enzymes is poorly understood, as it has not been studied extensively. Therefore, we investigated the effect of CellGevity dietary supplement on selected rat liver microsomal cytochrome P450 (CYP) enzymes, the most common drug-metabolizing enzymes. We also determined the total antioxidant potential of this dietary supplement *in vitro*. *Methods*. To determine the antioxidant potential of CellGevity dietary supplement, 2,2-diphenyl-2-picryl-hydrazyl (DPPH), total phenolic, and flavonoid assays were used after initial preparation of a solution form of the supplement (low dose, LD; 4 mg/kg and high dose, HD; 8 mg/kg). Rats received oral administration of these doses of the supplement for 7 days, after which the effect of the supplement on selected liver CYP enzymes was assessed using probe substrates and spectroscopic and high-performance liquid chromatographic methods. Rats which received daily administration of 80 mg/kg of phenobarbitone and distilled water served as positive and negative controls, respectively. *Results*. The IC_{50} value of the supplement 0.34 ± 0.07 mg/ml compared to 0.076 ± 0.03 mg/ml of the BHT (positive control). The total phenolic content of the supplement at a concentration of 2.5 mg/ml was 34.97 g gallic acid equivalent (GAE)/100 g while its total flavonoid content at a concentration of 2.5 mg/ml was 6 g quercetin equivalent (QE)/100 g. The supplement significantly inhibited rat CYP2B1/2B2 (LDT 92.4%; HDT 100%), CYP3A4 (LDT 81.2%; HDT 71.7%), and CYP2C9 (LDT 21.7%; HDT 28.5%) while it had no significant inhibitory effect on CYPs 1A1/1A2, CYP1A2, and CYP2D6. *Conclusion*. CellGevity dietary supplement possesses moderate antioxidant activity *in vitro* and has an inhibitory effect on selected rat liver CYP enzymes, suggesting its potential interaction with drugs metabolized by CYP enzymes.

1. Introduction

Noncommunicable diseases (NCDs) such as cardiomyopathies, asthma, diabetes mellitus, and cancer are the most common causes of death globally, with a higher percentage of premature deaths happening in developing nations than in developed nations [1]. This highlights the crucial need for simple and effective preventive strategies and treatments to reduce the current inequities within and among countries. At least half of these NCDs-related deaths are caused by common risk factors including malnutrition, a condition that represents a critical public health concern [2, 3]. Malnutrition occurs when the nutritional needs for growth (protein and calories) are not met within the context of either undernutrition or overnutrition and lead to deficiencies of essential micronutrients, with detrimental and sometimes irreversible effects [4].

The use of alternative therapies, in the form of dietary supplements, is becoming very common throughout the world, as many people nowadays are adopting a variety of lifestyle habits that contribute to healthy living [5]. Dietary supplements comprise a wide range of products intended for ingestion to meet essential nutritional needs. They may be individual components or combinations of vitamins, minerals, amino acids, or herbal products and have intermediate form between foods and drugs [6]. Thus, they possess both food and drug characteristics, a number of them being more food-like or drug-like, depending on their nature.

Dietary supplements are essential when nutritional needs are not covered by diet alone; however, the use of dietary supplementation when nutritional sufficiency has already been achieved remains controversial, as possible toxic effects of excessive intake have been reported for some micronutrients such as β-carotene and vitamin E [7, 8]. Whereas the quest for improved health with dietary supplements is commendable, there is a paucity of scientific data on some of the purported therapeutic efficacies of these dietary supplements.

Dietary supplements, including those of herbal origin, are known to alter the pharmacokinetics of concomitantly administered conventional drugs [9]. These supplements (or their constituents) often induce or inhibit drug-metabolizing enzymes such as cytochrome P450 (CYP), which play significant roles in phase I biotransformation reactions, converting lipophilic agents into hydrophilic metabolites and thereby facilitating excretion [10]. A typical example of a dietary supplement (herb) that modulates the activities of CYP enzymes is St. John's wort *(Hypericum perforatum)* [11, 12].

A number of dietary supplements currently available on the market have been reported to replenish levels of reduced glutathione (GSH), the most abundant naturally occurring antioxidant in the body [13]. Despite a scarcity of available scientific evidence, these GSH-enhancer dietary supplements are purported to play a potential role in the prevention of NCDs, especially those mediated by free radicals and characterized by depleted stores of tissue GSH [14]. One of such supplements, CellGevity, contains the GSH-precursor molecule, riboceine (D-ribose-L-cysteine), which has been reported to effectively deliver cysteine into the cell and enhance GSH level [15]. Riboceine has been shown to be significantly more effective than other glutathione enhancers [16], hence, the rationale for the choice of this dietary supplement in the present study.

In addition to riboceine, CellGevity contains an amalgam of constituents comprising turmeric root extract (curcumin), resveratrol, aloe extract, milk thistle, quercetin, broccoli seed extract, alpha lipoic acid, grape seed extract, vitamin C, selenomethionine, cordyceps, and piperine. Some of these constituents are known as inducers and/or inhibitors of CYP enzymes. Curcumin and resveratrol, for example, are potent inhibitors of CYP enzymes [17–19], while *aloe vera* induces CYP reductase and some Phase II enzymes [20].

Given the reported cases of induction and/or inhibition of CYP enzymes by some of its constituents and the potential supplement-drug interaction that may ensue, the present study investigates the effect of CellGevity dietary supplement on the activities of selected rat liver microsomal CYP enzymes and evaluates its total antioxidant potential.

2. Materials and Methods

Ethical Statement. All animal work was conducted according to the guidelines of the National Institute of Health for the Care of Laboratory Animals [21] and was approved by the Scientific and Technical Committee of Noguchi Memorial Institute for Medical Research, University of Ghana.

Experimental Animals. Prior to experiment, 20 male Sprague Dawley rats weighing 300 ± 50 g (≥8 weeks old) from the Animal Experimentation Unit, Center for Plant Medicine Research, Mampong-Akuapem, Ghana, were fed ad libitum using standard animal lab pellet (Sankofa Flour and Feeds, Accra, Ghana) and were housed in 4 groups of 5 animals per cage under standard laboratory conditions (25 ± 1°C ambient temperature, 60–70% relative humidity, and 12:12 h light : dark cycle) to acclimatize to the laboratory condition for 7 days.

Treatment Groups. Following acclimatization, rats were randomly assigned to one of the four experimental groups, being low dose supplement treatment (LDT; $n = 5$), high dose supplement treatment (HDT; $n = 5$), positive control (PC; $n = 5$), and negative control (NC; $n = 5$). The LDT group received a daily dose of 4 mg/kg of the supplement while the HDT group received 8 mg/kg. The PC group received a daily administration of 80 mg/kg of phenobarbitone, whereas the NC group was given distilled water daily. Each group received their respective treatment via oral route for 7 days. Following 7 days of treatment, animals were sacrificed by injection of an overdose of sodium pentobarbital intraperitoneally, and liver samples were harvested and snap-frozen in liquid nitrogen and stored at −80°C until further analysis.

2.1. Antioxidant Assays

2,2-Diphenyl-2-Picryl-Hydrazyl (DPPH) Assay. The DPPH method used was a modification of one reported by Blois [22]. Briefly, 20 mg of the supplement (CellGevity powder; Max International, Ghana) was dissolved in 1.0 ml of dimethyl sulfoxide (DMSO; Sigma Aldrich, USA) to obtain a stock solution of 20 mg/ml. Twofold serial dilutions of the stock were made to obtain concentrations of 10, 5, 2.5, 1.25, 0.625, and 0.3125 mg/ml. Twofold serial dilutions of the positive control, butylated hydroxyl toluene (BHT; St. Louis, MO, USA), were made to obtain concentrations of 0.5, 0.25, 0.125, 0.0625, 0.03125, and 0.015625 mg/ml. One hundred microliters of each of the samples and BHT dilutions was pipetted separately in triplicate into 96-well plates. A volume of 100 μL of 0.5 mM DPPH solution (Steinheim, Germany) was then added to each of the wells to obtain a total volume of 200 μL. The plates were incubated in the dark at room temperature for 20 minutes and absorbance was read at a wavelength of 517 nm.

Total Phenolic Assay. The assay used to estimate total phenols in the supplement was a modification of one reported by

Marinova et al. [23]. Briefly, a stock solution of the supplement was prepared by dissolving 20 mg of the sample in 1.0 ml of DMSO. Twofold serial dilutions of this stock were made to obtain concentrations of 10.0, 5.0, 2.5, and 1.25 mg/ml. The standard was prepared by dissolving 1.0 mg of gallic acid (generously provided by the Department of Nutrition and Food Science, University of Ghana) in 10% absolute ethanol. Twofold serial dilutions were made to obtain concentrations of 0.5, 0.25, 0.125, 0.0625, 0.03125, and 0.015625 mg/ml. One hundred microliters of each of the sample dilutions and the standard was pipetted separately in triplicate into 96-well plates. A volume of 100 μL of Folin-Ciocalteu reagent (Buchs, Switzerland) was then added to each well followed by 200 μL of sodium bicarbonate solution (0.2 g/ml) to obtain a total volume of 400 μL. The plates were incubated at room temperature for 120 minutes and absorbance read at a wavelength of 650 nm.

Total Flavonoid Assay. The total flavonoid assay used was one adapted from Ordoñez et al. [24]. Briefly, a stock solution of the supplement was prepared and diluted to obtain concentrations of 10.0, 5.0, 2.5, and 1.25 mg/ml. Quercetin standard (Buchs, Switzerland) was prepared and diluted to obtain concentrations of 0.1, 0.05, 0.025, 0.0125, 0.00625, 0.003125, and 0.0015625 mg/ml. One hundred microliters of each of the sample dilutions and the standard was pipetted separately in triplicate into 96-well plates. A volume of 100 μL of aluminum chloride solution (2% w/v; Sigma Aldrich, USA) was added to each of the wells to obtain a final volume of 200 μL per well. The plates were then incubated at room temperature for 20 minutes after which absorbance was read at a wavelength of 415 nm.

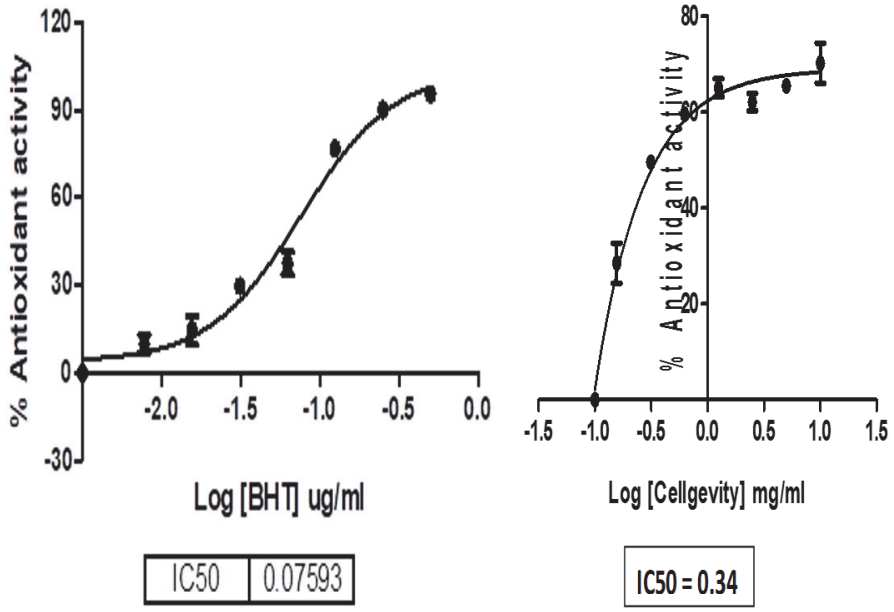
2.2. Rat Liver CYP Enzyme Induction/Inhibition Assays

Preparation of Microsomal Fractions and Protein Level Determination. Liver samples weighing 7.82 g were homogenized separately with two volumes of potassium phosphate buffer (pH 7.4) in a mortar with pestle. The homogenate was centrifuged at 4,500 rpm for 20 minutes at 4°C and the supernatant collected. Next, the supernatant was further centrifuged at 40,000 rpm for 60 minutes at 4°C with an ultra-centrifuge (Beckman Avanti J-25, USA). Following ultra-centrifugation, the resultant supernatant (cytosol) was separated from the pellet (microsomes). The microsomes obtained were then homogenized in potassium phosphate buffer (pH 7.4) to form a solution. Fourfold serial dilutions were carried out on the microsomal solutions using potassium phosphate buffer. Serial dilutions (2-fold, 6 dilutions) were also made with a protein standard, bovine serum albumin (BSA; St. Louis, MO, USA). Ten microliters of the BSA and 200 μL of Biorad reagent (Bio-Rad Laboratories Inc., USA) was added to each microsomal dilution in a 96-well plate and incubated at room temperature for 5 minutes, and absorbance was read at a wavelength of 590 nm.

CYP1A1/1A2-Ethoxyresorufin O-Deethylase (EROD), CYP1A2-Methoxyresorufin O-Demethylase (MROD), CYP3A4-BenzyloxyresorufinO-Debenzylase (BROD), and CYP2B1/2B2-Pentoxyresorufin O-Depentylase (PROD) Assays. Inhibition of CYP 1A1/1A2, 1A2, 3A4, and 2B1/2B2 enzymes by the supplement was determined using fluorimetric assays similar to ones described by Appiah-Opong et al. [17] and Umegaki et al. [25] but with slight modification. Briefly, 70 μL of potassium phosphate buffer (pH 7.4) was pipetted in triplicate into a 96-well plate followed by addition of 10 μL of each substrate (ethoxyresorufin, methoxyresorufin, benzyloxyresorufin, and pentoxyresorufin purchased from St. Louis, MO, USA). Next, 10 μL of the rat liver microsomal fraction obtained from each treatment group was added and incubated at 37°C for 5 min. Ten microliters (100 μM) of nicotinamide adenine dinucleotide phosphate (NADPH; St. Louis, MO, USA) was added to each of the wells and incubated at 37°C for 10, 20, and 30 min (for CYPs 1A1/1A2 and 1A2, 2B1/2B2 and 3A4, respectively). A volume of 40 μL of stopping solution (20% 0.5 M Tris and 80% acetonitrile) was added and the plate gently was shaken. Fluorescence was read at specific wavelengths at 586 nm.

CYP2D6-Dextromethorphan O-Demethylation Assay. The effect of the supplement on dextromethorphan O-demethylation by CYP2D6 was assayed as described by Appiah-Opong et al. [17]. Briefly, 350 μL of potassium phosphate buffer (pH 7.4) was pipetted into Eppendorf tubes in triplicate. Fifty microliters of 0.25 mM dextromethorphan (Milan, Italy) was added followed by 50 μL of microsomes obtained from each group. Preincubation was done at 37°C for 5 minutes in a water bath after which 50 μL of NADPH solution (100 μM) was added. Further incubation was done for 45 minutes, followed by the addition of 100 μL of stopping solution (300 mM zinc sulphate heptahydrate). The mixture was centrifuged at 4,000 rpm for 15 min at room temperature, and the supernatant was collected in vials and analyzed using an isocratic HPLC method with a C18 column (150 mm × 4.6 mm, VP-ODS). The mobile phase consisted of 24% (v/v) acetonitrile and 0.1% (v/v) trimethylamine adjusted to pH 3.0 with perchloric acid. The carrier flow rate was 0.8 ml/min and peaks were monitored at wavelengths of 280 nm (excitation) and 310 nm (emission).

CYP2C9-Diclofenac Hydroxylation Assay. The effect of the supplement on hydroxylation of diclofenac to 4-hydroxydiclofenac by CYP2C9 was determined as described by Appiah-Opong et al. [26]. Briefly, 350 μL of potassium phosphate buffer (pH 7.4) was pipetted into Eppendorf tubes followed by 50 μL of 0.05 mM diclofenac (Overrijse, Belgium). Next, 50 μL of the microsomal fraction obtained from each treatment group was added (in triplicate) and preincubated at 37°C for 5 minutes in a water bath. A volume of 50 μL of NADPH solution (100 μM) was added to each tube and further incubated in the water bath at 37°C for 10 minutes. The reaction was terminated by addition of 200 μL of stopping solution (ice-cold methanol) and the mixture was centrifuged at 12,000 rpm for 8 minutes at room temperature. The supernatants were collected in vials and analyzed using high-performance liquid chromatography (HPLC) [Agilent 1100 Series, Germany]. The HPLC conditions for the assay comprised an injection volume of 50 μL, a flow rate of 0.8 ml/min, a C18 column (150 mm × 4.6 mm, VP-ODS), a



(a) (b)

Figure 1: Concentration-response curves showing IC_{50} values for butylated hydroxytoluene (BHT; positive control (a)) and CellGevity dietary supplement (b).

temperature of 40°C, and a maximum pressure of 40 bar. A diode array served as the detector. Products formed were measured using an isocratic HPLC method. The mobile phase consisted of 60% of 20 mM potassium phosphate buffer (pH 7.4), 22.5% methanol, and 17.5% acetonitrile.

2.3. Statistical Analysis. All values are expressed as mean ± standard deviation (SD). Differences between groups were tested for significance using a One-Way ANOVA. p values < 0.05 were considered statistically significant. Significant differences were calculated with Bonferroni's Multiple Comparison Tests, and graphs were produced using Graph Pad Prism Software Version 7 (GraphPad Software, Inc., USA).

3. Results

3.1. Antioxidant Assays. To evaluate the antioxidant potential of CellGevity dietary supplement, DPPH, total phenolic, and flavonoid assays were used. The concentration of the supplement required to inhibit 50% of free radicals (IC_{50}) was 0.34 ± 0.07 mg/ml compared to 0.076 ± 0.03 mg/ml of the BHT (positive control). The total phenolic content of the supplement at a concentration of 2.5 mg/ml was 34.97 g gallic acid equivalent (GAE)/100 g while its total flavonoid content at a concentration of 2.5 mg/ml was 6 g quercetin equivalent (QE)/100 g. Figures 1(a) and 1(b) show the IC_{50} values of CellGevity as compared to BHT.

3.2. CYP Enzyme Assays. In order to determine the effect of CellGevity dietary supplement on rat liver microsomal cytochrome P450 (CYP) enzyme activities, selected CYP assays were used.

CYP1A1/1A2 and CYP1A2 Assays. There was no significant difference in the activity of CYP1A1/1A2 enzyme among NC, LDT, and HDT groups (Figure 2(a); $p > 0.05$). However, the CYP1A1/1A2 enzyme activity of these three groups markedly decreased compared to PC group (Figure 2(a); $p < 0.05$). A similar observation was made in CYP1A2 enzyme activity (Figure 2(b)).

CYP2B1/2B2 and CYP3A4 Assays. Unlike CYP1A1/1A2 and CYP1A2 enzyme activities, which showed no significant difference between NC, LDT, and HDT groups in the microsomal fractions, the activity of the CYP2B1/2B2 enzyme in LDT and HDT groups markedly decreased compared to NC group (Figure 2(c); $p < 0.001$). A similar pattern was observed in Figure 2(d). However, whereas the PC group showed markedly reduced CYP2B1/2B2 enzyme activity compared to NC group in Figure 2(c) ($p < 0.05$), that in CYP3A4 in Figure 2(d) showed no difference in comparison with the NC group ($p > 0.05$).

CYP2D6 and CYP2C9 Assays. CYP2D6 enzyme activity in LDT group increased significantly compared to HDT group (Figure 2(e); $p < 0.05$). In addition, NC group showed a markedly high CYP2D6 activity in comparison with PC group (Figure 2(e); $p < 0.001$). As seen in Figure 2(b), the activity of CYP2C9 followed a similar pattern in which no significant difference was observed between LDT and HDT groups while activity in NC group also markedly decreased (Figure 2(f); $p < 0.05$).

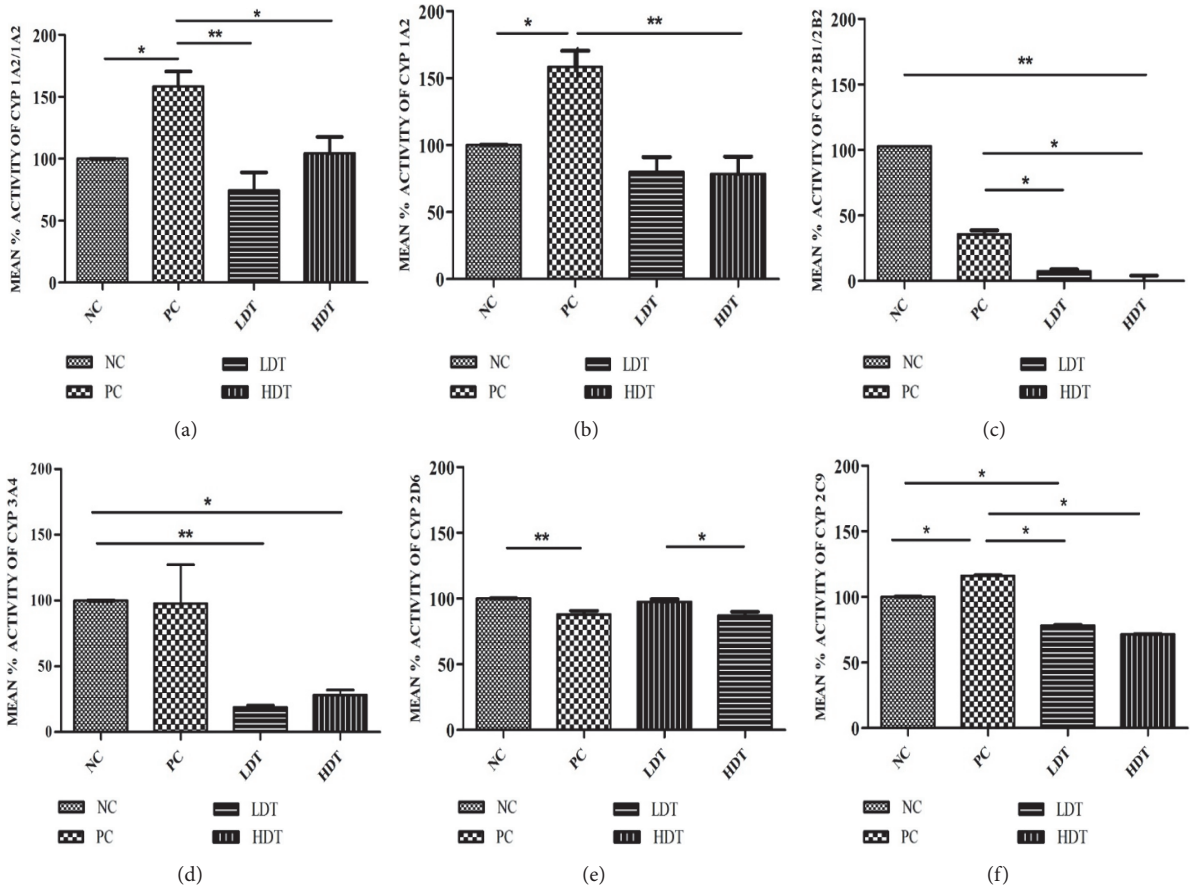


Figure 2: (a) *Effect of CellGevity dietary supplement on CYP1A1/1A2 activity in rat liver microsomes*. Negative control (NC; distilled water), positive control (PC; phenobarbitone 80 mg/kg), low dose treatment (LDT; 4 mg/kg supplement), and high dose treatment (HDT; 8 mg/kg supplement). Data represent mean ± standard deviations. ∗ and ∗∗ are values statistically different as indicated with $p < 0.05$ and $p < 0.001$, respectively. (b) *Effect of CellGevity dietary supplement on CYP1A2 activity in rat liver microsomes*. Negative control (NC; distilled water), positive control (PC; phenobarbitone 80 mg/kg), low dose treatment (LDT; 4 mg/kg supplement), and high dose treatment (HDT; 8 mg/kg supplement). Data represent mean ± standard deviations. ∗ and ∗∗ are values statistically different as indicated with $p < 0.05$ and $p < 0.001$, respectively. (c) *Effect of CellGevity dietary supplement on CYP2B1/2B2 activity in rat liver microsomes*. Negative control (NC; distilled water), positive control (PC; phenobarbitone 80 mg/kg), low dose treatment (LDT; 4 mg/kg supplement), and high dose treatment groups (HDT; 8 mg/kg supplement). Data represent mean ± standard deviations. ∗ and ∗∗ are values statistically different as indicated with $p < 0.05$ and $p < 0.001$, respectively. (d) *Effect of CellGevity dietary supplement on CYP3A4 activity in rat liver microsomes*. Negative control (NC; distilled water), positive control (PC; phenobarbitone 80 mg/kg), low dose treatment (LDT, 4 mg/kg supplement), and high dose treatment (HDT; 8 mg/kg supplement). Data represent mean ± standard deviations. ∗ and ∗∗ are values statistically different as indicated with $p < 0.05$ and $p < 0.001$, respectively. (e) *Effect of CellGevity dietary supplement on CYP2D6 activity in rat liver microsomes*. Negative control (NC; distilled water), positive control (PC; phenobarbitone 80 mg/kg), low dose treatment (LDT, 4 mg/kg supplement), and high dose treatment (HDT; 8 mg/kg supplement). Data represent mean ± standard deviations. ∗ and ∗∗ are values statistically different as indicated with $p < 0.05$ and $p < 0.001$, respectively. (f) *Effect of CellGevity dietary supplement on CYP2C9 in rat liver microsome*. Negative control (NC; distilled water), positive control (PC; phenobarbitone 80 mg/kg), low dose treatment (LDT, 4 mg/kg supplement), and high dose treatment (HDT; 8 mg/kg supplement). Charts represent mean ± standard deviations. ∗ are values statistically different as indicated with $p < 0.05$.

Overall Effect of the Supplement on Rat CYP Enzymes. The overall effect of this supplement on selected CYP enzymes is summarized in Table 1. Inhibition of CYP enzyme activity by the supplement was not dose-dependent. The general trend of enzyme inhibition (highest to lowest) by both doses of the supplement was CYP2B1 > CYP3A4 > CYP2C9 > CYP1A1/1A2 > CYP1A2 > CYP2D6.

4. Discussion

In the current study, we evaluated the antioxidant potential of CellGevity dietary supplement, comprising an aggregate of ingredients, and the effect of this supplement on the activities of selected rat liver microsomal enzymes. This study focuses on CYP enzymes (one of the conserved entities

Table 1: A summary of the effect of the supplement on rat CYP enzymes.

CYP isoform	Assay	Effect of supplement on CYP activity
CYP 1A1/1A2	EROD	No significant decrease in enzyme activity
CYP 1A2	MROD	No significant decrease in enzyme activity
CYP 2B1/2B2	PROD	Significant decrease in enzyme activity ($p < 0.001$; LDT and HDT)
CYP 2C9	Diclofenac hydroxylation	Significant decrease in enzyme activity ($p < 0.05$; LDT)
CYP 2D6	Dextromethorphan O-demethylation	No significant decrease in enzyme activity
CYP 3A4	BROD	Significant decrease in enzyme activity (LDT: $p < 0.001$; HDT: $p < 0.05$)

among species) which are the main enzymes involved in numerous oxidative reactions and often play a critical role in the metabolism and pharmacokinetics of xenobiotics. It is well established that some rat CYP enzymes are closely related to those of humans. For example, CYP1A shows a strong conservation among species with an identity to human > 80% in rat (83 and 80%, respectively, for CYP1A1 and -1A2) [27, 28].

Some constituents of CellGevity dietary supplement, such as curcumin, resveratrol, milk thistle, quercetin, and piperine, are known inhibitors of CYP3A4 [17–19, 29–31]. Hence, it is not surprising that this isoform was one of the enzymes significantly inhibited by the dietary supplement. CYP3A4 is one the most abundant CYP isoforms in human liver and is involved in the biotransformation of the majority of drugs [32]. However, some discrepancies between rats and human CYP3A4 isoforms, in the metabolism of drugs such as dihydropyridine calcium-channel blockers (e.g., nifedipine), have been reported, probably suggesting that rat is not a good model to study CYP3A4 induction [28, 33, 34]. Therefore, data from the current study, suggesting that CellGevity dietary supplement could alter the metabolism of some drugs that serve as human CYP3A4 substrates, should be interpreted cautiously.

Our study also showed that CellGevity dietary supplement significantly inhibited rat CYP2B1/2B2. Curcumin, one of the constituents of the supplement, is a less potent inhibitor of rat CYP2B1/2B2 compared to CYP1A1/1A2 enzyme [35]. This earlier report contradicts our finding, as we observed a significant inhibitory effect of the dietary supplement on CYP2B1/2B2 enzyme activity but not on the activities of CYP1A1/1A2 and CYP1A2 enzymes. As the dietary supplement has several constituents that affect CYP enzyme activity, it is possible that these refuting observations could be due to the synergistic inhibitory action of other constituents on CYP2B1/2B2 activity besides curcumin. It is important to note that the CYP2B subfamily is more abundant in rodents than in humans. In humans, however, the orthologous form of CYP2B1/2B2 is CYP2B6 [36]. Appiah-Opong et al. [17] reported that curcumin inhibited the human CYP2B6 enzyme, which is consistent with our observation in rats. This inhibitory effect on CYP2B activity suggests potential interaction when CellGevity dietary supplement is coadministered with drugs metabolized by this subfamily of CYP enzymes.

Another CYP enzyme, significantly inhibited by CellGevity dietary supplement, was CYP2C9. Although the effect of individual constituents of this dietary supplement on CYP enzyme activity was not investigated in our study, rodent and human microsome studies have shown that resveratrol, a constituent of this dietary supplement, is a potent inhibitor of CYP2C9 [37, 38]. A diet containing 0.5% w/w resveratrol fed to mice for 12 weeks was found to enhance the anticoagulant activity of warfarin, suggesting possible inhibition of CYP2C9 [37]. Using losartan as a probe drug, a daily dose of 1.0 g of resveratrol administered for 4 weeks was found to inhibit human CYP2C9 by 2.71-fold [38]. Previous reports also suggest that curcumin is a potent inhibitor of human recombinant CYP2C9 [17]. Additionally, two flavonolignans from milk thistle (another constituent of this dietary supplement) were found to inhibit human CYP2C9-mediated warfarin metabolism [39]. These pieces of evidence suggest that CellGevity dietary supplement could modulate human CYP2C9 enzyme activity.

In the current study, the EC_{50} value of CellGevity dietary supplement was 0.34 ± 0.07 mg/ml compared to 0.076 ± 0.03 mg/ml of the BHT. The total phenolic content of the supplement at a concentration of 2.5 mg/ml was 34.97 g gallic acid equivalent (GAE)/100 g while its total flavonoid content at a concentration of 2.5 mg/ml was 6 g quercetin equivalent (QE)/100 g. This antioxidant potential is moderately high compared to a related study where the authors reported a synergistic antioxidant activity of a green tea of herbal origin determined by an EC_{50} value of 33.5 mg/ml, a total phenolic, and flavonoid content of 2.5 g GAE/10 g and 1.2 g QE/10 g, respectively [40]. Furthermore, there have been studies including ours in which authors reported antioxidant potential of dietary supplements using *in vitro* assays [41]. However, a constituent of CellGevity dietary supplement, riboceine, is a prodrug which requires bioactivation *in vivo*. Once in circulation, riboceine is metabolized into cysteine and ribose, which are transported into cells [15]. It is noteworthy that cysteine is a substrate for GSH synthesis in the liver and other organs [42], suggesting that CellGevity

dietary supplement activates GSH pathway and possibly other endogenous antioxidants pathways, thereby bolstering the endogenous antioxidant defense system.

5. Conclusion

In conclusion, this study reports that CellGevity dietary supplement possesses antioxidant property *in vitro* and also inhibits activities of rat liver CYP2B1, CYP3A4, and CYP2C9 enzymes. Inhibition of these selected CYP enzymes by this dietary supplement suggests the possibility that CellGevity dietary supplement may contribute to supplement (herb-) drug interactions in humans.

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 declared that no conflicts of interest exist.

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Research Article

Which Benefits and Harms of Using Fenugreek as a Galactogogue Need to Be Discussed during Clinical Consultations? A Delphi Study among Breastfeeding Women, Gynecologists, Pediatricians, Family Physicians, Lactation Consultants, and Pharmacists

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Background. Breastfeeding women with hypogalactia are commonly recommended to use fenugreek as a galactogogue. This study aimed to achieve formal consensus among breastfeeding women and healthcare providers on which potential harms and benefits of using fenugreek need to be communicated and discussed during clinical consultations. *Methods*. A two-iterative round Delphi technique was used in two separate panels of breastfeeding women (n = 65) and healthcare providers (n = 56) to achieve formal consensus on a list of 24 and 16 items related to potential harms and benefits of fenugreek. *Results*. About 70% of the healthcare providers recommended quite often herbal remedies for breastfeeding women and about 68% of the women had been recommended to use herbal remedies many times by their healthcare providers. Consensus was achieved on 21 potential harms and 14 potential benefits of using fenugreek to enhance human milk supply that need to be discussed with breastfeeding women during consultations. *Conclusion*. Probably, potential harms and benefits of recommending fenugreek as herbal galactogogue for breastfeeding women seeking recommendations to increase their human milk supply need to be discussed during clinical consultations. Further observational studies are needed to assess what is being discussed in daily consultations when herbal remedies are recommended.

1. Introduction

Human milk has been recognized as the ideal form of enteral nutrition for term and preterm infants [1, 2]. Exclusive breastfeeding for the first six months of life has been shown to confer substantial benefits to both the mother and her infant [2]. Therefore, global health authorities recommend exclusive breastfeeding for all infants in the first six months of life which might then be continued alongside other solid foods as long as the mother and her infant desire [3]. According to recent estimates, only 37% of infants younger than six months of age are nourished exclusively on human milk in low and middle income countries [2]. In the US and Australia, about half of the infants were receiving human milk at all by 6 months and in the UK, only one-third were doing so [2]. These low figures cannot be explained merely by weak intentions to breastfeed because in the UK, more than 80% of women expressed their intention to breastfeed their infants [3, 4]. Certainly, some figures might be explained by insufficient human milk supply.

Insufficient breast milk supply was frequently reported as the main reason for discontinuing breastfeeding [5, 6]. Many women, particularly those who delivered preterm infants, suffer difficulties producing enough quantities of human milk. It is noteworthy mentioning here that even mothers of term infants under certain circumstances like maternal illness, cesarean delivery, excessive smoking, breast surgery, separation between mother and her infant, and psychosomatic illnesses might suffer insufficient human milk supply [3].

Nonpharmacological interventions remain the first line in managing insufficient human milk supply, although prescribing medications and recommending herbal galactogogues are common [7]. Women who discontinue breastfeeding as a result of insufficient human milk supply might be provided with adequate educational interventions regarding breastfeeding practices and/or might then be prescribed pharmacological agents to increase their human milk supply. Agents used to increase human milk supply are called galactogogues [5]. Metoclopramide and domperidone are the most commonly prescribed pharmacological galactogogues [5, 8, 9]. However, these agents have not received approval as safe and effective galactogogues from any health regulatory authority and currently are being used "off-label" [10, 11]. In addition, these agents are excreted in human milk and thus bear potential side effects and harms to infants [10–12]. Moreover, little guidance is available on the appropriate dosage of these agents when used as galactogogues [9, 13, 14]. Therefore, interventions to increase human milk supply using pharmacological agents might be complicated by safety concerns to women and their infants. Traditionally, herbal remedies have been viewed as good alternatives to prescription medications [15, 16].

In classical views, herbal remedies have been regarded as safe. Probably, this belief has emerged by advertising herbal remedies as mild, gentle, safe, and having unique attributes that are not found in prescription medications [15]. This myth was perpetuated by some healthcare providers when labeling herbal remedies as "natural" which are in turn mistakenly regarded as safe or in the worst case scenario, safer than prescription medications [17–19]. The myth that herbal remedies can never be harmful is perpetuated and commonly believed by many patients. However, this myth lacks scientific evidence. Herbal remedies contain chemicals that could resemble some active ingredients present in many prescription medications; thus, these chemicals would act by similar pharmacological mechanisms of action and have the ability to cause side effects and harm [15, 20]. It is noteworthy mentioning that herbal remedies are like prescription medications, have intended indications, are contraindicated in some cases, should be used with caution in some patients, and are associated with side effects [17, 18]. Therefore, herbal remedies should be recommended considering the 5 rights (right person, time, dose, frequency, and route of administration).

Herbal galactogogues have received considerable attention across different societies and cultures. Anecdotal reports of many herbal remedies supported their potential to enhance human milk supply. These herbal remedies include fenugreek, anise, caraway, fennel, milk thistle, and many others [16, 18, 21]. Fenugreek (*Trigonella arabica* Delile) which belongs to the pea family (Leguminosae) is the most widely used herbal galactogogue to enhance human milk supply in many countries [22]. Seeds of fenugreek, which is an annual herbaceous plant, are traditionally used as condiment and in folk medicine in many countries including the Indian subcontinent, China, and the Middle East [22]. A recent study in Kuwait showed that fenugreek was recommended for breastfeeding women with insufficient breast milk supply [23]. Anecdotal reports of the successful use of fenugreek as an herbal galactogogue have surfaced in 1940s. Little is known of the mechanism of action explaining how fenugreek might enhance milk supply. A theory suggested that fenugreek stimulate sweat production, and as the breast is a modified form of sweat gland, fenugreek might be able to stimulate the breast to supply an increasing amount of milk [21, 24]. There have been anecdotal reports of fenugreek increasing human milk supply in some 1200 breastfeeding women within 24–72 hours after consumption [24, 25]. Once the breast is stimulated, fenugreek consumption can be stopped as far the breast is stimulated and emptying continued. Fenugreek as galactogogue might be consumed in 2-3 capsules 3 times daily and each capsule might contain a variable quantity of fenugreek. At present, requirements for herbal products have not been standardized for consumption by patient [24]. It is noteworthy mentioning that the use of fenugreek is not free from side effects and has been associated with health related effects like excessive sweating, diarrhea, and worsening of asthma symptoms.

In modern healthcare delivery, patients are informed about the potential harms and benefits of therapeutic alternatives in order to develop their preferences. In general, making a decision on therapeutic alternatives involves balancing their potential benefits against their potential harms, taking into account the preferences of the patients. The benefits of informing patients are multifold, including better experienced quality of life, coping with side effects, and prevention of overestimation of the impact of therapy on cure [15]. Therefore, healthcare providers like gynecologists/obstetricians, pediatricians, lactation consultants, family physicians, and pharmacists who are often consulted by breastfeeding women seeking recommendations to enhance their human milk supply should discuss herbal galactogogues balancing their potential benefits again potential harms in case they wanted to opt for herbal remedies considering the preferences of the women concerned. Little was narrated on the potential harms and benefits of using fenugreek to enhance human milk supply in breastfeeding women that should be discussed during clinical consultations from the viewpoints of breastfeeding women, gynecologists/obstetricians, pediatricians, family physicians, lactation consultants, and pharmacists who are often consulted by breastfeeding women seeking recommendations to enhance their human milk supply. In general, recommendations on which potential harms and benefits of using fenugreek to communicate to and discuss with patients during clinical consultations are lacking. The aim of this study was to fill this gap in the literature.

The aims of this study was to achieve consensus among breastfeeding women, gynecologists/obstetricians, pediatricians, family physicians, lactation consultants, and pharmacists who are often consulted by breastfeeding women seeking recommendations to enhance their human milk supply on which potential harms and benefits of using fenugreek as a galactogogue that need to be communicated to and discussed with breastfeeding women during clinical consultations in which a decision to use fenugreek would be taken. This consensual core list of potential harms and benefits might promote congruence in daily healthcare delivery.

2. Materials and Methods

2.1. Gathering Information on Herbal Galactogogues Recommended in Clinical Practice. We contacted and interviewed 10 key contact healthcare providers who were often consulted by breastfeeding women seeking recommendations to use herbal galactogogues to enhance their human milk supply. We also interviewed 5 women who previously have sought recommendations and used herbal galactogogues to enhance their human milk supply.

The key contact healthcare providers were asked to provide their consent to include their initials and details as experts who were interviewed in this study. Participants were given the option to remain anonymous upon their desire. Key contacts provided their age, gender, academic degrees, specialty, number of years in practice, approximate number of breastfeeding women cared for on a monthly basis, herbal galactogogues they often recommend, the potential harms, and benefits of herbal galactogogues that need to be communicated to and discussed with breastfeeding women during the clinical consultations.

The key contact women were asked to provide their consent to include their initials and details as experts who were interviewed in this study. Women were also given the option to remain anonymous upon their desire. Women were asked to provide their age, academic degrees, employment status, and the potential harms and benefits of galactogogues that need to be communicated to and discussed with breastfeeding women during the clinical consultations. The detailed sociodemographic and practice details of the interviewees are provided as Supplementary Materials (Table S1).

Healthcare providers and women narrated their experience with herbal galactogogues in terms of benefits and harms. Herbal galactogogues mentioned by the interviewees are listed in Supplementary Materials (Table S2). All interviewees (healthcare providers and women) mentioned fenugreek as one of the most frequently recommended herbal galactogogues. As all interviewees mentioned fenugreek as a galactogogue, we decided to gather all potential harms and benefits of this herbal galactogogue that need to be communicated to and discussed with breastfeeding women during the clinical consultations between breastfeeding women and their caring healthcare providers in which fenugreek is to be recommended. All potential harms and benefits mentioned by the interviewees were collected. An extensive literature review was then conducted to gather other potential harms and benefits of using fenugreek that could be found in other studies [4, 6, 12, 13, 17, 18, 21, 22, 24–46]. All potential harms and benefits found in the previous studies were noted. Potential harms and benefits collected were rephrased into statements. We discarded all potential harms and benefits related to costs, convenience, and inconvenience. Statements were piloted for clarity and comprehensibility with 5 medical students and 5 lay persons. Some statements were revised based on the feedback of the pilot and all statements were compiled into a questionnaire.

2.2. The Consensual Technique. In this study, we used the Delphi technique as a tool to achieve formal consensus among panelists on which potential harms and benefits of using fenugreek by breastfeeding women to enhance their human milk supply should be communicated to and discussed with breastfeeding women during the clinical consultations between breastfeeding women and their caring healthcare providers. Recently, this formal consensus technique has evolved as one of the most frequently employed techniques in achieving consensus on issues lacking consensus in healthcare [15, 47–49]. This technique has many advantages over other techniques like round table meeting, focus, and nominal groups. The advantages of this technique include guarding the anonymity of the participants, ability to recruit panelists from different locations, convenience, saving the costs of bringing the panelists to a round table meeting, and immunity against individual domination of the discussion and influencing opinions of other panelists. The Delphi technique combines both quantitative as well as qualitative methods in which a multiround questionnaire system is completed in two or more iterative stages, known as rounds, over a period of time within one or more panels until consensus is achieved [50]. The panelists are often requested to express the level of their disagreement or agreement with a list containing items in a questionnaire. Consensus is defined a priori and items on which consensus was not reached in one round are included in a revised questionnaire for a subsequent round and the process is continued until reaching a conclusion that consensus on the remaining items is no longer likely to be achieved [15, 47–49]. Sharing statistical summaries and comments with the panelists in a trial to decrease the number of rounds needed to reach consensus on the items included is commonly practiced.

As the views and opinions of women and healthcare providers could be different from each other, we sought consensus in two separate panels [15]. A panel included healthcare providers who are often consulted by breastfeeding women seeking recommendations to increase their human milk supply and the other panel was composed of women who sought recommendations and used herbal galactogogues to enhance their human milk supply.

2.3. Panel of Healthcare Providers. A judgmental sampling technique was used to recruit panelists who were healthcare providers that were often consulted by breastfeeding women seeking recommendations to increase their human milk supply. Potential panelists were identified by personal contacts in the field. As breastfeeding women seeking recommendations

to increase their human milk supply often consult gynecologists/obstetricians, lactation consultant nurses, pediatricians, family medicine specialists, and pharmacists, we aimed to recruit panelists with these specialties. Because the Delphi technique implies that the panelists have to be rich with experience and information to narrate, it is well-established that selection and recruitment of the panel members are among the most captious steps in the Delphi technique [15]. In the current study, panelists were approached and invited to participate as panel members based on their qualifications, specialty, and experience in the field of recommending herbal galactogogues for breastfeeding women seeking recommendations to enhance their human milk supply. Field researchers approached in person and invited the potential panelists to participate as panel members in the current study. Field researchers explained the design and objectives of the study to potential panelists and obtained their verbal consent before participation. The inclusion criteria were (1) having a basic or advanced qualification in a healthcare specialty related to being consulted by breastfeeding women seeking recommendations to enhance their human milk supply, (2) having a license to practice in Palestine, (3) having 5 or more years of practicing experience in a healthcare establishment attended by breastfeeding women seeking recommendations to enhance their human milk supply, and this was important as possessing previous knowledge of the subject being researched is a critical prerequisite for a panelist to take part in the Delphi technique [15], (4) consultation with 5 or more breastfeeding women on a monthly basis, (5) knowledge of the use of herbal galactogogues in enhancing human milk supply. In this study, 56 panelists were recruited and participated in the panel of healthcare providers. The panelists were not offered any financial incentives.

2.4. Panel of Women. In this study, snowball sampling was used to identify and recruit women who sought recommendations and used herbal galactogogues to enhance their milk supply. Potential panelists were identified using personal contacts in the field. Potential panelists were approached by field researchers in person and invited them to participate in this study. The field researchers explained the design and objectives of the study to the potential panelists and obtained their verbal consent before they were recruited to the panel. Women were invited and recruited to the panel when they met the inclusion criteria of (1) having breastfed at least one infant, (2) having been recommended at least once to use herbal galactogogues to enhance their human milk supply, (3) using one or more herbal galactogogue to enhance human milk production, and (4) willingness to take part in the current study. In this study, 65 women were recruited to the panel. Again, participants were not offered any financial incentives.

2.5. The Iterative Delphi Technique Rounds

2.5.1. Delphi Round 01. In the first Delphi round, the questionnaire was given by hand to all 56 healthcare providers and 65 women. The questionnaire consisted of 2 sections. In the 1st section, the panelists were requested to disclose their sociodemographic details. The healthcare professionals provided their gender, age, academic qualifications, number of years in practice, specialty, how often they recommended herbal galactogogues for breastfeeding women in their clinical practice, and how often they communicated and discussed harms and benefits of herbal galactogogues that breastfeeding women might be consuming during clinical consultations. Female healthcare professionals were also requested to provide if they have breastfed before, and the number of infants they breastfed. Women were requested to provide their age, educational level, employment status, number of infants they breastfed, how often they have been recommended by their healthcare providers to use herbal remedies to enhance their human milk supply, and if they liked to have enough discussion with their healthcare providers on the potential harms and benefits of using herbal remedies during breastfeeding. The 2nd section of the questionnaire contained a list of 24 and 16 items related to potential harms and benefits, respectively, of using fenugreek as a herbal galactogogue to enhance human milk supply and the panelists were requested to express the degree to which they disagree or agree that each presented item needs to be communicated to and discussed with breastfeeding women during consultations on a Likert scale of 9 points [15, 47–49]. When the panelists scored 1–3, this indicated that they disagree with the importance of communicating and discussing the presented potential harm or benefit during the clinical consultation; that is, they are of the opinion that the presented potential harm or benefit should not be communicated to and discussed with breastfeeding women during the consultations. When the panelists scored 7–9, this indicated that they agree with the importance of communicating and discussing the presented potential harm or benefit to breastfeeding women during the clinical consultation; that is, they are of the opinion that the proposed potential harm or benefit should be communicated to and discussed with breastfeeding women during the consultation. When the panelists scored 4–6, this indicated that the panelists partially agreed with the importance of communicating and discussing the presented potential harm or benefit during the clinical consultation; that is, the panelists are inconclusive either the presented potential harm or benefit should be communicated to and discussed with breastfeeding women or not during the consultations. In this study, the panel members were requested and encouraged to add written comments to justify and/or qualify their scores on the Likert scale as in previous studies [15, 47–49].

2.5.2. Definition of Consensus and Analysis of the Scores. Scores were analyzed using an Excel Sheet (Microsoft Excel 2013). The first quartile (Q1), median (Q2), third quartile (Q3), and the interquartile range (IQR) were computed for each item. Scores of both panels were analyzed separately. The data were analyzed using the same definitions of consensus used in previous studies [15, 47–49]. Briefly, the item included the list of important harms or benefits that need to be communicated to and discussed with breastfeeding women during the consultation when the median score fell between 7 and 9 and the interquartile range (IQR) fell between 1 and 2 and the item was excluded from the list of important harms

or benefits that need to be communicated to and discussed with breastfeeding women during the consultation when the median score fell between 1 and 3 and the IQR fell between 1 and 2. However, the item was considered equivocal when the median score fell between 4 and 6 or the IQR was larger than 2. Equivocal items were included in a revised questionnaire for a subsequent Delphi round. In this study, consensus was based on at least 80% of the scores of the panelists in each panel separately.

2.5.3. Delphi Round 02. A revised questionnaire containing all equivocal items was subjected to a second Delphi round. In a trial to reduce the number of Delphi rounds needed to reach consensus, we provided the panelists with (1) the median score and the IQR for each potential harm or benefit, (2) reminder of their own scores in the previous Delphi round, and (3) summary of the comments made by the panelists either to justify or qualify their scores.

Scores in this round were computed and analyzed according to the same definitions used in the previous Delphi round. After analyzing the scores and comments obtained in the second Delphi round, we came to a conclusion that it was unlikely that consensus would be achieved if we would conduct further Delphi rounds.

2.6. Ethical Considerations. This study received ethical approval from the Institutional Review Board (IRB) committee of An-Najah National University. We obtained verbal consent from all panelists before they participated in the current study. All views, opinions, and scores of the panelists weighed equally in the analysis.

3. Results

3.1. Response Rate. Questionnaires were completed by 56 healthcare providers who are often consulted by breastfeeding women and 65 women who breastfed before in the first Delphi round; therefore, the response rate was 100%. However, in the second Delphi round, 48 (85.7%) of the healthcare providers and 40 (61.5%) of the women completed and returned the questionnaire.

3.2. Characteristics of the Panelists Who Took Part in the Study

3.2.1. The Panel of Healthcare Providers. In this study, the panelists who were healthcare providers were of different age groups, belonged to both genders, had variable number of years in practice, had different academic qualifications, and had various specialties. More than half of the panelists were male in gender, physicians, and 40 years, and older. About 56% of the panelists were either gynecologists/obstetricians, pediatricians, or family medicine specialists. About 59% of the panelists were in practice for 10 or more years. The detailed characteristics of the panelists are shown in Table 1.

3.2.2. The Panel of Women. The women who took part as panelists in this study were of different age groups and had different educational levels and employment status. The majority of the women (about 85%) had a university degree and were 25 years and older. About 43% of the women breastfed 3 or more infants. The detailed variables of the women panelists who participated in this study are shown in Table 2.

Table 1: Sociodemographic and practice details of the healthcare providers who are often consulted by breastfeeding women ($n = 56$).

Variable	n	%
Gender		
Male	30	53.6
Female	26	46.4
Age (years)		
<40	30	53.6
≥40	26	46.4
Have you breastfed before[a]		
Yes	19	73.1[b]
No	7	26.9[b]
Number of infants breastfed[a]		
0	7	26.9[b]
1	4	15.4[b]
2	3	11.5[b]
≥3	12	46.2[b]
Academic qualifications		
B.S.	21	37.5
M.S.	5	8.9
M.D.	28	50.0
Ph.D.	2	3.6
Specialty		
Gynecology/obstetrics	10	17.9
Pediatrics	5	8.9
Family medicine	16	28.6
Lactation consultant nurse	13	23.2
Pharmacist	12	21.4
Number of years in practice		
5–9	23	41.1
≥10	33	58.9
How often do you recommend herbal galactagogues for breastfeeding women?		
Quite often	39	69.6
Sometimes	17	30.4
How often do you discuss herbal galactagogues that breastfeeding women could be using during your consultations with them?		
Quite often	32	57.1
Sometimes	24	42.9

[a]The question was for healthcare providers who were female in gender.
[b]Percentages were based on the number of female panelists; B.S.: Bachelor of Science, M.S.: Master of Science, M.D.: Doctor of Medicine, and Ph.D.: Doctor of Philosophy.

3.3. Use of Fenugreek for Enhancing Human Milk Supply. About 70% of the healthcare provider panelists stated that

Table 2: Sociodemographic details of the women who participated in this study ($n = 65$).

Variable	n	%
Age (years)		
<25	10	15.4
≥25	55	84.6
Educational level		
School	16	24.6
Bachelor's degree	37	56.9
Master's degree	12	18.5
Employment status		
Employed	39	60.0
Unemployed	26	40.0
Number of infants breastfed		
1	22	33.8
2	15	23.1
≥3	28	43.1
How often have you been recommended by your healthcare provider to use herbal remedies for enhancing your human milk supply?		
Many times	44	67.7
Once or a few times	21	32.3
Do you like to have enough discussion with your healthcare provider on the potential harms and benefits of using herbal remedies?		
Always	43	66.2
Sometimes	22	33.8

they recommended quite often herbal remedies for breastfeeding women. About 68% of the women had been recommended many times by their healthcare providers to use herbal remedies for enhancing their human milk supply.

About 57% of the panelists discussed quite often herbal remedies that breastfeeding women could be using during their consultations with them. About 66% of the women stated that they would always like to have enough discussion with their healthcare providers on the potential harms and benefits of using herbal remedies for enhancing their human milk supply.

3.4. Potential Harms of Using Fenugreek to Enhance Human Milk Supply That Need to Be Communicated to and Discussed with Breastfeeding Women during the Clinical Consultation. In this study, consensus was achieved in both panels on 21 potential harms of using fenugreek to enhance human milk supply that need to be communicated to and discussed with breastfeeding women during the consultation. The detailed list of these items is shown in Table 3.

In general, there was consensus on 6 potential harms related to the anticoagulant effects of fenugreek, 3 potential harms related to the increased risk of abortion associated with using fenugreek, 4 potential harms related to comorbidities, 3 potential harms related to the effects of fenugreek on the blood pressure, 2 potential harms related to the effects of fenugreek on the blood glucose level, and 3 other potential harms related to the side effects of fenugreek.

3.5. Potential Benefits of Using Fenugreek to Enhance Human Milk Supply That Need to Be Communicated to and Discussed with Breastfeeding Women during the Consultation. In this study, consensus was achieved in both panels on 14 potential benefits of using fenugreek to enhance human milk supply that need to be communicated to and discussed with breastfeeding women during the consultation. A detailed list of these potential benefits is shown in Table 4.

In general, there was consensus on the potential benefits of fenugreek related to enhancing human milk supply and fertility. Consensus was also achieved to communicate and discuss other potential benefits of fenugreek related to its antioxidant, chemoprotective, immunomodulatory, antidepressant, and anti-infective properties with breastfeeding women.

3.6. Potential Harms and Benefits of Using Fenugreek to Enhance Human Milk Supply That Need or Need Not to Be Communicated to and Discussed with Breastfeeding Women during the Consultation Depending on the Individual Clinical Situation's Need. Consensus was not achieved on 3 potential harms and 2 potential benefits of using fenugreek to enhance human milk supply. These equivocal items are listed in Table 5. Whether to communicate and discuss these items during a clinical consultation was left to the choice of the healthcare provider and depending on the individual's needs.

4. Discussion

In the present study, we developed a consensual core list of important potential harms and benefits of using fenugreek as herbal galactogogue that should be communicated to and discussed with breastfeeding women seeking recommendations to increase their human milk supply from their caring healthcare providers in daily practice in two separate panels of women and healthcare providers. To the best of our knowledge, this consensual core list is the first attempt to develop guidance for healthcare providers to consult when recommending fenugreek-based herbal remedies to promote human milk supply in breastfeeding women seeking recommendations to enhance their human milk supply.

When gold standards are not existent, consensual techniques might provide alternative methods to reduce bias, enhance transparency, and validity of judgmental methods when developing certain criteria [15]. We believe that this consensual core list should appeal to healthcare providers and might be consulted to guide communicating and discussing potential harms and benefits of using fenugreek to promote human milk supply in breastfeeding women seeking recommendations to enhance their milk supply. Judgmental sampling was used to recruit panelists for the panel of healthcare providers and snowball sampling was used to recruit panelists for the panel of women. These nonprobability sampling techniques have long been regarded as biased [51]. However, for this study design and objectives, probability randomized

Table 3: Potential harms of using fenugreek to enhance human milk supply that need to be communicated to and discussed with breastfeeding women during the clinical consultation.

Item #	Potential harms	Round on which consensus was achieved	
		Panel of healthcare providers	Panel of women
	Fenugreek has anticoagulant effects		
1	Breastfeeding women who have a history of any clotting related disorder need to be warned not to take fenugreek	2	1
2	Breastfeeding women who have a history of vaginal bleeding disorder need to be warned not to take fenugreek	1	1
3	Breastfeeding women who are at risk of any bleeding disorder need to be warned not to take fenugreek	1	1
4	Breastfeeding women need to be warned that fenugreek might be associated with menstrual breakthrough bleeding	2	1
5	Breastfeeding women who are on anticoagulants need to be warned not to take fenugreek	2	1
6	Breastfeeding women who are on non-steroidal anti-inflammatory drugs (NSAIDs) need to be warned not to take fenugreek	2	1
	Fenugreek might be associated with abortion		
7	Women planning to become pregnant need to be warned that fenugreek is a potential utero-stimulant and might cause spontaneous abortion	2	2
8	Women with a history of previous miscarriage need to be warned not to take fenugreek	1	1
9	Women planning to become pregnant need to be warned that fenugreek might impair fetal development	1	1
	Risks associated with using fenugreek on other co-morbidities		
10	Breastfeeding women need to be warned that fenugreek might cause nausea and vomiting	2	2
11	Breastfeeding women need to be warned that fenugreek might cause diarrhea in the mother and her breastfed infant	2	1
12	Breastfeeding women with a history of asthma need to be warned that fenugreek might worsen the symptoms of their asthma	1	1
13	Breastfeeding women need to be warned that fenugreek might cause dehydration	1	1
	Fenugreek could be associated with hypotension		
14	Breastfeeding women with a history of or at risk of hypotension need to be warned not to take fenugreek	1	1
15	Breastfeeding women with a history of or at risk of dizziness need to be warned not to take fenugreek	2	1
16	Breastfeeding women who are on anti-hypertensive medications need to be warned not to take fenugreek	1	1
	Fenugreek could be associated with hypoglycemia		
17	Breastfeeding women with a history of or at risk of hypoglycemia need to be warned not to take fenugreek	2	1
18	Diabetic breastfeeding women whose disease is controlled by medications or insulin need to be warned not to take fenugreek	1	1
	Other adverse effects		
19	Breastfeeding women need to be warned that fenugreek might cause fever	2	1
20	Breastfeeding women need to be warned that fenugreek might cause excessive sweating	2	2
21	Breastfeeding women taking diuretics, laxatives, mineralocorticoids, and/or other hypokalemic agents need to be warned that fenugreek may worsen hypokalemia	2	1

Table 4: Potential benefits of using fenugreek to enhance human milk supply that need to be communicated to and discussed with breastfeeding women during the clinical consultation.

Item #	Potential benefits	Round on which consensus was achieved	
		Panel of healthcare providers	Panel of women
1	Breastfeeding women might be informed that fenugreek can be beneficial in enhancing their human milk production	1	1
2	Breastfeeding women might be informed that fenugreek might improve their fertility	2	2
3	Breastfeeding women might be informed that fenugreek has antioxidant properties	2	2
4	Breastfeeding women might be informed that fenugreek has estrogenic effects	2	1
5	Breastfeeding women might be informed that fenugreek has immunomodulatory effect	1	1
6	Breastfeeding women might be informed that fenugreek has chemo-protective effect against breast cancer	1	1
7	Breastfeeding women might be informed that fenugreek may decrease plasma cholesterol and triglycerides levels	1	1
8	Breastfeeding women might be informed that fenugreek may have antidepressant activity	2	1
9	Breastfeeding women might be informed that fenugreek may have antibacterial activity	1	1
10	Breastfeeding women might be informed that fenugreek may have antifungal activity	1	1
11	Breastfeeding women might be informed that fenugreek could decrease their appetite, especially those with a history of eating disorders	2	1
12	Breastfeeding women might be informed that fenugreek can enhance weight loss	2	1
13	Breastfeeding women might be informed that fenugreek might have antipyretic activity	2	1
14	Breastfeeding women might be informed that fenugreek may alleviate symptoms of ulcer	2	1

Table 5: Potential harms and benefits of using fenugreek to enhance human milk supply that need or need not to be communicated to and discussed with breastfeeding women during the consultation depending on the individual clinical situation's need.

Item #		Panel of healthcare providers				Panel of women			
		Round 1		Round 2		Round 1		Round 2	
		M	IQR	M	IQR	M	IQR	M	IQR
	Potential harms								
1	Breastfeeding women need to be warned that fenugreek may induce thirst	5	2	5	3	6	2	5	3
2	Breastfeeding women need to be warned that fenugreek may be associated with maple syrup like urine	4	3	5	2	5	2	6	3
3	Breastfeeding women need to be warned that fenugreek may be associated with maple syrup like sweat	5	2	4	3	4	3	4	2
	Potential benefits								
1	Breastfeeding women might be informed that fenugreek may have antiparkinsonian activity	4	4	5	3	6	2	6	3
2	Breastfeeding women might be informed that fenugreek may improve memory and cognition	4	2	4	3	5	3	5	3

M: median, IQR: interquartile range.

sampling techniques were not feasible. Moreover, judgmental and snowball sampling techniques permitted the recruitment of panelists with prior knowledge of the subject being investigated who were rich in experience to narrate [15, 47–49]. The panel of healthcare providers was composed of gynecologists/obstetricians, pediatricians, family physicians, lactation consultants, and pharmacists. Those healthcare professionals would normally be consulted by breastfeeding women seeking recommendations to increase their human milk supply [17, 52]. Women who were recruited for the panel of women experienced inadequate human milk supply sought recommendations from healthcare providers and used herbal galactogogues.

The number of panelists in the panel of healthcare providers and panel of women was slightly larger than those used in previous studies in which consensus was sought on issues in healthcare [15, 47–49]. Currently, there is no consensus on the number of panelists in a panel of experts. Panel sizes varied greatly in previous studies and the sizes ranged from 10 over 1000 panel members [51].

In this study, a consensual core list of potential harms and benefits of using fenugreek as herbal galactogogue was developed to guide healthcare providers on what harms and benefits to discuss and/or address during the clinical consultation when opting to recommend fenugreek for breastfeeding women seeking recommendations to increase their human milk supply. Guidelines on what healthcare providers should communicate and discuss in terms of potential harms and benefits are currently lacking. We believe this consensual core list should help healthcare providers and change their behaviors during consultations with breastfeeding women seeking recommendations to increase their human milk supply. It has been argued that professionals would change behavior in response to recommendations they agree with rather than recommendations they do not agree with [15, 47–49].

The use of herbal remedies was reported to be high among women in Palestine [31, 53]. In this study, about 68% of the women reported that they were recommended to use herbal galactogogues many times. Similarly, about 70% of the healthcare providers reported that they recommend quiet often herbal galactogogues for breastfeeding women seeking recommendations to increase their human milk supply. Our findings were consistent with those previously reported by Bazzano et al. in the US, in which 70% of the healthcare providers surveyed indicated that they often recommend galactogogues [52]. Similarly, fenugreek was the most frequently recommended herbal galactogogue in Bazzano's study. In this study, about 68% of the women reported that they always wanted to have enough discussion with their caring healthcare providers on the potential harms and benefits of herbal remedies. Findings of this study were consistent with those reported in a previous study in which 76% of pregnant women stated that they would like to have enough discussion on the benefits and harms of ginger when recommended to alleviate symptoms of nausea and vomiting of pregnancy [15]. In this study, inclusion women who experienced human milk insufficiency and used herbal galactogogues in the panel of women ensured inclusion of the insecurities and concerns breastfeeding women would like their caring healthcare providers to address during clinical consultations. Interestingly, about 57% of the healthcare providers reported that they quite often address potential harms and benefits of herbal remedies during consultations with breastfeeding women.

In this study, the response rate was high in both Delphi rounds. This was consistent with other studies seeking consensus on issues in healthcare using the Delphi technique [15, 47–49]. This strength adds to the validity of the findings reported in this study. The panel of healthcare providers included panelists of both genders, different age groups, geographical locations, practice settings, specialties, and number of years in practice (Table 1). The panel of women included panelists from different geographical locations, age groups, number of breastfed infants, educational levels, and employment status (Table 2). This diversity adds to the strength and validity of the findings reported in this study.

In this study, consensus was achieved on potential harms related to the anticoagulant potential of fenugreek that need to be discussed and/or addressed during the clinical consultation (Table 3). These findings were consistent with those reported in another study in which consensus was achieved among healthcare professionals on addressing the potential harms and benefits of using ginger to manage nausea and vomiting of pregnancy, especially harms related to the anticoagulant potential of ginger [15]. Not surprisingly, patients were previously reported to want to hear more from their healthcare providers on the best ways to make out of the therapies they are taking [54, 55]. The anticoagulant effects of fenugreek were previously reported. A recent study showed that aqueous extract of fenugreek inhibited blood coagulation process *in vitro* and increased prothrombin time in a dose dependent manner in blood samples obtained from healthy individuals [41]. Drug-herb interaction between fenugreek and warfarin was also reported [26]. Professional groups like the American Society of Anesthesiologists have advised patients to stop consuming herbal therapies 2-3 weeks prior to surgery as a safety precaution to avoid risks of bleeding [15, 32]. Findings of this study suggested that both healthcare providers and women wanted the risks of bleeding associated with the use of fenugreek by breastfeeding women to communicate and discuss during the consultation in which fenugreek is recommended to be used. Informed breastfeeding women could be in a better position to decide whether to use fenugreek or opt for another safer alternative.

In this study, the panelists were of the opinion that the risks of abortion associated with using fenugreek should be communicated to and discussed with breastfeeding women during the consultations. Again, these findings were consistent with those reported in a previous study in which pregnant women and gynecologists agreed that the risks of abortion associated with using ginger for nausea and vomiting of pregnancy should be addressed during clinical consultations [15]. Previous studies showed that aqueous extract of fenugreek had potential teratogenic effects in humans and animals [33, 39]. Health regulatory bodies tend to recommend avoidance of herbal remedies even when the risks associated with their use are inconclusive. As a

good example here, the German E Commission and the Finnish Food Safety Authority recommended that pregnant women should avoid ginger even though the risks of abortion associated with using ginger by pregnant women were largely inconclusive [56]. There could be cases in which breastfeeding women could become pregnant. The panelists in this study were of the opinion to warn women of these potential risks during the clinical consultations. Conservative views imply that women should be warned even when the potential risks are still inconclusive [38, 42].

The use of fenugreek could worsen the symptoms of some comorbidities. For example, fenugreek could worsen the symptoms of asthma [38, 42]. It has been recommended that individuals with chronic asthma and allergy should avoid consumption of fenugreek [28, 38]. Therefore, in this study, the panelists were of the opinion that this risk should be communicated to and discussed with breastfeeding women during consultations. Many breastfeeding women could be asthmatics and should be warned of this potential harm of using fenugreek. Again, breastfeeding women should be warned that fenugreek could cause nausea and vomiting which could be disturbing to the breastfeeding women and could have negative effects on their reported quality of life [39]. Fenugreek could be associated with diarrhea and excessive sweating for the breastfeeding women and their breastfed infants [34]. Severe diarrhea and excessive sweating could result in huge fluid loss that might lead to dehydration as well as serious consequences on the health of infants. These risks should be communicated to and discussed with breastfeeding during the consultations.

The findings of this study suggested that the risks associated with the effects of fenugreek on the blood pressure, blood glucose, and potassium levels should be communicated to and discussed with breastfeeding women during the consultations [29, 30, 37, 40]. Some breastfeeding women could be at risk of hypotension or hypoglycemia and should be warned against these risks when using fenugreek. The blood pressure and blood glucose levels of some breastfeeding women might be controlled by medications. Using fenugreek might have negative consequences of these controlled levels and hence, breastfeeding women at risk should be warned. Similarly, some breastfeeding women could be taking diuretics, laxatives, mineralocorticoids, or other hypokalemic agents. The panelists in this study were of the opinion that breastfeeding women should be warned that fenugreek might worsen their hypokalemia.

The panelists in this study agreed that benefits related to enhancing human milk supply should be communicated to and discussed with breastfeeding women during the consultations [22]. Enhancing human milk supply would be the primary anticipated effect of using fenugreek as a galactogogue. The panelists were of the opinion of informing the breastfeeding women recommended to use fenugreek of its antioxidant, estrogenic, and immunomodulatory properties [35, 43]. Chemoprotective effects against breast cancer and antidepressant effects of fenugreek might also be communicated to and discussed with breastfeeding women [27, 35, 43]. Many breastfeeding women might be concerned with breast cancer and postpartum depression and could be interested in learning about these potential benefits of fenugreek. Breastfeeding women might also be informed of the antibacterial, antifungal, and antipyretic effects of fenugreek [42]. Fenugreek might also be beneficial in controlling appetite, promoting weight loss, alleviate ulcer, and decreasing cholesterol and triglycerides levels. Many breastfeeding women could have gained weight during pregnancy and might be interested in decreasing their weight. Fenugreek might offer some help toward this end.

The opinions of the panelists were divisive on the importance of communicating and discussing potential effects of fenugreek in inducing thirst, marble like urine and sweat. Similarly, the opinions of the panelists were divisive whether to communicate to and discuss with breastfeeding women potential benefits of fenugreek related to enhancing cognition, memory, and its antiparkinsonian effects [36, 43]. These potential harms and benefits might be or might not be discussed depending on the needs of each individual case.

In general, care should be taken when breastfeeding women are recommended treatments as many medications and herbal remedies are excreted into the human milk. Therefore, both breastfeeding women and their breastfed infants could be vulnerable. In all cases, potential benefits should be weighed against potential risks considering other available safe alternatives. Similar measures should be applied when fenugreek-based herbal remedies are intended to be recommended as galactogogues for breastfeeding women seeking recommendations to enhance their human milk supply.

The findings of this study could be interpreted considering a number of limitations. First, this was an observational consensual study. Observing healthcare provider's recommendations of fenugreek in daily clinical practice and why it was recommended for breastfeeding women could have shown other findings. Second, in this study, we did not classify potential harms and benefits into major harms and minor harms. However, this classification goes beyond the scope and objectives of this study. Third, we did not hierarchize the potential harms and benefits in order of importance. The hierarchy would have helped healthcare providers to prioritize the information to be communicated and discussed in case they did not have enough time to go over all potential benefits and harms. Fourth, judgmental and snowball sampling techniques were used to recruit panelists for this study. These nonprobability sampling techniques are viewed as biased in conservative views. However, these techniques are commonly used for this type of studies as probability sampling techniques are not practically feasible. Finally, the number of panelists who participated in each panel was relatively small. However, there is no consensus on the number of panelists required for a Delphi technique. The number of panelists used in this study was slightly larger than sizes used in previous studies seeking consensus on issues in healthcare.

5. Conclusion

Panelists in this study were of the opinion that potential harms and benefits of recommending the use of fenugreek

as herbal galactogogue for breastfeeding women seeking recommendations to increase their human milk supply need to be discussed during the clinical consultations. This could be important in promoting congruence in daily healthcare delivery, improving patient's experience with therapy, coping with side effects of the therapy, and enhancing patient reported quality of life. In this study, consensus was achieved on a core list of potential harms and benefits of using fenugreek as herbal galactogogue in breastfeeding women seeking recommendations to enhance their human milk supply that need to be communicated to and discussed with breastfeeding women during the consultations in which fenugreek-based herbal remedies are to be recommended. This consensual list might be consulted as guidance by healthcare providers who are often consulted by breastfeeding women seeking recommendations to enhance their human milk supply. Further randomized clinical trials are still required to establish evidence-based benefits and harms of fenugreek in breastfeeding women. More observational studies are needed to assess what is being communicated and discussed in daily consultations when herbal remedies are recommended.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Supplementary Materials

Supplementary Table S1 provides the sociodemographic and practice details of the key contacts who were interviewed in this study ($n = 15$). Supplementary Table S2 provides details of the plants cited by the key contacts who were interviewed in this study ($n = 15$). *(Supplementary Materials)*

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Research Article

Glehnia littoralis Root Extract Inhibits Fat Accumulation in 3T3-L1 Cells and High-Fat Diet-Induced Obese Mice by Downregulating Adipogenic Gene Expression

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Glehnia littoralis has been reported to have several pharmacological properties but no reports describing the antiadipogenic effect of this plant have been published. This study was conducted to investigate the effects of *Glehnia littoralis* root hot water extract (GLE) and its underlying mechanism on 3T3-L1 cell adipogenesis and in high-fat diet- (HFD-) induced obese mice. We measured intracellular lipid accumulation using oil red O staining *in vitro*. For *in vivo* study, twenty-eight C57BL/6J male mice were randomly divided into four groups, Control, HFD, HFD + 1% GLE, and HFD + 5% GLE, which was performed for eight weeks. We determined the expression levels of the adipogenesis-related proteins by RT-PCR and western blotting in HFD-induced obese mice. The GLE dose-dependently inhibited 3T3-L1 adipocyte differentiation and intracellular lipid accumulation in differentiated adipocytes. Further, body weight gain and fat accumulation were significantly lower in the GLE-treated HFD mice than in the untreated HFD mice. GLE treatment suppressed the expression of adipogenic genes such as peroxisome proliferator-activated receptor (PPAR) γ, CCAAT/enhancer-binding protein (C/EBP) α, fatty acid synthase (aP2), and fatty acid synthase (FAS). These results suggest that the GLE inhibits adipocyte differentiation and intracellular lipid accumulation by downregulating the adipogenic gene expression both *in vitro* and *in vivo*.

1. Introduction

The prevalence of obesity has increased dramatically worldwide owing to lifestyle and diet changes and is rapidly becoming a threat to human health. Obesity has recently attracted increasing attention owing to its association with several metabolic diseases including type II diabetes, cardiovascular disease, and hypertension [1].

Obesity is caused by excess adipose tissue mass, which is the major energy reserve in the body [2]. As the adipose tissue mass can be modulated by inhibiting adipogenesis (differentiation of preadipocytes to mature adipocytes) [3], obesity treatments are usually targeted at suppressing energy or food intake, preadipocyte differentiation and proliferation, and lipogenesis, while increasing energy expenditure, lipolysis, and fat oxidation [4]. However, no effective treatment options are currently available for obesity. Therefore, plant-based bioactive materials are being isolated and their pharmacological properties are being actively researched [5, 6]. Several studies suggest that phytochemical treatments can regulate adipose tissue mass by inhibiting adipogenesis [3, 7, 8].

Glehnia littoralis Fr. Schmidt ex Miq. (Umbelliferae) is a perennial herb that grows on the sandy beaches of eastern China, Korea, Japan, and North-west America [9]. Its roots and rhizomes, which are listed in the Korean, Chinese,

and Japanese Pharmacopoeias [10], have traditionally been used for their diaphoretic, antipyretic, antiphlogistic, and analgesic properties. Further, the aqueous extract of *G. littoralis* has been reported to have several pharmacological properties including antioxidant [11], anticancer [12, 13], anti-inflammatory [10], and some immunomodulatory properties [14, 15]. The major components of the underground parts of *G. littoralis* have been identified as quercetin, isoquercetin, rutin, chlorogenic acid, and caffeic acid [11].

To date, no reports describing the antiadipogenic effect of this plant have been published. High-fat diet- (HFD-) induced animal models of obesity and 3T3-L1 cells have been widely used for studying the antiobesity properties of various compounds [16]. Therefore, this study was conducted to elucidate the effects of the *Glehnia littoralis* root extract (GLE) on the adipogenic differentiation of 3T3-L1 cells by measuring intracellular lipid accumulation. We also investigated the mechanism underlying the inhibitory effects of GLE on adipocyte differentiation in HFD-induced obese mice to determine the potential medicinal benefits of *G. littoralis* as an antiobesity agent.

2. Materials and Methods

2.1. Preparation of Glehnia littoralis Root Extract (GLE). *G. littoralis* roots obtained from Fine Food Tech Co., Ltd. (Gongju, Korea), were air-dried at 50°C at an air velocity of 1.5 m/s for 4 days, blended, and further ground, to obtain a fine powder. The powder (300 g) was soaked in 3 L of distilled water and then heated at 100°C for 4 h. The crude extract was collected, filtered with a sterilized cloth, freeze-dried at −60°C, and stored in a deep freezer (−70°C) until use.

2.2. Determination of the Polyphenol Components of GLE by High-Performance Liquid Chromatography (HPLC). The HPLC analysis was performed on a Dionex Summit™ system (Thermo Scientific, Waltham, MA, USA) equipped with an UVD 340U-photodiode array detector (Dionex, Sunnyvale, CA, USA) using a reverse-phase C18 analytical column (4.6 × 250 mm i.d., 5 μm, Shiseido Capcell Pak MG). The mobile phase was solvent A (methanol, acetic acid, and water at 10 : 2 : 88 v/v/v) and solvent B (methanol, acetic acid, and water at 90 : 3 : 7 v/v/v). The analysis was performed under the following gradient conditions: 100% A to 0% B (0–30 min), 100% B (30–40 min), 100% B to 0% A (40–42 min), and 100% A (42–60 min), with a flow rate of 1 mL/min and a detection wavelength of 280 nm with 1 nm bandwidth. All standards were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Cell Culture and Differentiation. Murine 3T3-L1 preadipocytes were obtained from the Korean Cell Bank (Seoul, Korea) and cultured to confluence in Dulbecco's modified Eagle's medium (DMEM, Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) and 1% penicillin-streptomycin (Gibco, Rockville, MD, USA) in a humidified 5% CO_2 atmosphere at 37°C. On day 2 after confluence (designated as day 0), cell differentiation was induced with the MDI differentiation medium containing 1 μM dexamethasone (DEX, Sigma-Aldrich, St. Louis, MO, USA), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich, St. Louis, MO, USA), 10 μg/mL insulin (INS, Sigma-Aldrich, St. Louis, MO, USA), and DMEM supplemented with 10% FBS. After 48 h (day 2), the culture medium was replaced with DMEM supplemented with 10% FBS, and this was repeated every 48 h until day 8. The cells were treated with different concentrations of the GLE (0, 50, 100, 200, and 400 μg/mL) from day 0 to 8, and untreated cells were used as a control.

2.4. Determination of Cell Viability. The effect of different concentrations of the GLE on 3T3-L1 preadipocyte viability was determined by the cell counting kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Tokyo, Japan). Briefly, the cells were seeded in a 96-well plate at a density of 1×10^4 cells/well and treated with the GLE (0–400 μg/mL) for 24 h. 10 μL of CCK-8 reagent was then added to each well and the absorbance was measured at 450 nm using an Infinite® F50 microplate reader (Tecan, Männedorf, Switzerland). The viability of the GLE-treated cells was expressed as a percentage of the control cell viability.

2.5. Oil Red O Staining and Estimation of the Intracellular Lipid Content. The lipid accumulation in adipocytes, which indicates the extent of differentiation, was measured using oil red O staining. Briefly, differentiated 3T3-L1 cells were fixed in 10% formaldehyde in PBS for 1 h, washed with distilled water, and dried completely. The cells were then stained with 0.5% oil red O solution in 60 : 40 (v/v) isopropanol : triple distilled water for 15 min at room temperature, washed four times with triple distilled water, and dried. The treated cells were observed under an Olympus microscope (BX51, Tokyo, Japan), and representative images were captured using an Olympus DP70 camera. The cell differentiation was quantified by elution of the stain with isopropanol and measurement of the absorbance at 520 nm.

2.6. Animals and Diets. C57BL/6J male mice (6- to 8-week-old) were purchased from Samtako Bio Korea Co., Ltd., (Osan, Korea) and initially acclimated to laboratory conditions for 1 week, prior to experimental use. After acclimatization, 28 mice were randomly divided into four groups, namely, the American Institute of Nutrition- (AIN-) 93G diet (control, C), high-fat diet (HFD), HFD with 1% GLE (HFD + 1% GLE), and HFD with 5% GLE (HFD + 5% GLE) groups. The HFD contained 45.5% fat (as soybean oil and lard), 20% protein, and 34.5 % carbohydrate (Table 1).

The mice were housed under a 12 : 12 h light-dark cycle at 22 ± 2°C and 55 ± 5% relative humidity with ad libitum access to the specified diets and sterile drinking water for 8 weeks. The food intake and body weight were measured every week, and the feed efficiency ratio (FER) was calculated as the total weight gain/total food intake. All experiments on animals were carried out in accordance with the institutional guidelines of the Hankyong National University, Anseong, Korea. This study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication number 85-23, revised 1996, latest revision in 2011), and was approved by the

Table 1: Composition of experimental diets.

Ingredient	HFD	HFD + 1% GLE	HFD + 5% GLE
Casein	23.31	23.31	23.31
Sucrose	20.14	20.14	20.14
Dextrose	11.65	11.65	11.65
Corn starch	8.48	7.48	3.48
Cellulose	5.83	5.83	5.83
Soybean oil	2.91	2.91	2.91
Lard	20.69	20.69	20.69
Mineral mix[(1)]	5.24	5.24	5.24
Vitamin mix[(1)]	1.17	1.17	1.17
L-Cysteine	0.35	0.35	0.35
Choline bitartrate	0.23	0.23	0.23
GLE[(2)]		1.00	5.00

HFD: high-fat diet, HFD + 1% GLE: HFD containing 1% *Glehnia littoralis* root extract (GLE); HFD + 5% GLE: HFD containing 5% GLE. [(1)] Mineral and vitamin mixtures were based on the AIN-93 standard diet for rodents. [(2)] *Glehnia littoralis* root extract powder.

Table 2: List of primers used in RT-PCR analysis.

Gene	Forward primer	Reverse primer
PPARγ	GATGGAAGACCACTCGCATT	AACCATTGGGTCAGCTCTTG
C/EBPα	TGGACAAGAACAGCAACGAG	TCACTGGTCAACTCCAGCAC
SREBP-1c	GCTGTTGGCATCCTGCTATC	TAGCTGGAAGTGACGGTGGT
aP-2	TCAGCGTAAATGGGGATTTGG	GTCTGCGGTGATTTCATCGGA
FAS	CCCTTGATGAAGAGGGATCA	ACTCCACAGGTGGGAACAAG
Leptin	TGAGTTTGTCCAAGATGGACC	GCCATCCAGGCT CTCTGG
β-Actin	CAC CCC AGC CAT GTA CGT	GTCCAGACGCAGGATGGC

Hankyong National University Animal Welfare Committee (Hankyong. 2015-2).

At the end of the experimental period, the animals were fasted overnight and administered mild ether anesthesia, and blood was collected via puncture of the retroorbital sinus in ethylenediaminetetraacetic acid- (EDTA-) coated vials. The animals were then euthanized by cervical dislocation under mild ether anesthesia and the abdominal, perirenal, and epididymal fat pads were excised. The fat samples were rinsed with saline and stored at −70°C until further analysis.

2.7. RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA was isolated from the epididymal fat samples of the experimental mice using the RNAiso Plus reagent (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of the total RNA in a 20 μL reaction volume using a Maxime RT PreMix kit (iNtRON Biotechnology, Seongnam, Korea) containing the OptiScript™ reverse transcriptase and i-StarTaq™ DNA polymerase, following the manufacturer's recommended protocol. The oligonucleotide primers are shown in Table 2. The PCR conditions consisted of an initial denaturation step at 95°C for 5 min, followed by 30 amplification cycles consisting of denaturation for 40 s at 95°C, annealing for 40 s (temperature 56–62°C), and extension for 1 min at 72°C. The PCR products were separated on an agarose gel (1.5%) by electrophoresis for 30 min at 100 V. The bands were visualized, and their relative intensities were analyzed using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.8. Western Blot Analysis. Proteins were extracted from the epididymal fat samples using a protein extraction kit (iNtRON Biotechnology, Seongnam, Korea). The lysates were centrifuged at 15,000 rpm for 15 min at 4°C, and the protein content of the supernatant was determined by Bio-Rad™ assay kit (Hercules, CA, USA). Diluted protein samples (30 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10%) and transferred to nitrocellulose membranes. The membranes were blocked overnight with 5% skim milk in Tris-buffered saline-Tween 20 (TBST, 20 mM Tris-HCl, pH 7.6, 140 mM NaCl, and 0.1% Tween 20) and incubated with the following primary antibodies (1 : 1000 dilution): PPARγ, C/EBPα, SREBP-1c, aP2, leptin, FAS, and β-actin (Abcam, Cambridge, UK). The membranes were then washed four times with TBST buffer and incubated with the corresponding horseradish-peroxidase- (HRP-) conjugated secondary antibody (1 : 2000 dilution). The immunoreactive protein bands were visualized using an enhanced chemiluminescence plus

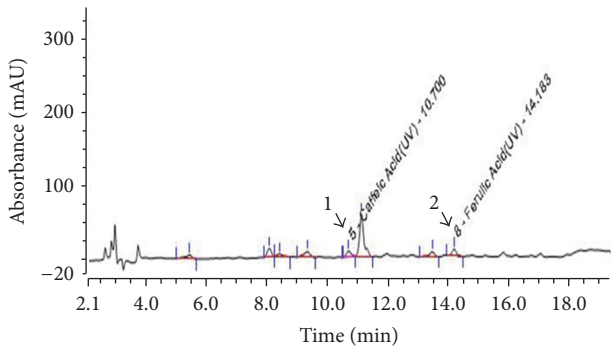

Figure 1: *HPLC profile and chemical structures of the polyphenol components of the Glehnia littoralis root extract (GLE)*. Caffeic acid (peak 1) and ferulic acid (peak 2).

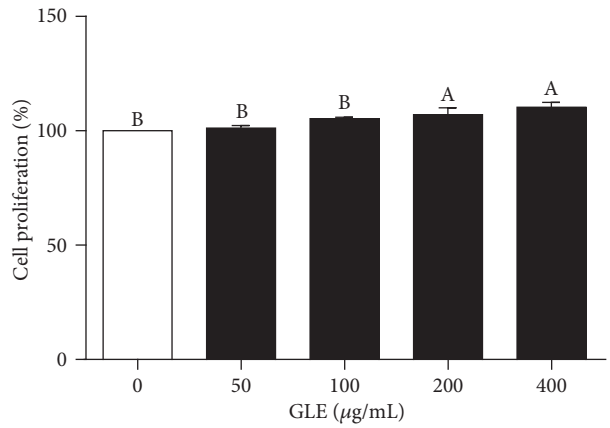

Figure 2: *Effect of the GLE on 3T3-L1 cell proliferation*. 3T3-L1 preadipocytes were cultured in serum-free medium with GLE (0–400 μg/mL) for 24 h. Posttreatment cell viability was determined by cell counting kit- (CCK-) 8 assay. Values are expressed as mean ± SD ($n = 3$). Viability of untreated controls is set to 100%. Means with different superscript letters are significantly different by Duncan's multiple range test ($p < 0.05$). GLE, *Glehnia littoralis* root extract.

kit (Amersham Pharmacia Biotech, Buckinghamshire, UK), and their relative intensities were quantified using the ImageJ 1.41 software.

2.9. Statistical Analysis. The results are expressed as the mean ± standard deviation (SD) of at least three independent experiments. Statistical differences between the groups were evaluated by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Values of $p < 0.05$ were considered statistically significant. The statistical analysis system (SAS) software package version 9.2 (SAS Institute Inc., Cary, NC, USA) was used for the analysis.

3. Results and Discussion

3.1. Determination of Active Components of GLE. When the composition of the GLE was investigated by comparing its HPLC profile with that of nine standard compounds including cnidilide, ligustilide, neocnidilide, butylphthalide, senkyunolide, tetramethylpyrazine, caffeic acid, ferulic acid, and perlolyrine eluted under the same conditions, two compounds, namely, caffeic acid and ferulic acid, were identified as the active constituents of the GLE (Figure 1).

3.2. Effect of the GLE on 3T3-L1 Cell Proliferation. The cytotoxicity of the GLE was evaluated prior to the investigation of its antiadipogenic effects on 3T3-L1 cells. Treatment with different concentrations (50–400 μg/mL) of the GLE for 24 h stimulated the proliferation of 3T3-L1 cells with no cytotoxicity observed following the treatment with 400 μg/mL of the GLE for 24 h (Figure 2).

3.3. Effect of the GLE on 3T3-L1 Preadipocytes Differentiation. We evaluated the effect of the GLE on postconfluent 3T3-L1 preadipocytes that were induced to differentiate in MDI differentiation medium for 2 days. Oil red O staining was used to monitor the changes in lipid accumulation during preadipocyte differentiation. Representative images of the oil red O-stained, GLE-treated cells acquired on day 8 of the differentiation period showed a dose-dependent suppression of intracellular lipid accumulation (Figures 3(a) and 3(b)). The lipid content decreased by 31 and 52% in response to 200 and 400 μg/mL of the GLE, respectively. Adipogenesis, the stage of the cell differentiation process during which preadipocytes mature into adipocytes, is accompanied by lipid accumulation as well as changes in gene expression and hormone sensitivity [17]. These results show the inhibitory effect of the GLE on adipocyte differentiation.

3.4. Effect of the GLE in HFD-Induced Obese Mice. We further elucidated the antiadipogenic effects of the GLE by performing an *in vivo* experiment with HFD-induced obese mice. As shown in Figure 4(a), the body weights of mice in the HFD and HFD + 1% GLE groups were significantly higher than those of mice in the control and HFD + 5% GLE groups after 6 weeks of treatment ($p < 0.05$). At the end of the experiment, mice in the HFD + 5% GLE group exhibited a drastic reduction in body weight gain compared to that reported for the HFD group mice (8.2 ± 3.4 versus 17.3 ± 2.6 g). However, the antiadipogenic effect in the HFD + 1% GLE group was not as pronounced as that in the HFD + 5% GLE group. The feed efficiency ratio (FER) of the HFD + 5% GLE group was significantly lower than that of the HFD and HFD + 1% GLE groups (Figure 4(b)) ($p < 0.05$). The fat weight, which comprises the abdominal, perirenal, and epididymal fat pad weights, of mice in the HFD + 5% GLE group (8.2 ± 0.3 g) was approximately 50% lower than that of mice in the HFD (16.3 ± 0.3 g) and HFD + 1% GLE (15.8 ± 0.2 g) groups. The fat weight per 100 g body weight of mice in the HFD + 5% GLE group (27.7 ± 1.0 g) was significantly lower than that of mice in the HFD (42.7 ± 0.8 g) and HFD + 1% GLE (40.3 ± 0.5 g) groups (Figure 4(c)) ($p < 0.05$).

It is well-known that an imbalance between energy intake and energy expenditure leads to body fat storage owing to increased lipogenesis and adipogenesis [18]. However, this study showed that supplementing the diet with 5% GLE effectively inhibited the body fat accumulation in HFD-induced obese mice compared with that in the untreated HFD

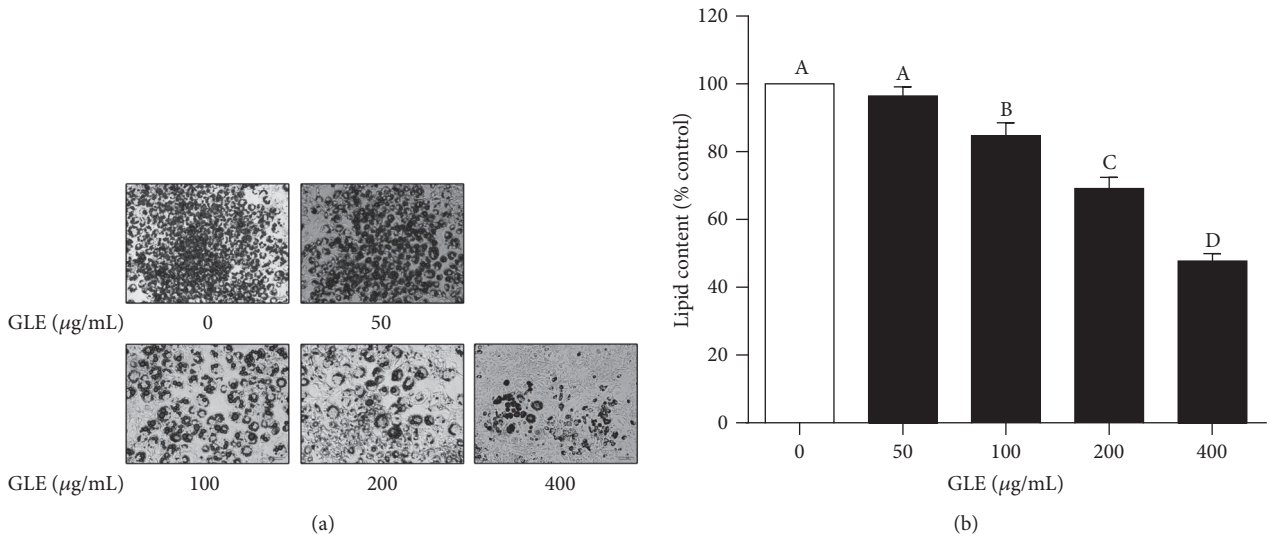

Figure 3: *Effect of the GLE on 3T3-L1 adipocyte differentiation.* (a) Oil red O staining showing the differentiation of induced 3T3-L1 preadipocytes. Black color indicates stained cytoplasmic lipids. (b) Quantification of lipid accumulation in differentiated 3T3-L1 cells based on the absorbance at 520 nm of destained oil red O extracted from the adipocytes. Lipid content in untreated control cells is set to 100%. Values are expressed as mean ± SD ($n = 3$). Means with different superscript letters are significantly different by Duncan's multiple range test ($p < 0.05$). GLE, *Glehnia littoralis* root extract.

group. Therefore, the GLE could be useful for treating obesity by reducing body fat accumulation.

3.5. Effects of the GLE on Critical Adipogenic Gene and Protein Expression in HFD-Induced Obese Mice. In order to investigate the molecular mechanisms underlying the antiadipogenic effect of the GLE in HFD-induced obese mice, we analyzed the gene and protein expression of various transcription factors associated with preadipocyte differentiation and fat accumulation via RT-PCR and western blotting, respectively. The GLE treatment markedly decreased the expression of adipogenic markers such as PPARγ, C/EBPα, and SREBP-1c and lipid metabolism genes such as aP2, leptin, and FAS (Figure 5). The mRNA levels of PPARγ, C/EBPα, and SREBP-1c in the GLE-treated groups were significantly lower than those in the HFD group ($p < 0.05$), with the levels in the HFD + 5% GLE group being reduced by 59.5, 118.3, and 41.3%, respectively, compared to those in the HFD group (Figures 5(b)–5(d)).

Preadipocyte differentiation is regulated by transcriptional activators including members of the C/EBP and PPARγ families [19–21]. Currently, C/EBPα and PPARγ are considered the primary mediators of adipogenesis. These transcription factors have been shown to activate adipocyte-specific genes and are also involved in the growth arrest required for preadipocyte differentiation [22]. The complex process of adipogenesis commences with PPARγ production, which is controlled and activated by C/EBPα and SREBP-1c [17]. C/EBPα also activates the promoters of the adipocyte genes leptin and aP2 [23], while both PPARγ and C/EBPα coordinate the expression of genes involved in generating and maintaining aP2 and leptin levels. The expression of aP2 and FAS mRNA in the HFD group was 134.1 ± 4.6% and 192.4 ± 4.6%, while that in the 5% GLE-treated group was 89.7 ± 3.9% and 80.7 ± 2.5%, respectively, compared to the expression in the control group (100%) (Figures 5(e) and 5(g)). The mRNA expression of leptin, which serves as a major adipostat by suppressing the urge to eat and promoting energy expenditure [24], decreased by 19 and 107.7% in a dose-dependent manner compared with that in the HFD group, following the treatment with 1 and 5% GLE, respectively (Figure 5(f)). Interestingly, the 5% GLE treatment significantly decreased the expression of aP2, leptin, and FAS mRNA, compared to the expression in the control group ($p < 0.05$). In particular, the leptin mRNA expression in the HFD + 5% GLE group decreased by 44.6 ± 2.7%. The GLE treatment also suppressed the expression of SREBP-1c and FAS. SREBP-1c accelerates adipogenesis by inducing the expression of FAS. Leptin, which is one of the best-known hormone markers for obesity, was also downregulated following the ingestion of an HFD with 5% GLE. These findings also indicate that GLE might contain FAS or leptin inhibitors and present its efficiency against fat accumulation through this pathway in addition to adipogenesis inhibition. It has been reported that caffeic acid phenethyl ester suppresses the production of leptin during differentiation of 3T3-L1 preadipocytes [25]. Therefore, one of components of GLE such as caffeic acid may be responsible inhibitor for both FAS and leptin.

PPARγ and C/EBPα are major regulators of the preadipocyte differentiation process, and C/EBPα mediates the transactivation of leptin transcription [26]. C/EBPα, which is expressed rather late in the adipogenesis process, has been widely reported to be both necessary and sufficient for the differentiation of 3T3-L1 preadipocytes to adipocytes [23, 27, 28] and appears to promote the differentiation in conjunction with PPARγ by cross-regulation [29]. SREBP-1c

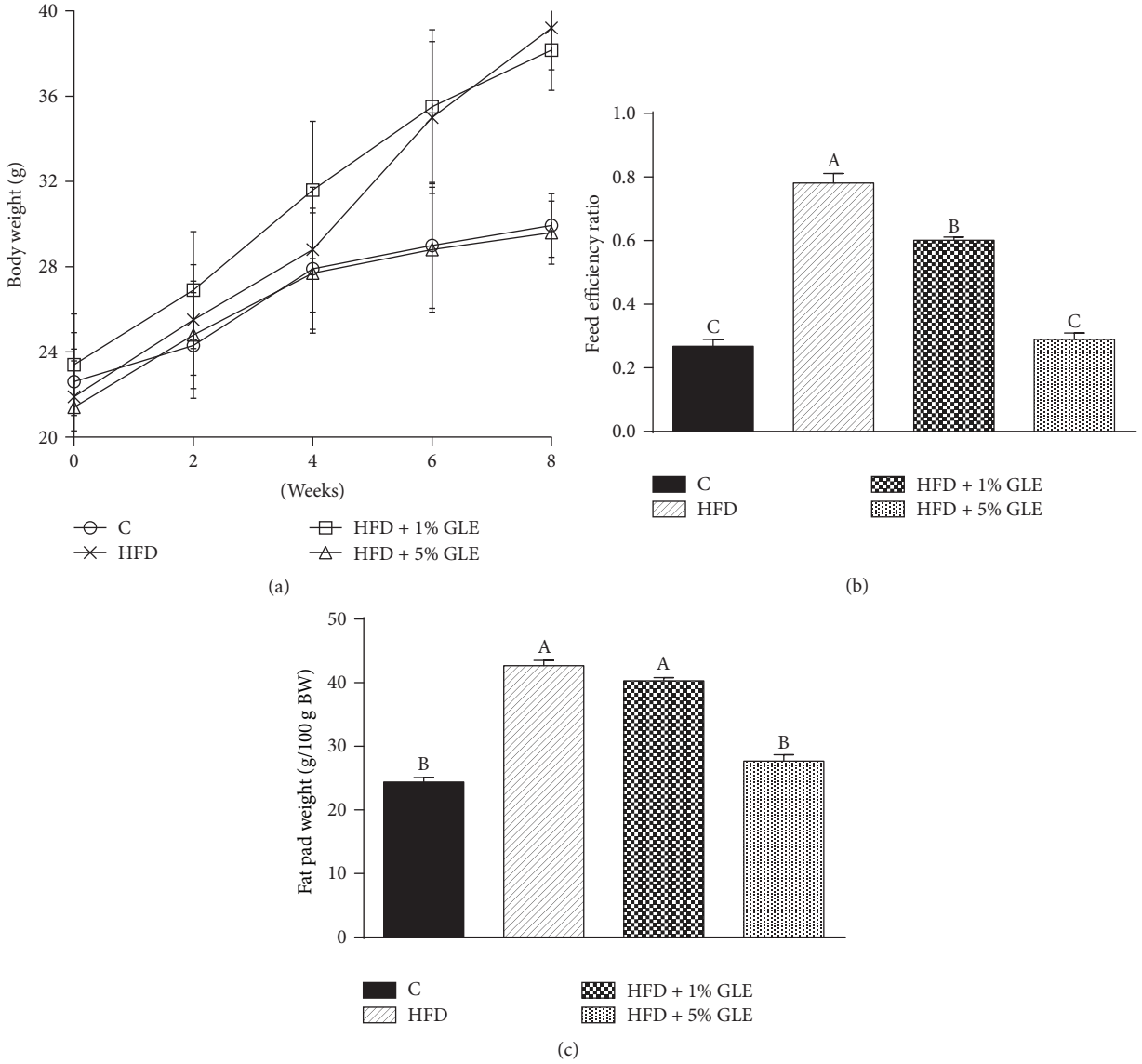

Figure 4: *Effect of the GLE on the growth of and fat accumulation in HFD-induced obese mice*. (a) Body weight of the mice that were fed experimental diets. (b) Feed efficiency ratio (FER) calculated as the total weight gain/total food intake. (c) Fat weight per 100 g body weight. Fat weight includes the abdominal, renal, and epididymal fat pad weights of mice that were fed experimental diets. Values are presented as mean ± SD ($n = 7$). Each bar with different superscript letters is significantly different by Duncan's multiple range test ($p < 0.05$). Experimental groups: Control, fed basic diet; HFD, fed high-fat diet; HFD + 1% GLE, fed HFD containing 1% GLE; HFD + 5% GLE, fed HFD containing 5% GLE. GLE, *Glehnia littoralis* root extract; HFD, high-fat diet.

regulates the lipogenic gene expression associated with fatty acid synthesis, which promotes increased triglyceride synthesis and the expression of PPARγ ligands [30]. The results of our study suggest that the GLE downregulates the expression of SREBP-1c, leading to decreased PPARγ expression. SREBP-1c also reportedly binds to the promoter region of FAS to activate its transcription [31]. The expression of aP2 and FAS genes, which are involved in lipid metabolism, was significantly downregulated in the GLE-treated HFD mice. aP2, which is expressed in adipocytes and is also known as the fatty acid binding protein 4 (FABP4), has profound effects on insulin sensitivity and glucose metabolism and plays an important role in adipocyte differentiation [32]. Additionally, aP2 is activated by PPARγ, C/EBPα, and SREPB-1c [32]. Furthermore, the protein levels of the adipogenic transcription factors and lipid metabolism genes, namely, PPARγ, C/EBPα, SREPB-1c, aP2, leptin, and FAS, in the epididymal fat of the GLE-treated HFD mice followed the same trend as their respective mRNA levels (Figures 6(a)–6(g)). Thus, the expression of the critical adipogenic proteins PPARγ and C/EBPα decreased following the treatment with 1 and 5% GLE (Figures 6(b) and 6(c)). In connection with the discussion before, it has been suggested that GLE might suppressed the secretion of adipocytokines

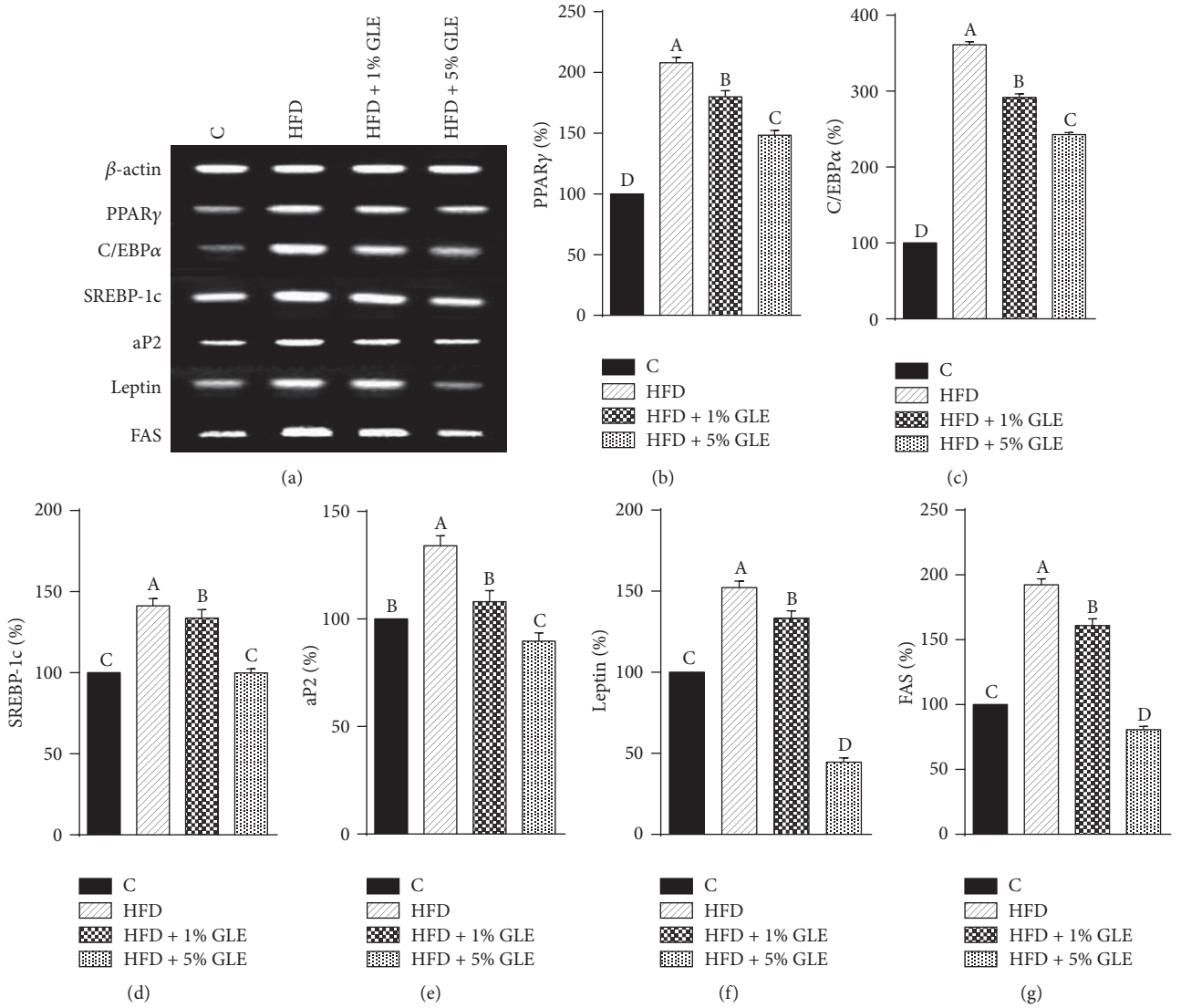

Figure 5: *Effect of the GLE on the mRNA expression of major adipogenic transcription factors in HFD-induced obese mice*. (a) A representative image of the RT-PCR results. mRNA levels of (b) PPARγ, (c) C/EBPα, (d) SREBP-1c, (e) aP2, (f) Leptin, and (g) FAS as determined by RT-PCR. Values are presented as a percentage of the levels in controls. Data are expressed as mean ± SD ($n = 7$). Bars with different superscript letters are significantly different by Duncan's multiple range test ($p < 0.05$). Experimental groups: Control, fed basic diet; HFD, fed high-fat diet; HFD + 1% GLE, fed HFD containing 1% GLE; HFD + 5% GLE, fed HFD containing 5% GLE. PPAR, peroxisome proliferator-activated receptor; C/EBP, CCAAT/enhancer-binding protein; FAS, fatty acid synthase; aP2, adipose fatty acid binding protein; SREBP, sterol regulatory element binding protein; RT-PCR, reverse transcription-polymerase chain reaction; GLE, *Glehnia littoralis* root extract; HFD, high-fat diet.

such as leptin through the suppression of PPARγ expression [25].

Obesity is related to adipocyte differentiation and excess fat accumulation [18]. In our study, GLE administration reduced fat accumulation in 3T3-L1 adipocytes and HFD-induced obese mice by suppressing the expression of key transcription factors and genes at both the mRNA and protein level. SREBP-1c is known to accelerate adipogenesis by inducing the expression of FAS, which is an adipogenic enzyme [33]. Additionally, triglyceride accumulation in the livers of SREBP-1c-deficient ob/ob mice has been reported to decrease by approximately 50% compared with that in ob/ob mice livers [34].

Our results showed that the abdominal, perirenal, and epididymal fat weight of 5% GLE-treated mice was less than half of that of the untreated HFD-induced obese mice, which may have been due to the GLE-mediated inhibition of the mRNA and protein expression of SREBP-1c and FAS. We also demonstrated that the antiobesity effects of the GLE on various genes involved in adipogenesis, which is a differentiation pathway, are mediated via the downregulation of major transcription factors including PPARγ, C/EBPα, and SREBP-1c. The consequent downregulation of lipid metabolizing mediators such as aP2, leptin, and FAS, which are involved in the transport, uptake, and synthesis of lipids, resulted in the reduced fat accumulation in adipocytes.

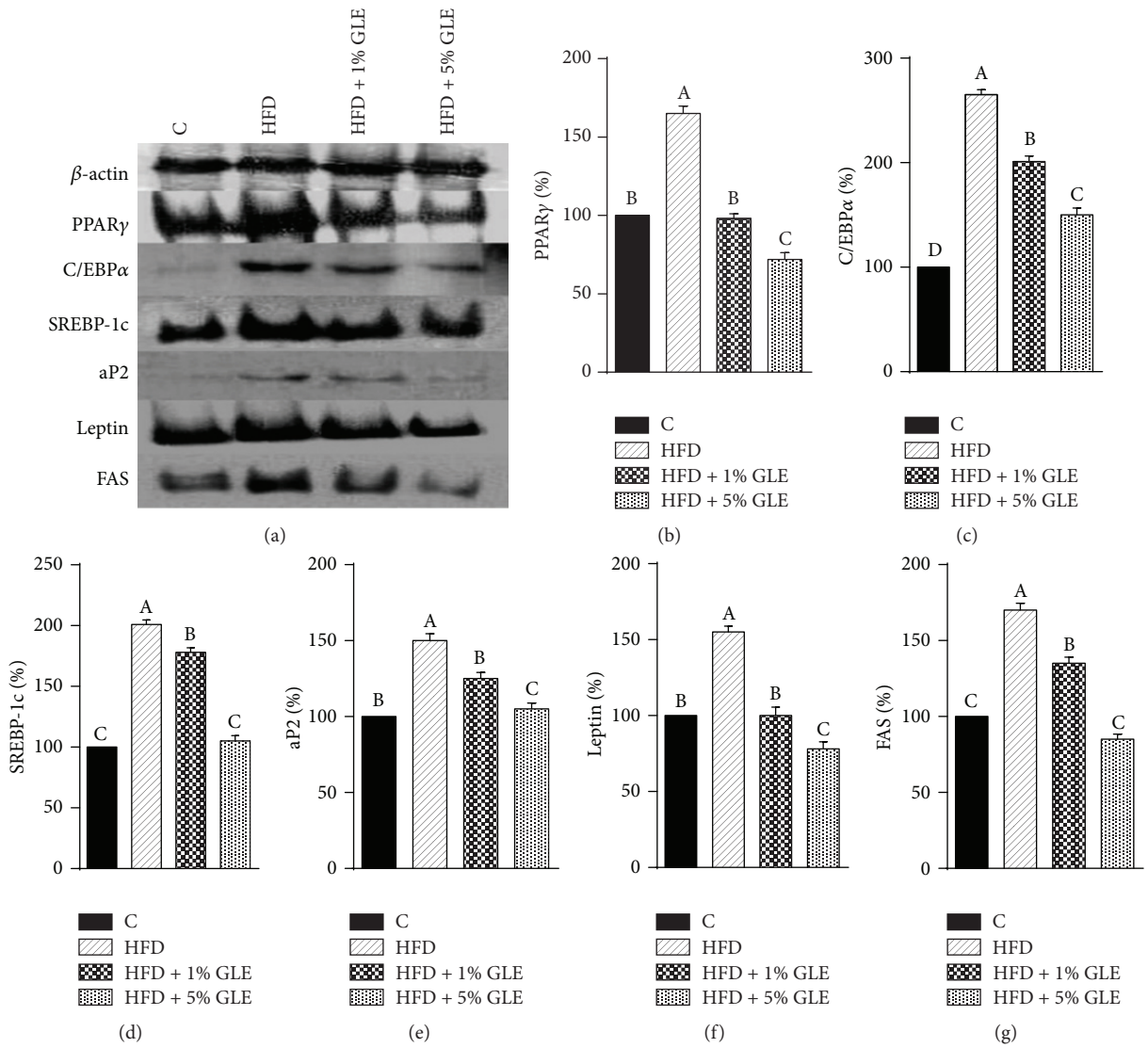

Figure 6: *Effect of the GLE on the protein expression of major adipogenic transcription factors in HFD-induced obese mice*. (a) A representative image of the western blotting results. Protein levels of (b) PPARγ, (c) C/EBPα, (d) SREBP-1c, (e) aP2, (f) Leptin, and (g) FAS as determined by western blotting. Values are presented as a percentage of the levels in controls. Data are expressed as mean ± SD ($n = 7$). Bars with different superscript letters are significantly different by Duncan's multiple range test ($p < 0.05$). Experimental groups: Control, fed basic diet; HFD, fed high-fat diet; HFD + 1% GLE, fed HFD containing 1% GLE; HFD + 5% GLE, fed HFD containing 5% GLE. PPAR, peroxisome proliferator-activated receptor; C/EBP, CCAAT/enhancer-binding protein; FAS, fatty acid synthase; aP2, adipose fatty acid binding protein; SREBP, sterol regulatory element binding protein; GLE, *Glehnia littoralis* root extract; HFD, high-fat diet.

4. Conclusion

In conclusion, the GLE strongly inhibited adipogenesis by reducing the expression of adipogenesis-related transcription factors. Therefore, the GLE may act as an effective nutraceutical for the treatment of obesity by suppressing either adipocyte differentiation or lipid accumulation.

Abbreviations

GLE: *Glehnia littoralis* root extract
HFD: High-fat diet
PPARγ: Peroxisome proliferator-activated receptor γ
C/EBPα: CCAAT/enhancer-binding protein α
FAS: Fatty acid synthase
aP2: Adipose fatty acid binding protein
SREBP-1c: Sterol regulatory element binding protein-1c.

Conflicts of Interest

The authors declare that they have no conflicts interest.

Acknowledgments

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Research Article

Treatment of Urolithiasis with Medicinal Plant *Salvia miltiorrhiza*: A Nationwide Cohort Study

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Salvia miltiorrhiza Bunge (Danshen), a common medicinal plant in traditional Chinese medicine, has been tested effectively to prevent urolithiasis in animals; nevertheless, the clinical application for urolithiasis remains unclear. We thus investigated the clinical effect of Danshen by analyzing the database from the Taiwan National Institute of Health. The cohort "Danshen-users" was prescribed Chinese herb medicine Danshen after the initial diagnosis of calculus. The control group (non-Danshen-users) was not given Danshen after the initial diagnosis of calculus. The date of first using Danshen after new diagnosis date of calculus was considered as index date. The outcome variables were categorized into two categories: the first category included calculus surgical treatment, including extracorporeal shock wave lithotripsy, ureteroscopy, percutaneous nephrostomy with fragmentation, and ureterolithotomy; the second category included any bleeding disorders, including gastrointestinal bleeding, intracranial hemorrhage, and blood transfusions. The incidence of calculus surgical treatment in the Danshen-users was less than that in the non-Danshen-users: 1.071% in 1,000 person-years (200 people followed up for 5 years) and 3.142% in 1,000 person-years, respectively. The adjusted hazard ratio for calculus surgical treatment in the Danshen-users was 0.34 (95% confidence intervals: 0.31–0.38) as compared to the non-Danshen-users. When stratified by sex, the incidence of calculus surgical treatment in Danshen-users was 0.685% in 1,000 person-years and 1.575% in 1,000 person-years for women and men, respectively, which was lower than that in non-Danshen-users. Danshen decreased the ratio of subsequent stone treatment after the first treatment in the study population; there was no increased bleeding risk due to long-term Danshen use.

1. Introduction

Urolithiasis is a common urological disorder with an annual incidence of 7–13% in North America, 1–5% in Asia [1, 2], and 6.4% in Taiwan [3]. Urolithiasis is also a disease with high recurrence. Over 50% of the patients with stone experience stone episode recurrence after 5 years of their first treatment [4]. Therefore, seeking drugs for the prevention of stone recurrence is an important issue.

Salvia miltiorrhiza Bunge (Danshen) is a common medicinal plant in traditional Chinese medicine (TCM) with its roots (dried) possessing pharmacological properties [5]. Danshen is a classical Huoxue Huayu herb (a TCM term used for activating blood circulation, relieving pain, activating blood to promote menstruation, clearing heart fire, tranquilizing, and treating blood stasis) that has been prescribed clinically for one thousand years [6]. In modern medicine, Danshen is used for the treatment of cardiovascular diseases [7, 8], osteoporosis [9], anticancer [5], and hepatoprotective effect [9]. Danshen is one of the tested effective TCM herbs for prevention of stone disease in our previous study [10, 11]. We chose Danshen (as an herb to be tested) because of its effectiveness in the treatment of blood disorders. According to TCM, blood stasis is one of the major pathogeneses of stone disease, and hematuria is frequently observed in patients with stones.

We used the database from the National Institutes of Health (NIH), Taiwan, to study the clinical effect of the potential TCM herb on urolithiasis. Although Danshen has been tested effectively for the prevention of urolithiasis in animal models [11], the clinical application in the prevention of urolithiasis is still under investigation [12]. The objective of the present study was to investigate the preventive effect of Danshen clinically by analyzing the NIH database. The surrogate outcome will be a decrease in the number of stone surgeries in a cohort. We have also studied the possible effects of increased bleeding tendency due to the antiplatelet effect of Danshen used for treating blood stasis.

2. Materials and Methods

2.1. Database. For this retrospective cohort study, our data source was from National Health Insurance Research Database (NHIRD) in Taiwan. Taiwan's National Health Insurance (NHI) program is a compulsory insurance that has been providing comprehensive coverage to 99% of 23 million individuals since 1996. The NHIRD included information of sex, birthday, outpatient care, inpatients care, western and traditional Chinese medicine (TCM) prescription, medical institutions, and registration files with scrambled identifications. We used the LHID 2000 (Longitudinal Health Insurance Database 2000), which contains medicine information between 1996 and 2013 of 1 million beneficiaries randomly sampled from the registry of all beneficiaries in 2000. The sampled patients exhibit no significant difference in age, sex, birth year, or average insured payroll-related amount from the general population. The International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM) codes were used for diagnoses. Because the NHIRD contains identified secondary data for research, the present study was waived from informed consent. A disease diagnosis without valid supporting clinical findings may be considered a medical fraud by NHI with a penalty of 100 times of the payment claimed by the treating physician or hospital. This study was approved by the Institutional Review Board of China Medical University (CMUH104-REC2-115).

2.2. Study Population. All cases diagnosed with calculus (ICD-9-CM: 592.0, 592.1, and 592.9) from January 2000 to December 2010 and aged ≥18 years were the study cohort population. The case cohort population was defined as patients who were orally given (either single or formula form) herbal medicine powder Danshen after initial diagnosis of calculus. Patients did not use Danshen after initial diagnosis date of calculus as compared to cohort group. The date of first using Danshen after new diagnosis date of calculus was considered as index date.

2.3. Covariate Assessment. Sociodemographic factors included age and sex. Age was divided into 3 groups: 18–39 years old, 40–64 years old, and ≥65 years old. Baseline comorbidities were considered if ICD-9-CM codes appeared at least once in outpatients or inpatients before initial fibromyalgia diagnosis, including diabetes mellitus (ICD-9-CM: 250), hypertension (ICD-9-CM: 401–405), urinary tract infection (ICD-9-CM: 599.0), chronic kidney disease (ICD-9-CM: 585), and gout (ICD-9-CM: 274.9).

2.4. Primary Outcome. The outcome variables were two: one was calculus surgical treatment, including extracorporeal shock wave lithotripsy (ESWL), ureteroscopy, percutaneous nephrostomy with fragmentation (PCNL), and ureterolithotomy, and the other was any bleeding disorders, including gastrointestinal bleeding (ICD-9-CM: 578.0, 578.1, 578.9), intracranial hemorrhage (ICH, ICD-9-CM: 432.0, 432.9), and blood transfusions (OP code: 99.0). Each individual was assessed from the index date to 31 December 2013 (end of the study) until the time of diagnosis of calculus surgical treatment or any bleeding disorders or until the patients were censored for withdrawal from insurance or lost to follow-up (which one first occurs).

2.5. Statistical Analyses. Student's t-tests for continuous variables and Chi-square test for categorical variables were used to compare the two study groups. We estimated hazard ratios (HRs) and 95% confidence intervals (CI) of calculus surgical treatment and bleeding disorder for the cohort using Danshen compared to the cohort not using Danshen by Cox proportional hazard model. Statistical analysis was performed and figures were created using SAS 9.4 (SAS Institute, Cary, NC) and R software. $P < 0.05$ in two-tailed tests indicated statistical significance.

3. Results

Our study included a total of 8,568 patients using Danshen (Danshen-users) and 56,502 patients not using Danshen (non-Danshen-users) suffering from calculus disease [after frequency matching (1 : 1) through sex, age (per 5 years), initial diagnosis year of calculus, and index year]. There were 8,536 Danshen-users and non-Danshen-users in each cohort. Table 1 shows the characteristics of both groups. The mean age (standard deviation, SD) for Danshen-users and non-Danshen-users was 46.40 (14.29) years and 46.42 (14.30) years, respectively. After frequency matching, the distribution of sex and age was not significantly different (P =

Table 1: Characteristics of calculus patients according to use or no use of Danshen.

Variable	Using Danshen in calculus patients				P value*
	No (n = 8536)		Yes (n = 8536)		
	n	%	n	%	
Sex					0.99
Female	4723	55.33	4723	55.33	
Male	3813	44.67	3813	44.67	
Age group, years					0.99
18–39	3018	35.36	3018	35.36	
40–64	4508	52.81	4508	52.81	
≥65	1010	11.83	1010	11.83	
Mean ± SD (years)	46.42 (14.30)		46.40 (14.29)		0.9157[a]
Baseline comorbidities					
Diabetes mellitus	2992	35.05	3339	39.12	<0.0001
Urinary tract infection	4897	57.37	5241	61.4	<0.0001
Hypertension	4402	51.57	4561	53.43	0.0148
Chronic kidney disease	657	7.7	753	8.82	0.0076
Gout	1608	18.84	1811	21.22	0.0001
Duration between diagnosis date of calculus and index, days (mean, median)	1120 (923)		1130 (921)		0.4553[a]

*Chi-square test; [a]t-test. The mean (median) of follow-up period was 6.27 (5.98) years and 5.09 (4.86) years for cohort group using Danshen and cohort group not using Danshen, separately.

0.99 for both) between Danshen-users and non-Danshen-users. The proportion of baseline comorbidities in Danshen-users was higher than that in non-Danshen-users ($P < 0.05$ for all). The mean (median) follow-up period for Danshen-users and non-Danshen-users was 6.27 (5.98) years and 5.09 (4.86) years, respectively.

The incidence of calculus surgical treatment in the Danshen-users was less than that in the non-Danshen-users, 1.071% in 1,000 person-years (200 people followed up for 5 years) and 3.142% in 1,000 person-years, respectively. The adjusted hazard ratio (HR) for calculus surgical treatment in the Danshen-users was 0.34 (95% CI: 0.31–0.38) as compared to the non-Danshen-users. When stratifying by sex, the incidence of calculus surgical treatment in the Danshen-users was 0.685% in 1,000 person-years and 1.575% in 1,000 person-years for women and men, respectively, which was lower than that in the non-Danshen-users (2.454% in 1,000 person-years and 4.070% in 1,000 person-years for women and men, resp.).

When stratifying by age, the incidence of calculus surgical treatment in Danshen-users was 1.087% in 1,000 person-years, 1.137% in 1000 person-years, and 0.690% in 1,000 person-years for 18–39 years age group, 40–64 years age group, and above 65 years age group, respectively, which was lower than that in non-Danshen-users (2.675% in 1,000 person-years, 3.514% in 1,000 person-years, and 2.989% in 1,000 person-years for 18–39 years age group, 40–64 years age group, and above 65 years age group, resp.). The adjusted HRs revealed a significantly lower risk of calculus surgical treatment in the Danshen-users as compared to the non-Danshen-users in all the categories: women, men, age group of 18–39 years, age group of 40–64 years, and age group above 65 years population (Table 2). The estimated cumulative incidence of calculus surgical treatment in the Danshen-users was lower than that in the non-Danshen-users ($P < 0.0001$, log-rank test) (Figure 1).

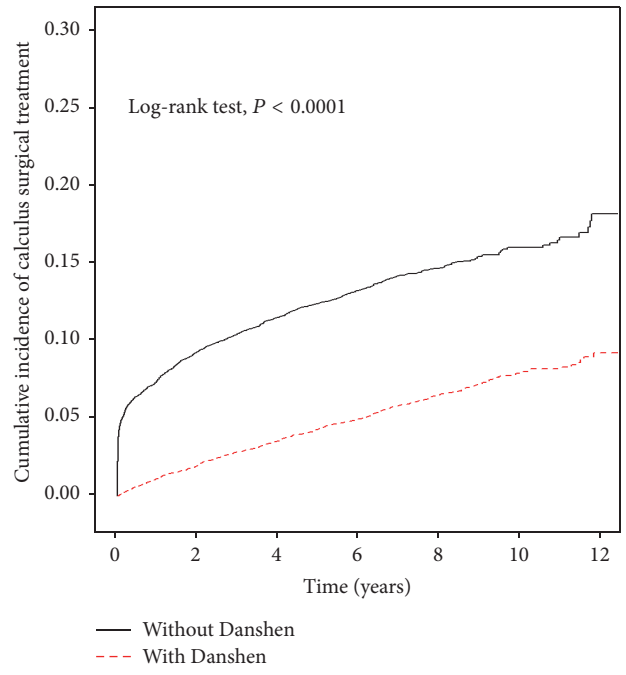

Figure 1: The estimated cumulative incidence of calculus surgical treatment in the Danshen-users was lower than that in the non-Danshen-users by Kaplan-Meier analysis.

The incidence of any bleeding disorder in the Danshen-users was less than that in the non-Danshen-users (1.708% in 1000 person-years and 2.577% in 1,000 person-years, resp.).

Table 2: Incidence rates, hazard ratio, and confidence intervals of calculus surgical treatment and any bleeding disorder for calculus patients using and not using Danshen in the stratification of sex and age.

Variables	Using Danshen in calculus patients						Crude HR	Adjusted HR†
	No (n = 8536)			Yes (n = 8536)				
	Event	Person-years	IR	Event	Person-years	IR	(95% CI)	(95% CI)
Outcome: calculus surgical treatment								
Total	1370	43605	31.42	574	53589	10.71	0.36 (0.33–0.4)***	0.34 (0.31–0.38)***
Sex								
Female	615	25057	24.54	208	30354	6.85	0.3 (0.26–0.35)***	0.29 (0.24–0.34)***
Male	755	18548	40.70	366	23235	15.75	0.41 (0.36–0.47)***	0.39 (0.34–0.44)***
Age group, years								
18–39	449	16784	26.75	215	19782	10.87	0.43 (0.36–0.5)***	0.4 (0.34–0.47)***
40–64	799	22739	35.14	320	28151	11.37	0.35 (0.3–0.39)***	0.33 (0.29–0.38)***
≥65	122	4082	29.89	39	5656	6.90	0.26 (0.18–0.37)***	0.25 (0.17–0.35)***
Outcome: any bleeding disorders								
Total	1138	44166	25.77	917	53678	17.08	0.66 (0.61–0.72)***	0.61 (0.56–0.67)***
Sex								
Female	572	25341	22.57	463	30379	15.24	0.68 (0.6–0.77)***	0.63 (0.56–0.71)***
Male	566	18824	30.07	454	23299	19.49	0.64 (0.57–0.73)***	0.59 (0.52–0.67)***
Age group, years								
18–39	202	16862	11.98	162	19786	8.19	0.68 (0.56–0.84)***	0.65 (0.53–0.8)***
40–64	629	23102	27.23	516	28238	18.27	0.67 (0.59–0.75)***	0.63 (0.56–0.71)***
≥65	307	4202	73.07	239	5653	42.28	0.58 (0.49–0.69)***	0.55 (0.46–0.65)***

IR, incidence rates per 1,000 person-years; HR, hazard ratio; CI, confidence interval. Adjusted HR† represented adjusted hazard ratio: mutually adjusted for Danshen drug used, sex, age, diabetes mellitus, urinary tract infection, hypertension, chronic kidney disease, and gout in Cox proportional hazard regression.
*** $P < 0.001$.

The adjusted HR for calculus surgical treatment in Danshen-users was 0.61 (95% CI: 0.56–0.67) as compared to the non-Danshen-users. When stratifying by sex, the incidence of any bleeding disorder in the Danshen-users was 1.524% in 1,000 person-years and 1.949% in 1,000 person-years for women and men, respectively, which was lower than that in the non-Danshen-users (2.557% in person-years and 3.007% in 1,000 person-years for women and men, resp.).

When stratifying by age, the incidence of calculus surgical treatment in Danshen-users was 0.819% in 1,000 person-years, 1.827% in 1,000 person-years, and 4.228% in 1,000 person-years for 18–39 years age group, 40–64 years age group, and above 65 years age group, respectively, which was lower than that in the non-Danshen-users (1.198% in 1,000 person-years, 2.723% in 1,000 person-years, and 7.307% in 1,000 person-years for 18–39 years age group, 40–64 years age group, and above 65 years group, resp.). The adjusted HRs revealed a significantly lower risk of any bleeding disorder in the Danshen-users as compared to the non-Danshen-users in all the categories: females, males, age group of 18–39 years, age group of 40–64 years, and above 65 years age group population (Table 2). The estimated cumulative incidence of any bleeding disorder in the Danshen-users was lower than that in the non-Danshen-users ($P < 0.0001$, log-rank test) (Figure 2). The HRs and 95% CI of calculus surgical treatment and any bleeding disorder associated with cumulative dose per year of Danshen among calculus patients with Danshen-users were shown in Table 3.

4. Discussion

We observed that Danshen significantly reduces the subsequent surgical treatment after the first stone episode, with a hazard ratio of 0.34. This effect was consistent in both sexes and among all age groups. Danshen may prove to be clinically effective for those having stone disease and seeking a measure to prevent their further surgical treatment. Danshen poses a concern regarding the increased bleeding tendency due to its enhanced blood stasis effect. However, we did not find any incidence involving hemorrhage or any transfusion event in this cohort. This result suggests that long-term use of Danshen may prove to be safe without any bleeding disorder.

The idea of using Danshen in the present study originated from our previous data [11]. Danshen revealed its preventive results for the crystal formation in a fruit fly (as observed in an animal study). *Salvia miltiorrhiza* appeared in the classic herbal book named "Shennong Ben Cao Jing" more than 2000 years ago (about 200 and 250 AD) [13]. It was described as a noble and nontoxic herb. It is often used to treat cardiovascular diseases [14, 15], hypertension, and ischemic stroke due to its efficacy on blood circulation [16, 17]. Till date, more than thirty pharmaceutical preparations for the treatment of cardiovascular diseases have been developed in clinical practice [18]. Cardiac and renal dysfunctions often occur simultaneously due to the shared causes and pathogenesis [19]. Furthermore, oxidative stress is considered as the most important determinant of the common cause [20].

Table 3: Hazard ratios and 95% confidence intervals of calculus surgical treatment and any bleeding disorder associated with cumulative dose per year of Danshen among calculus patients using Danshen.

Used Danshen dose (g) per year	n	Number of events	Hazard ratio (95% CI)	
			Crude	Adjusted†
Event: calculus surgical treatment				
Less than 417 g per year	2813	203	1 (reference)	1 (reference)
417–1096 g per year	3025	179	0.75 (0.62–0.92)**	0.76 (0.62–0.93)**
More than 1096 g per year	2698	192	0.92 (0.75–1.12)	0.94 (0.77–1.14)
Event: any bleeding disorders				
Less than 417 g per year	2813	306	1 (reference)	1 (reference)
417–1096 g per year	3025	308	0.84 (0.71–0.98)*	0.83 (0.71–0.97)*
More than 1096 g per year	2698	303	0.94 (0.80–1.10)	0.95 (0.81–1.11)

Adjusted HR† represented adjusted hazard ratio: mutually adjusted for sex, age, diabetes mellitus, urinary tract infection, hypertension, chronic kidney disease, and gout in Cox proportional hazard regression; * $P < 0.05$; ** $P < 0.01$; tertiles are two cut points that will divide a dataset into three equal-sized groups. 417 g was 33rd percentage point and 1096 g was 66th percentage.

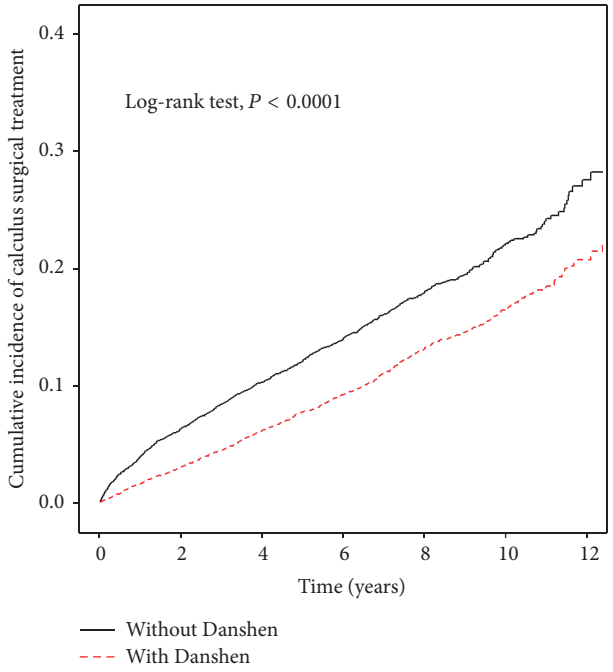

Figure 2: The estimated cumulative incidence of any bleeding disorder in the Danshen-users was lower than that in the non-Danshen-users by Kaplan-Meier analysis.

The hypertensive patients have a greater risk of kidney stones than those with normal blood pressure. The patients with kidney stones are more likely to suffer from hypertension than those without urolithiasis. Thus, there exists a positive correlation between hypertension and renal stones [21]. According to a recent study, Danshen is the most frequently prescribed single herb drug for hypertension [22]. In addition, previous animal studies revealed that overproduction of reactive oxygen species causes kidney damage, and *Salvia miltiorrhiza* helps to improve the renal function and prevent oxidative stress in the renal tissues, thereby treating the renal damage [23–26].

Our previous study conducted with an emerging translational model to screen antilithic herbal drugs revealed the inhibitory effect of Danshen on the formation of CaOx crystals in the Malpighian tubules of *Drosophila* [11]. According to the epidemiological studies, urolithiasis is associated with many chronic diseases including obesity, metabolic syndrome, diabetes, hypertension, chronic kidney disease, and coronary artery disease [27–32]. The correlation between these chronic diseases and renal stones is most likely the result of a common pathophysiological mechanism. Reactive oxygen species (ROS) and oxidative stress are the common features between kidney stones and venereal diseases [33]. Further evidences showed that ROS is also produced in idiopathic CaOx kidney stones. A kidney stone is not only a physical-chemical event but also a metabolic disorder. The chronic diseases associated with oxidative stress are related to each other. Oxidative stress is usually caused by one disorder, which in turn contributes to the development of another disorder, particularly hypertension and diabetes. Both these effects may lead to oxidative stress, kidney damage, and inflammation, along with the changes in the urinary environment which promote crystallization [21]. Therefore, the treatment of urinary tract stones should not be overlooked, and the original source must be cured completely. Furthermore, TCM focuses on the reconstruction of the homeostasis prior to the formation of stones, along with acting as a treatment of urolithiasis after kidney disease and stone formation [34]. Therefore, Danshen may play an important role in the prevention of urolithiasis.

The limitations of this study include limited patient number, a surrogate outcome instead of recurrence, and unknown stone site and number. In addition, calculus surgical treatment option depends on stone size. However, LHID 2000 does not provide the information of stone size.

5. Conclusions

Danshen decreased the ratio of subsequent stone treatment after the first treatment in a population study from Taiwan's database. Long-term use of Danshen may prove to be safe

with a reduced risk of a bleeding event. Therefore, Danshen is a safe herb having a potential for the prevention of calculus.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Effect of Resveratrol Dry Suspension on Immune Function of Piglets

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Resveratrol, a polyphenolic plant antitoxin, has a wide range of pharmacological activities. In this study, we systematically evaluated the effects of resveratrol dry suspension (RDS) on immune function in piglets that were treated with different doses of RDS for 2 weeks. The results showed that the RDS has significant effects on the development, maturation, proliferation, and transformation of T lymphocytes. RDS could regulate humoral immune responses by upregulating the release of IFN-γ and downregulating the release of TNF-α. After piglets were vaccinated against classical swine fever virus and foot-and-mouth disease virus, the antibody titers were significantly increased. RDS treatment showed an excellent resistance to enhance T-SOD activity. Values of blood routine and blood biochemistry showed no toxicity. These results suggested that RDS could be considered as an adjuvant to enhance immune responses to vaccines, as well as dietary additives for animals to enhance humoral and cellular immunity.

1. Introduction

The immune system is a vital barrier against the invasion of microorganisms, and it assumes enormous importance in fight against diseases and malignant abnormal cells [1]. Modern medical research has brought natural products into people's vision to enhance or restore the immune system. It is shown that some phytochemicals are beneficial to the health of the body by promoting the immune function, reducing inflammation, and activating enzymes [2]. As a result, natural plants with pharmacological activities are recommended as dietary supplements or therapeutic agents to effectively care for the organism.

Resveratrol (trans-3,4,5-trihydroxystilbene), a natural polyphenolic compound extracted from *Polygonum cuspidatum*, was first found in red wine because of the beneficial effect on the heart [3]. It has been exposed to a variety of biological activities, including anticancer, antioxidative, anti-inflammatory, antimicrobial, and estrogenic activities [4]. By interacting with multiple molecular targets, resveratrol could regulate innate and adaptive immunity [5]. It has attracted increasing attention due to the rich biological activities and has been recognized for its benefits to human health and used as a healthcare product in some people's diet [6].

Resveratrol supplementation in rat diets showed an increase in IgM concentration and splenocyte proliferation and a decrease in the triglyceride level [7]. In chickens, resveratrol could promote growth and inhibit antigen-induced apoptosis [8]. In ducklings infected with virulent duck enteritis virus, resveratrol supplementation could increase the survival rate, relieve tissue lesions, and reduce viral load in blood [9].

Although the function of resveratrol to regulate the immune response has been demonstrated in various animal models, it has been rarely reported in piglets. Pigs can be used as animal models for human diseases because of the great similarity between pigs and humans in lipid metabolism, cardiovascular physiology [10], and digestive system [11].

In our previous research, resveratrol was prepared into a dry suspension with the presence of suitable excipients to solve the trouble of poor water solubility in our laboratory. Therefore, in this study, the piglets were given resveratrol dry suspension (RDS) and the immune-regulating function was determined for the purpose of development of a new additive for piglets.

2. Materials and Methods

2.1. Chemicals. The resveratrol dry suspension (RDS) was prepared in Natural Medicine Research Center of Sichuan Agricultural University (Chengdu, China), and the content of resveratrol was 3%. Resveratrol was purchased from Sigma Co., Ltd. (USA). *Echinacea purpurea* powder was purchased from Qilu Animal Health products Co., Ltd. (Jinan, China).

2.2. Animals. Animal experiments were conducted under the principles of proper laboratory animal care and were approved by the ethical committee of the Laboratory Animals Care and Use of Sichuan Agriculture University (Chengdu, China; license number SCXK (Sichuan) 2014-187). 40 cross-bred weaned piglets (Duroc × Landrace × Big White) at 28 days of age were randomly divided into five groups of 8 animals each group (4 females and 4 males). The 5 groups were as follows: saline control group (Group I), low dose of RDS-treated group (0.1 g/kg/d; Group II), middle dose of RDS-treated group (0.33 g/kg/d; Group III), high dose of RDS-treated group (1.0 g/kg/d; Group IV), and *Echinacea purpurea*-treated group (0.05 g/kg/d; Group V), respectively. The RDS and *Echinacea purpurea* (positive control) were suspended in water and fed to animals at 9 a.m. every morning for 14 days. The standard diet of animals was formulated based on the NRC (2012) recommendation for the nutrient requirements of 7–11 kg pigs [12]. The piglets were bred at a stationary temperature of 20–25°C, a stable relative humidity of 50 ± 10%, and illumination of 12 h per day in accordance with the International Committee on Laboratory Animals. The animals were domesticated for 4 days before experiments. It is assured that all animals are treated humanely in the laboratory and that the fewest numbers of animals are used to achieve the desired objectives.

2.3. Growth Performance and Visceral Index Assay. During treatment period, piglets were weighed under limosis. The states of the animals were observed and recorded every day. The average daily feed intake (ADFI), average daily gain (ADG), and ratio of feed to gain (F : G) were measured.

Within 24 hours of the last administration, piglets were sacrificed and the organs were weighed, including heart, lung, liver, kidney, spleen, and inguinal lymph nodes. The indexes were calculated according to the following formula: index (mg/g) = (the weight of organ)/the body weight.

2.4. Vaccine Treatment and Detection of Serum Antibody Level. Each piglet was inoculated with classical swine fever vaccine (CSFV) in the first day of the trial reference to the recommended immunization program [13]. A week later, the piglets were inoculated with foot-and-mouth disease vaccine (FMDV) again. The delay of second vaccination time was to eliminate or mitigate the stress response of piglets to FMDV [14].

Blood samples from anterior vein were collected to determine the serum antibody level at 0 d, 7 d, and 14 d during the trial, respectively. The antibody levels of CSFV and FMDV in serum were analyzed by ELISA kits (Shenzhen finder Biotech Co., Ltd., China) in accordance with the manufacturer's instructions.

2.5. T Lymphocyte Subsets Assay. Within 24 hours of the last administration, 2 ml of blood sample of each piglet from anterior vein was collected and dealt with EDTA. The lymphocytes were separated by lymphocyte separation medium (Beijing Solarbio, China). Then, the cells were incubated with CD3e-FITC, CD4α-PRE, and CD8α-SPRD monoclonal antibodies (BD Biosciences, USA) at temperature 37°C for 0.5 h in the darkness, followed by centrifugation and resuspending in PBS. T lymphocyte subsets were analyzed by flow cytometry (BD Biosciences, USA).

2.6. Proliferative Activity of Peripheral Blood Lymphocyte and Spleen Lymphocytes. Within 24 hours of the last administration, blood sample of each piglet from anterior vein was collected with anticoagulation. Then 3 ml of blood sample was slowly injected into 6 ml of porcine peripheral blood lymphocyte separation solution (Beijing Solarbio, China) and centrifuged to obtain the intermediate white cell layer. The cells were washed and centrifuged by PBS three times and then suspended in RPMI-1640 medium (Beijing Solarbio, China) at the concentration of 2×10^6 cells/L. Blastogenic response of lymphocytes to the mitogen of ConA (Beijing Solarbio, China) was assessed by CCK-8 (Dojindo Laboratories, Japan). Lymphocyte suspension was incubated with ConA (10 μg/mL) in 150 μL RPMI 1640 medium containing 10% fetal bovine serum (FBS, Gibco Company, USA) at 37°C with 5% CO_2. After incubation for 48 h, 10 μL CCK-8 was added to each well. After incubation for 2 h, the absorbance at 450 nm was measured by a microplate reader (Bio-Rad, USA).

Within 24 hours of the last administration, 3 piglets from each group were sacrificed and the spleen was isolated in a sterile environment. Spleen tissue with the weight of 5 g was disrupted, and spleen cell suspensions were passed through sterile nylon mesh. Red blood cells were lysed by Erythrocyte Lysate (Beijing Solarbio, China). The spleen cells were suspended in RPMI-1640 medium and the methods of culture and detection were identical to those described above.

2.7. Determination of Serum Immunoglobulin Levels. The blood of piglets was collected from the anterior vein at the end of the trial. The serum was isolated by centrifugation. The serum concentrations of IgG, IgA, and IgM were measured by ELISA kits (Shanghai MLBIO, China).

2.8. The Antioxidant Capacity of Serum. The serum total antioxidant capacity (T-AOC), malondialdehyde level (MDA), and superoxide dismutase (T-SOD) in serum were

Table 1: Growth performance and visceral index.

Items	Group I	Group II	Group III	Group IV	Group V
Initial body weight (kg)	6.52 ± 0.07	6.52 ± 0.18	6.75 ± 0.41	6.57 ± 0.38	6.61 ± 0.16
Final body weight (kg)	7.82 ± 0.37	7.65 ± 0.31	8.5 ± 0.4	8.13 ± 0.75	8.41 ± 0.13
Average daily feed intake (g)	242.86 ± 23.26	193.33 ± 12.03	248.07 ± 42.6	183.34 ± 33.35	128.09 ± 3.92
Average daily gain (g)	93.33 ± 29.65	80.95 ± 9.71	149.05 ± 27.64	111.9 ± 27.92	226.45 ± 26.95
Ratio of feed to gain	3.25 ± 1.04	2.42 ± 0.16	1.67 ± 0.03	1.68 ± 0.17	1.78 ± 0.24
Heart coefficient	5.37 ± 0.34	4.99 ± 0.24	5.45 ± 0.27	5.18 ± 0.11	5.51 ± 0.39
Lung coefficient	27.32 ± 2.52	26.5 ± 1.92	24.28 ± 2.05	24.47 ± 1.73	20.67 ± 1.25
Liver coefficient	27.32 ± 2.52	26.5 ± 1.92	24.28 ± 2.05	24.47 ± 1.73	20.67 ± 1.25
Kidney coefficient	5.94 ± 0.49	6.05 ± 0.18	6.3 ± 0.54	5.62 ± 0.22	6.11 ± 0.34
Spleen coefficient	1.94 ± 0.14	1.52 ± 0.18	1.68 ± 0.04	1.52 ± 0.16	2.01 ± 0.19
Lymph nodes coefficient	1.45 ± 0.13	1.84 ± 0.26	1.76 ± 0.22	1.44 ± 0.15	1.63 ± 0.16

Group I, saline control; Group II, RDS 0.1 g/kg treated group; Group III, RDS 0.33 g/kg treated group; Group IV, RDS 1.0 g/kg treated group; Group V, *Echinacea purpurea* powder 0.05 g/kg treated group. Data are represented as means ± SE; $n = 6$; comparison was made with the model group; one-way ANOVA followed by Duncan test.

Table 2: T lymphocyte subsets.

Items	Group I	Group II	Group III	Group IV	Group V
CD3+ (%)	65 ± 4.71	63.43 ± 5.02	71.17 ± 0.89	61.57 ± 4.87	62.1 ± 4.75
CD3+CD4+ (%)	27.97 ± 3.89	35.9 ± 5.71	43.3 ± 4.56	35.8 ± 3.39	29.37 ± 2.59
CD3+CD8+ (%)	23.67 ± 3.88	22.23 ± 2.63	24.23 ± 1.5	20.7 ± 2.01	18.1 ± 2.01
CD3+CD4+/CD3+CD8+	1.25 ± 0.29	1.6 ± 0.07	1.78 ± 0.09	1.74 ± 0.1	1.45 ± 0.17

Group I, saline control; Group II, RDS 0.1 g/kg treated group; Group III, RDS 0.33 g/kg treated group; Group IV, RDS 1.0 g/kg treated group; Group V, *Echinacea purpurea* powder 0.05 g/kg treated group. Data are represented as means ± SE; $n = 6$; comparison was made with the model group; one-way ANOVA followed by Duncan test.

determined by ELISA kits (Nanjing Jiancheng Bioengineering Institute, China).

2.9. Determination of Serum Cytokine Levels. The serum cytokine levels of interleukin, interferon, and tumor necrosis factor were determined by ELISA kits (Shanghai MLBIO, China).

2.10. Hematologic Examination and Serum Biochemical Examination. The blood samples obtained at the end of the trial were collected into a precalibrated tube containing sodium citrate. The hematological parameters included white blood cell count (WBC), red blood cell count (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), MCH concentration (MCHC), platelet count (PLT), and leukocyte differential count (lymphocytes, neutrophils, and monocytes) [15].

Serum biochemical indicators were detected, including albumin (ALB), total protein (TP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), urea nitrogen (BUN), creatinine (CRE), glucose (GLU), calcium (Ca), phosphorus (P), total bilirubin (TBIL), and total cholesterol (CHO).

3. Results

3.1. Growth Performance and Visceral Coefficients. The growth performance and visceral index of piglets were shown in Table 1. Animals were randomly grouped and showed no difference in initial body weight. While the animals gained weight during experiment, the average daily feed intake and average daily gain of all drug treatments did not significantly differ in comparison to the saline control group ($p > 0.05$). The RDS and *Echinacea purpurea* treatment had no effect on coefficients of organs when compared to the saline control group ($p > 0.05$).

3.2. Percentage and Ratio of T Lymphocyte Subsets. The percentage of T lymphocytes in the peripheral blood of piglets was shown in Table 2, as well as the percentage of CD3+CD4+ and CD3+CD8+ labeled T cells and the ratio of the two. The percentages of T lymphocyte, including CD3+, CD3+CD4+, and CD3+CD8+, and the ratio of CD3+CD4+/CD3+CD8+ did not show any difference ($p > 0.05$) among all the groups. In RDS treatment, these T lymphocyte subsets were slightly higher than positive control ($p > 0.05$).

3.3. Proliferative Activity of Peripheral Blood Lymphocyte and Spleen Lymphocytes. The proliferation of peripheral blood lymphocytes and splenic lymphocytes under the stimulation of ConA was shown in Figure 1. Compared with saline control group, RDS treatment (0.33 g/kg) significantly ($p < 0.01$) stimulated the proliferation of peripheral blood lymphocytes, while the other treatment groups did not show any differences. In splenic lymphocytes, all RDS treatments significantly increased ($p < 0.05$) lymphocyte proliferation,

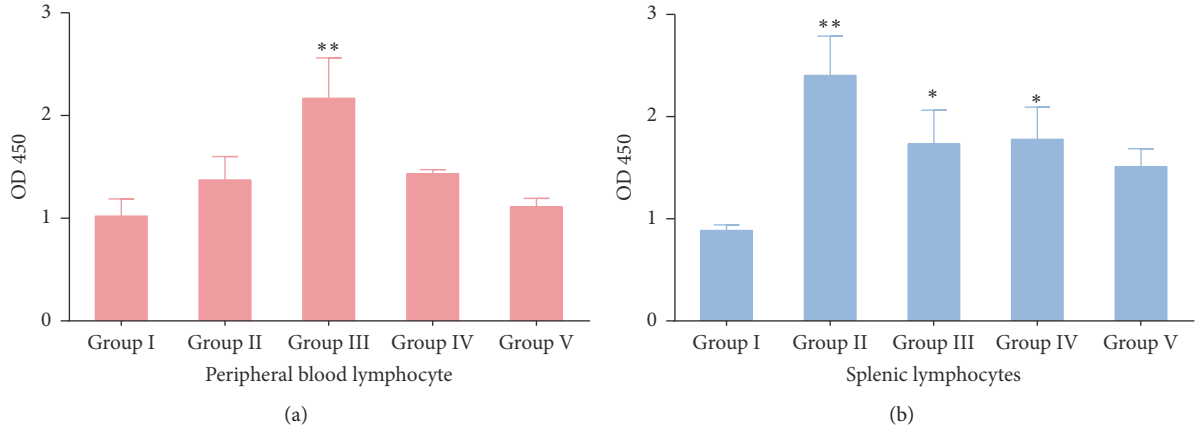

Figure 1: Proliferative activity of peripheral blood lymphocyte and spleen lymphocytes under the stimulation of ConA. (a) Proliferation of peripheral blood lymphocytes; (b) proliferation of splenic lymphocytes. Group I, saline control; Group II, RDS 0.1 g/kg treated group; Group III, RDS 0.33 g/kg treated group; Group IV, RDS 1.0 g/kg treated group; Group V, *Echinacea purpurea* powder 0.05 g/kg treated group. Data are represented as means ± SE; $n = 6$; comparison was made with the saline control group; one-way ANOVA followed by Duncan test. The symbols represent statistical significance at $^{*}p < 0.05$ and $^{**}p < 0.01$.

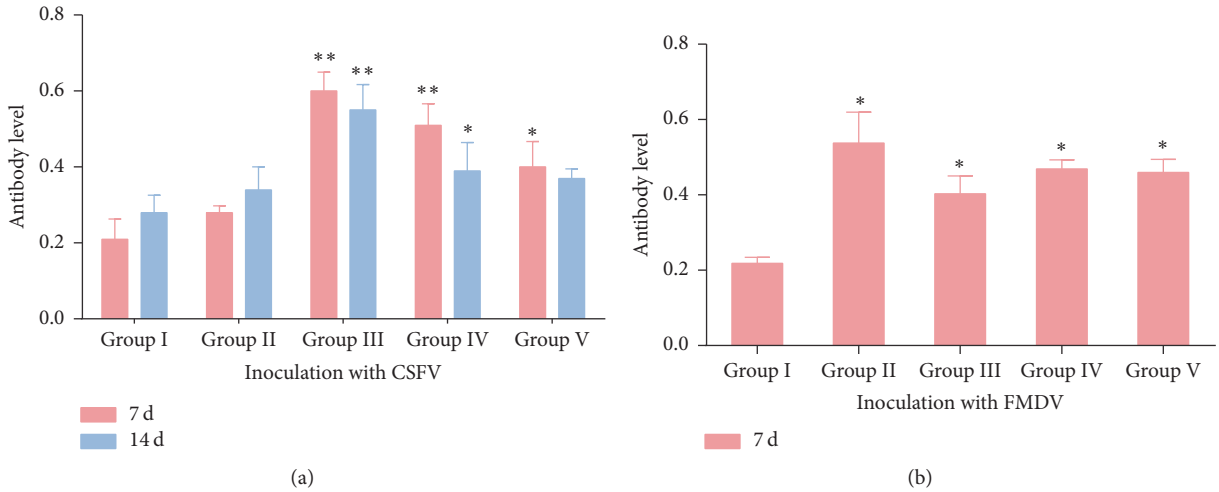

Figure 2: Antibody levels in serum. (a) The antibody level of CSFV; (b) the antibody level of FMDV. Group I. saline control; Group II, RDS 0.1 g/kg treated group; Group III, RDS 0.33 g/kg treated group; Group IV, RDS 1.0 g/kg treated group; Group V, *Echinacea purpurea* powder 0.05 g/kg treated group. RDS, resveratrol dry suspension; CSFV, classical swine fever vaccine; FMDV, foot-and-mouth disease vaccine. Data are represented as means ± SE; $n = 6$; comparison was made with the saline control group; one-way ANOVA followed by Duncan test. The symbols represent statistical significance at $^{*}p < 0.05$ and $^{**}p < 0.01$.

which showed RDS possessed potent effect on lymphocyte activity.

3.4. Antibody Levels in Serum. The detection of antibody levels in piglets was shown in Figure 2. The levels of CSFV antibody produced after 7 days of inoculation in piglets were significantly increased ($p < 0.01$) in RDS treatment (0.33 g/kg and 1.0 g/kg) compared to the saline control group, while the antibody level in *Echinacea purpurea* powder-treatment was also remarkably higher ($p < 0.05$) than that of saline control group. After 14 days of inoculation CSFV, only RDS treatment (0.33 g/kg and 1.0 g/kg) differed significantly in the saline control group ($p < 0.01$ or $p < 0.05$). Detection results after a week of vaccination with FMDV showed that all drug treatments significantly ($p < 0.05$) improved the antibody levels in piglets. These data demonstrated the positive effects of RDS on the secretion of antibodies.

3.5. Immunoglobulin Levels in Serum. The immunoglobulins levels of serum in piglets were measured in the first and second weeks of the trial, respectively, and the results were shown in Figure 3. At 7 d of the trial, all RDS treatments significantly increased ($p < 0.05$ or $p < 0.01$) the levels of IgG and IgM in the serum, while the RDS treatment (0.33 g/kg) and *Echinacea purpurea* powder treatment significantly increased ($p < 0.01$) the content of IgA. At 14 d, the RDS treatment (0.33 g/kg) significantly promoted ($p < 0.01$) the secretion of IgA in serum, yet the other drug-treatment groups had no effect on the changes of immunoglobulin content compared with the saline control group.

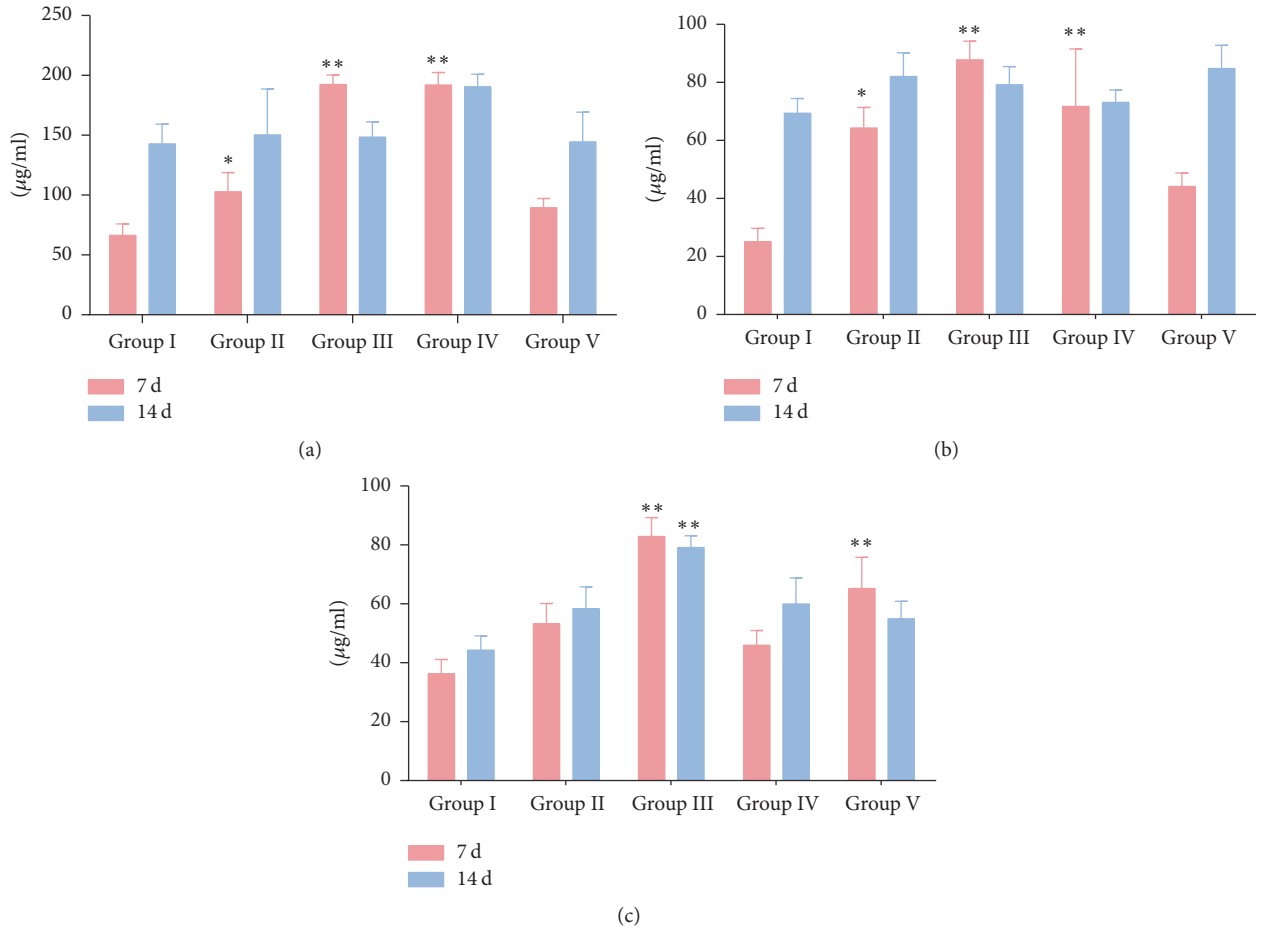

Figure 3: Immunoglobulin levels in serum. (a) Immunoglobulin G levels; (b) immunoglobulin M levels; (c) immunoglobulin A levels. Group I, saline control; Group II, RDS 0.1 g/kg treated group; Group III, RDS 0.33 g/kg treated group; Group IV, RDS 1.0 g/kg treated group; Group V, *Echinacea purpurea* powder 0.05 g/kg treated group. Data are represented as means ± SE; $n = 6$; comparison was made with the saline control group; one-way ANOVA followed by Duncan test. The symbols represent statistical significance at $^{*}p < 0.05$ and $^{**}p < 0.01$.

3.6. Antioxidant Capacity of Serum. The result (Figure 4) showed that, at 7 d of the trial, RDS treatment (0.33 g/kg and 1.0 g/kg) and *Echinacea purpurea* powder treatment significantly improved ($p < 0.01$) the total antioxidant capacity of serum. Similarly, the RDS treatment (0.33 g/kg) and the *Echinacea purpurea* treatment significantly increased the total antioxidant capacity at 14 d, while the other groups were not significantly different compared with the saline control group. All the drug treatments had no effect on MDA production. RDS-treatment groups (0.33 g/kg and 1.0 g/kg) and positive control group significantly improved the activity of serum T-SOD after 7 d ($p < 0.01$ or $p < 0.05$), and only the RDS-treatment (0.33 g/kg) and positive control group significantly improved the activity of serum T-SOD after 14 d. The results confirmed that RDS had a good antioxidant capacity at the dose of 0.33 g/kg.

3.7. Cytokine Levels in Serum. The result (Figure 5) showed that all RDS treatments and *Echinacea purpurea* treatment reduced the release of TNF-α ($p < 0.01$ or $p < 0.05$) at 7 d, while the RDS treatment (0.1 g/kg and 0.33 g/kg) also reduced the release of IL-12 ($p < 0.05$). In the second week, all RDS-treatment and *Echinacea purpurea*-treatment groups increased the release of IFN-γ ($p < 0.05$) and the RDS treatment (1.0 g/kg) increased the release of IL-2 ($p < 0.01$).

3.8. Hematologic Examination and Serum Biochemical Examination. Tables 3 and 4 show the effects of RDS on blood and serum biochemical markers, respectively. RDS-treatment groups (0.33 g/kg and 1.0 g/kg) and positive control group significantly increased the number of white blood cells (WBC), neutrophils (NEUT), lymphocytes (LY), and monocytes (MONO). The creatinine (CRE) levels were significantly higher in the RDS medium and high dose groups than that of saline group ($p < 0.05$). The urea nitrogen (BUN) and triglyceride (TG) levels were increased in the RDS-treatment (0.1 g/kg) group ($p < 0.01$). Alanine aminotransferase (ALT) levels were increased in the median dose group; meanwhile blood sugar (GLU) levels were lower in the RDS-treatment (0.33 g/kg) group ($p < 0.01$).

4. Discussion

Our study systematically evaluated the effect of RDS on the immune function of piglets through various parameters. We

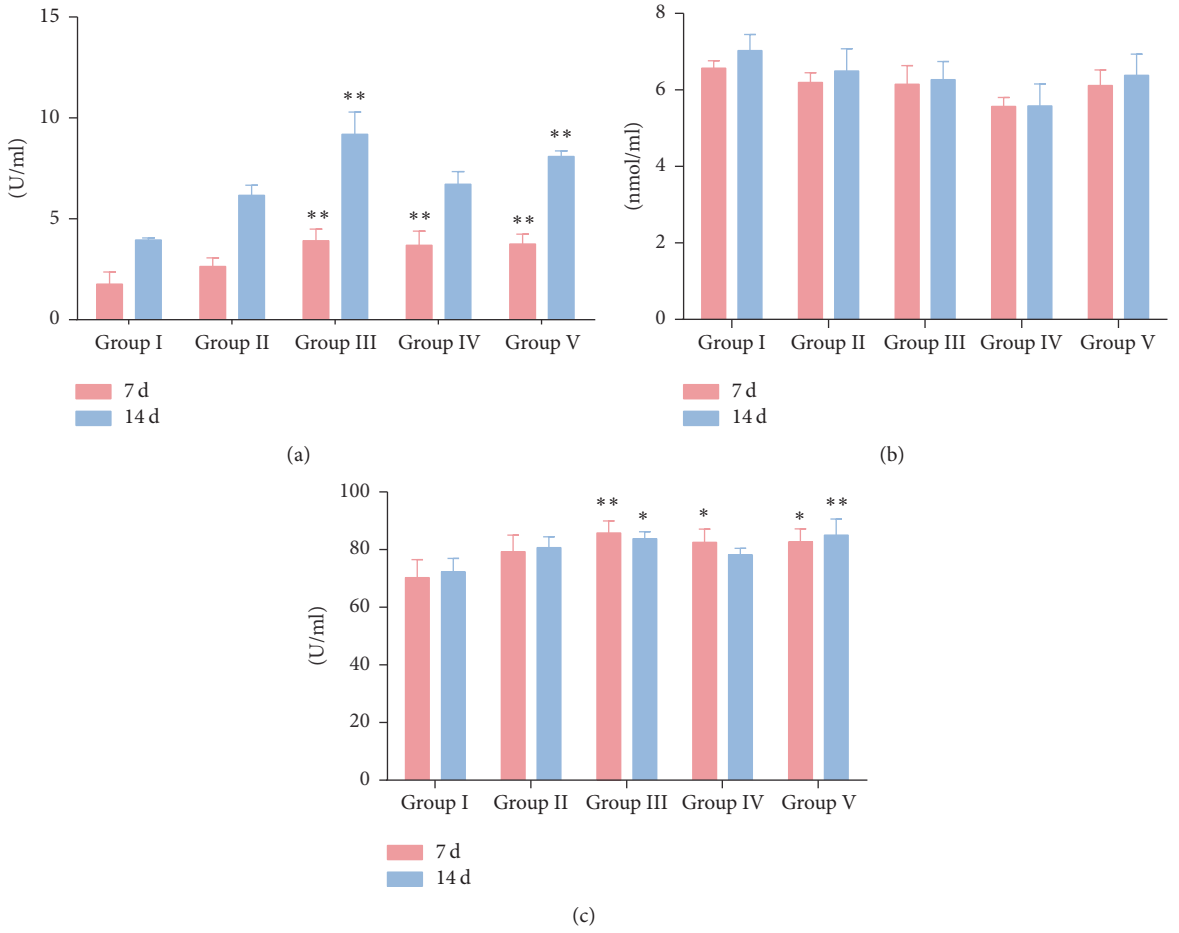

Figure 4: Serum total antioxidant capacity. (a) Serum T-AOC activity; (b) serum MDA activity; (c) serum T-SOD activity. Group I, saline control; Group II, RDS 0.1 g/kg treated group; Group III, RDS 0.33 g/kg treated group; Group IV, RDS 1.0 g/kg treated group; Group V, *Echinacea purpurea* powder 0.05 g/kg treated group. Data are represented as means ± SE; $n = 6$; comparison was made with the saline control group; one-way ANOVA followed by Duncan test. The symbols represent statistical significance at $^{*}p < 0.05$ and $^{**}p < 0.01$.

Table 3: Blood routine examination.

Items		Group I	Group II	Group III	Group IV	Group V
WBC	(10^9/L)	13.27 ± 0.71	13.04 ± 1.23	21.54 ± 3.29**	21.09 ± 0.96**	18.7 ± 1.18*
NEUT	(10^9/L)	5.02 ± 0.25	4.16 ± 0.77	11.2 ± 1.69**	7.65 ± 0.11*	7.8 ± 0.69*
LY	(10^9/L)	7.87 ± 0.55	8.43 ± 0.48	9.43 ± 1.61*	13.21 ± 0.75**	10.26 ± 0.51*
MONO	(10^9/L)	0.31 ± 0.04	0.27 ± 0.01	0.86 ± 0.15**	0.63 ± 0.08*	0.61 ± 0.09*
HB	(g/L)	111 ± 1.32	113.67 ± 3.51	115.33 ± 6.64	110.67 ± 0.56	113.33 ± 3.19
PLT	(10^9/L)	524.33 ± 55.85	448.67 ± 70.48	482.33 ± 52.38	430 ± 69.48	481.33 ± 52.59
RBC	(10^12/L)	6.9 ± 0.08	6.95 ± 0.14	6.61 ± 0.64	7.01 ± 0.09	6.78 ± 0.36

Group I, saline control; Group II, RDS 0.1 g/kg treated group; Group III, RDS 0.33 g/kg treated group; Group IV, RDS 1.0 g/kg treated group; Group V, *Echinacea purpurea* powder 0.05 g/kg treated group. Data are represented as means ± SE; $n = 6$; comparison was made with the model group; one-way ANOVA followed by Duncan test. The symbols represent statistical significance at $^{*}p < 0.05$ and $^{**}p < 0.01$.

found that RDS was the effective preparation of resveratrol and could significantly enhance immune function of piglets. *Echinacea purpurea* was shown to elicit an immune response by increasing the phagocytosis of granulocytes and the number of lymphocytes in fattening pigs as a feed additive [16]. Therefore, it was selected as a positive control drug to assess the effect on immune function of resveratrol. The results showed that RDS had a better immune-enhancing activity, suggesting that RDS had the potential to be used as an immunopotentiator.

In this study, RDS had no effect on the growth performance and organ coefficient of the piglets, which was similar to the previous study [17]. It was reported that standard diet supplemented with 300 or 600 mg resveratrol/kg significantly

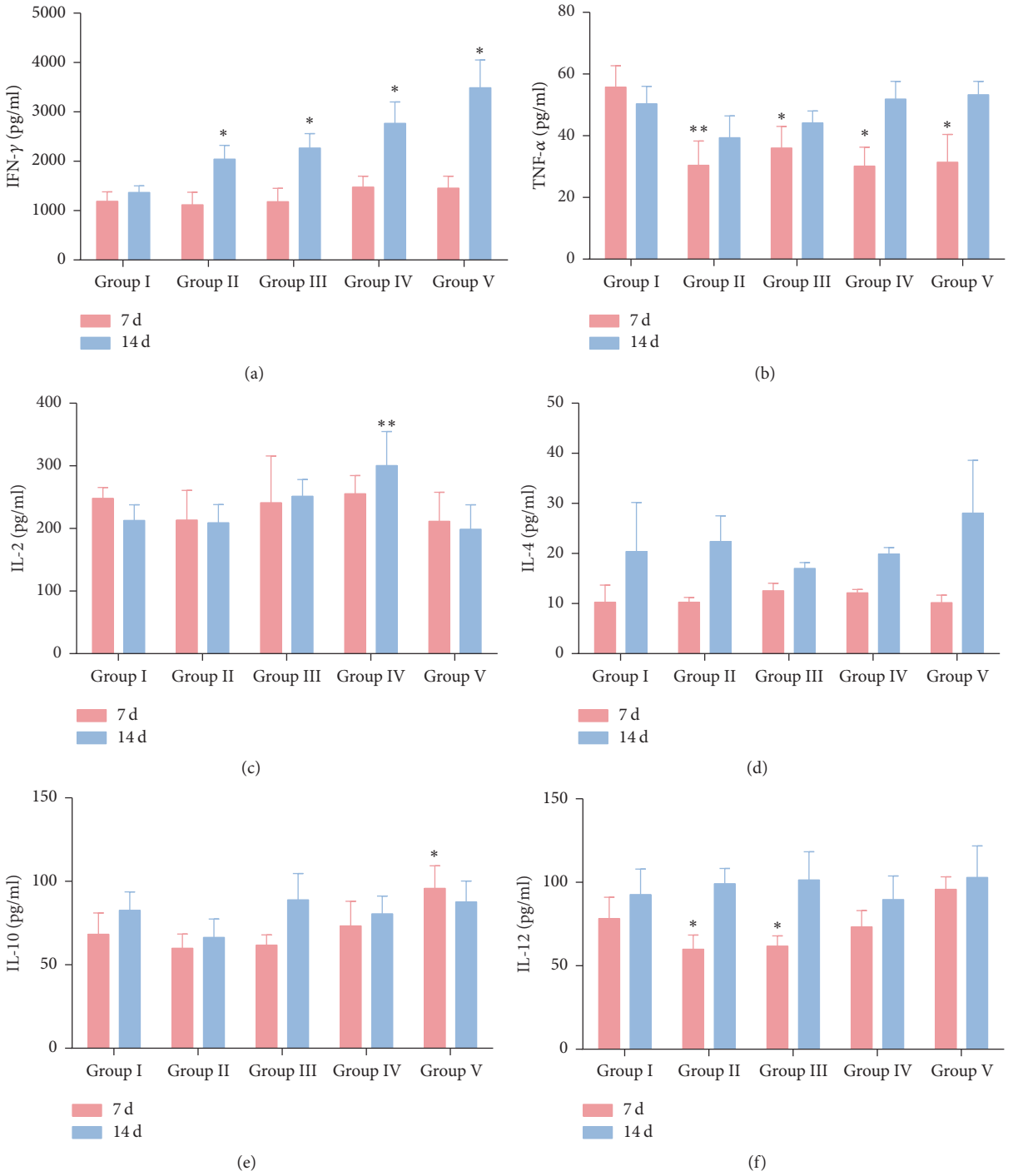

Figure 5: Cytokines levels in serum. (a) IFN-γ levels; (b) TNF-α levels; (c) IL-2 levels; (d) IL-4 levels; (e) IL-10 levels; (f) IL-12 levels; Group I, saline control; Group II, RDS 0.1 g/kg treated group; Group III, RDS 0.33 g/kg treated group; Group IV, RDS 1.0 g/kg treated group; Group V, *Echinacea purpurea* powder 0.05 g/kg treated group. Data are represented as means ± SE; $n = 6$; comparison was made with the saline control group; one-way ANOVA followed by Duncan test. The symbols represent statistical significance at $^{*}p < 0.05$ and $^{**}p < 0.01$.

reduced the pig's liver coefficient being probable due to the decrease of the visceral adipose tissue weight [18].

CD3+CD4+ cell as a T helper/inducing cell secretes a variety of lymphokines which can regulate other cells involved in the immune response, while CD3+CD8+ cell as a cytotoxic T cell can secrete IFN-γ and kill the target cells carrying the antigen when it was activated [19]. The effect of resveratrol increasing the ratio of CD3+CD4+/CD3+CD8+ was confirmed in the obese model of C57BL/6 mice [20]. The reduction in CD3+CD4+/CD3+CD8+ ratio was usually associated with malignancies or the attack of the virus such as HIV infection [21], and the reduction also existed in the

Table 4: Serum biochemical indexes.

Items		Group I	Group II	Group III	Group IV	Group V
TP	(g/L)	51.87 ± 0.27	53.3 ± 1.52	52.2 ± 2.2	49.43 ± 1.29	53.48 ± 0.79
ALB	(g/L)	36.07 ± 1.42	40.07 ± 2.24	36.43 ± 1.59	34.1 ± 1.12	38.72 ± 0.33
TBIL	(μmol/L)	1.37 ± 0.13	1.57 ± 0.18	2.6 ± 0.52	1.57 ± 0.06	1.47 ± 0.12
ALT	(IU/L)	30.67 ± 2.05	26.2 ± 1.45	37.93 ± 6.76**	29.7 ± 2.56	29.07 ± 1.03
AST	(I/L)	42.77 ± 1.38	50.23 ± 7.54	85.1 ± 10.79	59.1 ± 2.76	59.95 ± 2.48
ALP	(IU/L)	249.37 ± 11.59	250.67 ± 19.04	235.9 ± 30.28	265.13 ± 18.11	247.51 ± 5.78
γ-GT	(U/L)	47.47 ± 2.55	44.53 ± 1.7	49.9 ± 2.76	56.2 ± 2.72	54.62 ± 1.91
BUN	(mmol/L)	3.34 ± 0.45	4.73 ± 0.15**	3.79 ± 0.44	3.67 ± 0.19	3.83 ± 0.14
CRE	(μmol/L)	75.67 ± 3.94	76 ± 0.63	86.67 ± 5.42*	85.67 ± 0.76*	83.14 ± 2.43
GLU	(mmol/L)	5.44 ± 0.26	5.49 ± 0.19	4.69 ± 0.13**	5.45 ± 0.11	5.34 ± 0.17
TC	(mmol/L)	1.66 ± 0.2	1.92 ± 0.03	1.9 ± 0.15	1.56 ± 0.02	1.95 ± 0.07
TG	(mmol/L)	0.36 ± 0.07	0.61 ± 0.09**	0.48 ± 0.02	0.45 ± 0.02	0.48 ± 0.01
CK	(IU/L)	949 ± 330.45	816.33 ± 126.21	2493.33 ± 1061.58	1596 ± 360.01	743.33 ± 60.97
K	(mmol/L)	4.95 ± 0.18	4.79 ± 0.07	4.62 ± 0.4	5.27 ± 0.09	4.84 ± 0.15
Na	(mmol/L)	136.53 ± 2.21	132.1 ± 0.66	137 ± 2.18	136.47 ± 0.53	136.18 ± 1.21
Cl	(mmol/L)	96.7 ± 2.16	93.13 ± 1.48	98.33 ± 1.75	97.37 ± 0.14	98.47 ± 0.88
Ca	(mmol/L)	2.92 ± 0.07	3.07 ± 0.13	2.8 ± 0.12	2.83 ± 0.05	2.92 ± 0.1

Group I, saline control; Group II, RDS 0.1 g/kg treated group; Group III, RDS 0.33 g/kg treated group; Group IV, RDS 1.0 g/kg treated group; Group V, *Echinacea purpurea* powder 0.05 g/kg treated group. Data are represented as means ± SE; $n = 6$; comparison was made with the model group; one-way ANOVA followed by Duncan test. The symbols represent statistical significance at $^{*}p < 0.05$ and $^{**}p < 0.01$.

mouse model of systemic lupus erythematosus [22]. In our study, there was no significant difference between the normal and treated groups. When referring to the normal human range of 1.1–2 [23], the ratio of piglets was considered to have a normal fluctuation.

T lymphocytes can be transformed into lymphoblasts for cell division and proliferation in vitro culture under the stimulation of mitogen, such as concanavalin (ConA). Antigen stimulation changed from steady state of small lymphocytes into large lymphocytes, accompanied by increased cell volume and lighter nuclear staining, nucleolus, and cytoplasmic ribosome. Then, lymphocyte division and proliferation of effector cells took place [24]. Lymphocyte proliferation tests are often used to assess cellular immune function. It is reported that there was a trend for increased proliferation for cells treated with resveratrol [25]. Compared to the immunosuppressive mice, spleen lymphocyte proliferation was enhanced with resveratrol-treatment [26]. In our study, all RDS-treatment groups showed a positive effect on the activation and proliferation of T lymphocytes in spleen and in peripheral blood. Our study also demonstrated that RDS was effective in activating the function of T lymphocytes stimulated by antigens.

Natural products have been shown to serve as adjuvants that can enhance animal antibody levels under the stimulation of vaccines. Astragalus polysaccharide and oxymatrine have been reported to possess synergistical immunoenhancement in enhancing the immune efficacy of Newcastle disease vaccine [27]. The antibody titer against infectious bursal disease virus in broilers with treatment of *Echinacea purpurea* extract (0.1–1 g/kg) was significantly higher than that in control group [28]. Adding 0.5% *Echinacea* into diet had an enhancing effect on response of influenza vaccine [29]. Swine fever and swine foot-and-mouth disease are acute and infectious diseases which happened worldwide and brought huge losses to mankind [30]. In the present study, both RDS treatment (0.33 g/kg and 1.0 g/kg) and *Echinacea* treatment significantly improved the antibody titers against CSFV and FMDV, and the activity of RDS treatment was superior to *Echinacea* treatment. A recent study evaluated the effects of resveratrol on inflammatory response and antibody production against *Philasterides dicentrarchi* induced in turbot; the results showed a good regulatory effect of resveratrol on the inflammatory response the vaccine induced [31]. These results suggested that resveratrol could be considered as an adjuvant to enhance the immune response of vaccine in animals.

Immunoglobulins are formed in spleen and lymph nodes and secreted by mature plasma cells. They exist in the serum, body fluids, and tissues and can be directly involved in humoral immunity. Resveratrol supplementation remarkably promoted the production of immunoglobulin G in rats [32]. Similar studies also reported that dietary supplementation of 0.2% resveratrol improved the serum IgG levels in piglets [17]. In the first week of our trial, the levels of IgG, IgM, and IgA in serum were increased in varying degrees with different dose of RDS supplementation, while these effects could not be observed at the end of the second week. We speculate that this may be due to the improvement of the immune system in the growth process of piglets, and the impact of drug treatment on its immune response has diminished. These results suggested that RDS may be more effective in immunocompromised animals in regulating and participating in immune responses.

Recently, the antioxidant activity of resveratrol has been fully confirmed by various experiments. It has been shown

that resveratrol can exhibit prooxidant properties, leading to oxidative breakage of cellular DNA in the presence of transition metal ions, such as copper, which hinted the anticancer and chemopreventive properties of resveratrol [33]. Resveratrol may protect against oxidant injury due to its capacity to inhibit COX-2-derived PGE 2 synthesis [34]. A study in rats showed that resveratrol significantly and dose-dependently decreased brain MDA level and increased brain SOD, catalase, and peroxidase activities [35]. RDS has been proven to enhance the activities of T-AOC and SOD in our experiment, while it did not affect the level of MDA in the serum. These studies showed that RDS enhanced the ability to scavenge oxygen free radicals and improved the total antioxidant capacity.

Resveratrol can regulate the secretion of cytokines by mediating and activating immune cells. It was reported that TNG-α levels in diabetic rats treated with resveratrol (5 g/kg) have decreased significantly [36], and this trend was also be demonstrated in our study. The mechanism may be due to the downregulation of JAK-STAT pathway and decreasing the levels of activated STAT1 in the nucleus [37]. Besides, resveratrol could reduce the release of proinflammatory cytokines on human periodontal ligament cells, such as IL-12 stimulated by LPS [38]. In our study, RDS was involved in the regulation of humoral immune responses by upregulating the release of IFN-γ and downregulating the release of TNF-α.

Blood routine and biochemical tests are often used to assist in the diagnosis of diseases and to observe the toxicity of drugs. In our study, the increase in WBC, NEUT, LY, and MONO suggested that a slight inflammation may have taken place in the RDS-treatment groups (0.33 g/kg and 1.0 g/kg) and *Echinacea purpurea*-treatment group. Resveratrol suppressed oxidative and inflammatory stress response to a high-fat, high-carbohydrate meal [39]. In the present study, the blood glucose (GLU) levels in the RDS-treatment (0.33 g/kg) group were also reduced, which was similar to the report. RDS had no significant effect on liver function, renal function, and electrolyte and other biochemical indexes in comparison with blank control. A small number of indicators (rise or fall) were still within the normal range of fluctuations, which can be accepted when referring to normal levels [40]. These tests suggested that RDS was lowly toxic or nontoxic to piglets.

5. Conclusion

In summary, RDS significantly affects the development, maturation, proliferation, and transformation of T lymphocytes and is involved in the regulation of humoral immune responses by upregulating the release of IFN-γ and downregulating the release of TNF-α. It significantly increased the antibody titers of the piglets under the stimulation of CSFV and FMDV when immunized against the vaccine. It showed an excellent resistance to oxidation and enhanced the T-SOD activity, and it has low toxicity. These positive effects hint that RDS could be considered as an adjuvant to enhance the body's immune response to vaccines, as well as dietary additives for animals to enhance humoral and cellular immunity and to play antioxidant and antiaging effects.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Qiuting Fu, Qiankun Cui, and Yi Yang contributed equally to this work.

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