

Research Article

Bioassay-Guided Isolation and Antioxidant Evaluation of Flavonoid Compound from Aerial Parts of *Lippia nodiflora* L.

A. Sudha and P. Srinivasan

Department of Bioinformatics, Alagappa University, Karaikudi, Tamil Nadu 630004, India

Correspondence should be addressed to P. Srinivasan; sri.bioinformatics@gmail.com

Received 27 February 2014; Revised 16 April 2014; Accepted 16 April 2014; Published 25 May 2014

Academic Editor: Kota V. Ramana

Copyright © 2014 A. Sudha and P. Srinivasan. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The present study was designed to identify the DPPH (2, 2-diphenyl-1-picrylhydrazyl) free-radical scavenging constituents from methanol extract of *L. nodiflora* using bioassay-guided fractionation. The ethyl acetate fraction (EAF) revealed a strong antioxidant activity, compared to other fractions through *in vitro* DPPH radical-scavenging assay. The repeated fractionation of active EAF by silica gel column chromatography yielded a compound with strong antioxidant potential. The isolated bioactive compound was determined as 2-(3, 4-dimethoxyphenyl)-5-hydroxy-7-methoxy-4H-chromen-4-one (5-hydroxy-3', 4', 7-trimethoxyflavone), by comparing spectral data (UV, IR, ¹H NMR, ¹³C NMR, and MS) with literature reports. The isolated compound demonstrated an excellent antioxidant activity through all antioxidant assays and also significantly inhibited lipid peroxidation at a concentration of 50 µg/mL. The results obtained suggested that extracts from *L. nodiflora* or its derived phytochemical can be used potentially as a bioactive source of natural antioxidants by contributing beneficial health effects.

1. Introduction

Reactive oxygen species (ROS) are continuously formed as a by-product of metabolisms in aerobic organisms and are also produced on exposure to tobacco smoke, ozone, radiations, organic solvents, and other environmental pollutants [1]. ROS play an important role in various physiological processes, including energy production, phagocytosis, cellular signal transduction, cell proliferation, differentiation, and apoptosis. On the other hand, increasing evidence highlights that overproduction of ROS can induce oxidative damage to all biomolecules (lipids, carbohydrates, proteins, enzymes, DNA, and RNA) and acts as a mediator of numerous disorders, for example, inflammation, arthritis, diabetes, arteriosclerosis, cancer, genotoxicity, and neurological disorders such as Alzheimer's disease [2]. Antioxidants are very essential for averting degenerative reactions produced by free radicals and reactive oxygen species, which have been concerned with many diseases and in food deterioration and spoilage [3]. However, the safety of some of the synthetic antioxidants used in the food industry has been questioned,

because recent studies recognized that they might be carcinogenic [4]. Hence, there is an emerging interest in natural antioxidants, which might help to prevent oxidative damage [5].

Plants and plant products are magnificent sources of phytochemicals and have been found to hold an array of biological activities including antioxidant potential [6]. Plants synthesize antioxidant compounds, mostly flavonoids and polyphenols, which have been reported to protect the human body from various diseases by neutralizing ROS. Recently, phenolic compounds have received increasing significance among various phytochemicals, due to their wide distribution in the plant kingdom and for their biological activities, namely, anticarcinogenic, antiatherogenic, anti-inflammatory, antimicrobial, and antioxidant activities [7–9]. Antioxidants can be either used as dietary, food supplement or as a drug by the humans [10]. Several studies have revealed that the increased dietary intake of natural antioxidants, such as flavonoids and other phenolic compounds, almost present in plants, exhibits potential protective effects against many degenerative diseases [11–13].

Lippia nodiflora Linn. (Verbenaceae), commonly called Poduthalai in Tamil, is a creeping perennial herb and locally abundant in wet regions, and several medicinal properties are attributed to this plant in the traditional system of medicine. The infusion of the leaves and tender stalks is given to children suffering from indigestion and to women after delivery. The chutney made from the leaves and fruits are often taken to relieve the irritation of internal piles [14]. Numerous pharmacological properties of *L. nodiflora* including anti-inflammatory, antipyretic, antitussive, antidiabetic, and antimelanogenesis effects have been reported [15–18]. The ethnopharmacological relevance of *L. nodiflora* for skin diseases and in folk cosmetics, such as pimples, carbuncle, and skin burns, has also been revealed [19]. The phytochemical constituents of *L. nodiflora*, such as flavonoids [20, 21], flavone glycosides [22], alkaloids, essential oils, resin [23], quinol [24], and steroids [25, 26], have been previously reported. Hence, these phytochemicals are considered to be accountable for the pharmacological properties of this plant. Even though there is an evidence for the antioxidant activity of methanolic extract of *L. nodiflora*, the major antioxidative constituents present in the aerial parts have not been extensively investigated. Hence, the present study was undertaken to isolate active compounds responsible for the antioxidant property of methanol extract of *Lippia nodiflora* L. through bioassay-guided fractionation using *in vitro* DPPH assay.

2. Materials and Methods

2.1. General. Nuclear magnetic resonance spectra were recorded on BRUKER, Avance 400 MHz (Switzerland) NMR instrument, operating at 400 MHz for ^1H and 100 MHz for ^{13}C nuclei at room temperature and referenced to the residual solvent signal. The chemical shift and coupling constants (J) values are reported in ppm and Hz, respectively. HPLC analysis was performed using a C-18 column (250 × 4.6 mm, 5 μ) in a Shimadzu LC-8A chromatographic apparatus (Shimadzu, Singapore). The mobile phase consisted of methanol-0.5% phosphoric acid in water (60:40, v/v) and the flow rate was held constant at 1 mL/min. The peaks are detected at 280 nm, using variable wavelength UV detector. Silica gel 60 F₂₅₄ plates (20 × 20 cm, 0.2 mm thick; E-Merck, Germany) were used for thin-layer chromatography (TLC) analysis. The ultraviolet spectra were recorded using Varian Cary 500 scan spectrophotometer, λ_{max} (log ϵ) in nm, whereas the FTIR spectrum was obtained using a Nicolet 380 (Thermo Scientific, USA). The functional group was identified using potassium bromide (KBr) and scanned in the range of 4000–400 cm^{-1} . Sample was dissolved in methanol and ESI mass spectra were obtained with a Thermo Scientific Exactive Mass Spectrometer (Thermo Fisher Scientific, USA).

The reagents such as 2-deoxy-D-ribose, butylated hydroxyl toluene (BHT), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), phenazine methosulphate (PMS), nitroblue tetrazolium (NBT), sodium nitroprusside, and Griess reagent were obtained from (Sigma Chemicals St. Louis, MO, USA). 2, 4, 6-Tripyridyl-S-triazine (TPTZ), thiobarbituric acid (TBA),

trichloroacetic acid (TCA) ethylene diaminetetraacetic acid (EDTA), ferric chloride (FeCl_3), hydrogen peroxide (H_2O_2), and nicotinamide adenine dinucleotide-reduced (NADH) were obtained from M/s (Sisco Research laboratories, Mumbai, India). HPLC grade solvents and reagents used for extraction and silica gel (0.075–0.15 mm) for column chromatography were obtained from M/s (Sisco Research laboratories, Mumbai, India). All other chemicals and reagents used in this study were of analytical grade.

2.2. Plant Materials. The plant materials were freshly collected between August and September 2011, from Karaikudi, Sivagangai district, Tamil Nadu. The plant was taxonomically identified and authenticated by Dr. G. V. S. Murthy, Joint Director, Botanical Survey of India, Tamil Nadu Agricultural University Campus, Coimbatore (BSI/SRC/5/23/2012-13/Tech-19). A voucher specimen of the plant *L. nodiflora* (DBI-HP: Specimen-1) was prepared and preserved in the Molecular Biology Lab, Department of Bioinformatics, Alagappa University, Karaikudi, Tamil Nadu.

2.3. Extraction, Fractionation, and Isolation of Antioxidant Compound. The aerial parts (stem, leaves, and flowers) were washed with tap water, shade-dried, and reduced to fine powder. The powdered aerial parts of *L. nodiflora* (1.5 kg) were extracted with 90% methanol (4.5 L × 2) at room temperature. The mixture was filtered through Whatman number 1 filter paper and the solvents from the combined extract were concentrated using a vacuum rotary evaporator (Superfit, India), at 60°C to afford 64.7 g of crude methanol extract (4.31%). The solvent was selected based on its yield from preliminary extraction and phytochemical screening studies. The extract was dissolved in 500 mL of warm water, and the resulting aqueous portion was partitioned with ethyl acetate (EtOAc) (4 × 200 mL) using a separating funnel to afford ethyl acetate fraction (EAF) and aqueous portion. The aqueous phase was then successively partitioned with n-butanol (3 × 200 mL), thus obtaining n-butanol soluble fraction (BF) and water fraction (WF). All the fractions were collected separately and reduced using a vacuum rotary evaporator to remove the solvent and the resultant aqueous extract was lyophilized *in vacuo*. The samples were then tested for its antioxidant property using 2, 2-diphenyl-1-picrylhydrazyl (DPPH $^{\bullet}$) assay.

The DPPH $^{\bullet}$ active EAF (17.2 g) was loaded as a dried slurry of silica gel to column chromatography (45 × 3.5 cm) and eluted with petroleum ether: EtOAc gradient elution (100:0—0:100), in increasing order of polarity. A total of 92 fractions of 100 mL each were collected and analyzed by TLC (Silica gel F₂₅₄ plates 20 × 20 cm, Merck, Germany). TLC analysis was carried out using ethyl acetate: chloroform: formic acid (5:4:1) as the mobile phase and the separated bands were visualized using iodine vapors and vanillin-sulphuric acid reagent. These fractions were pooled to afford seven major fractions (Fr. A: 1–13, Fr. B: 14–35, Fr. C: 36–48, Fr. D: 49–60, Fr. E: 61–70, Fr. F: 71–80, and Fr. G: 81–92) based on TLC analysis. These fractions were tested for bioactivity using DPPH spectrophotometric assay.

For further purification, the highly active Fr. B (2.8 g) was loaded on a silica gel column, eluted with petroleum ether-EtOAc gradients, and the ethyl acetate content of the mixture was increased in a series of 5% steps. The inactive and less active proved fractions were discarded. Finally, the active Fr. B₂ eluted with petroleum ether-ethyl acetate (85:15) yielded 117 mg of compound. The purity of isolated compound was established by HPLC and its structure was confirmed through the interpretation of the spectral data (UV, FT-IR, ¹H, ¹³C NMR, and MS) and further tested for its antioxidant effects.

2.4. Antioxidant Activities

2.4.1. DPPH Radical Scavenging Assay. The DPPH radical scavenging activities of methanol extract, EAF, BF, and WF were tested according to Yamaguchi et al. [27]. Briefly, 0.2 mL of the sample solutions of different concentrations was added to 1 mL of 0.1 mM of freshly prepared DPPH solution. The reaction mixtures were shaken vigorously and absorbance at 517 nm was determined after 20 min at room temperature. Control sample was prepared containing the same volume without test compounds or reference antioxidants, while DMSO was used as blank. The reference antioxidant BHT was used as the positive control in all the assays. The radical scavenging activity was measured as a decrease in the absorbance of DPPH[•] and calculated as follows:

$$\text{Scavenging effect (\%)} = \left[\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right] \times 100, \quad (1)$$

where A_{control} is the absorbance of the control and A_{sample} is the absorbance of the extract or fractions or standard.

2.4.2. Superoxide Radical-Scavenging Assay. The superoxide radical-scavenging effect was determined by the method of Nishikimi et al. [28]. The reaction mixture with NBT (1 mM) in phosphate buffer (0.1 M, pH 7.4), NADH (1 mM) with or without samples, and PMS (0.1 mM) was incubated at room temperature for 5 min and the absorbance was recorded at 560 nm. The inhibition percentage was calculated against a control without the samples. The scavenging ability was calculated using the equation as described for DPPH assay.

2.4.3. Hydroxyl Radical Scavenging Assay. The capacity of the extract and compound to reduce hydroxyl radical-mediated peroxidation was carried out by the method of Hinneburg et al. [29]. Briefly, 0.5 mL of 5.6 mM 2-deoxy-D-ribose in KH_2PO_4 -NaOH buffer (50 mM, pH 7.4), 0.2 mL of 100 μM FeCl_3 , and 104 mM EDTA (1:1 v/v) solution were added to 0.1 mL of different concentrations of test samples, followed by 100 μL of 1.0 mM H_2O_2 and 0.1 mL of 1.0 mM aqueous BHT. The reaction mixtures were shaken vigorously and incubated at 50 °C for 30 min. Subsequently, 1 mL of 2.8% TCA and 1 mL of 1.0% TBA were added to each tube containing reaction mixture and samples were mixed well again and boiled in a water bath at 50 °C for 30 min. The absorbance of solution was read at 532 nm. The hydroxyl radical scavenging ability was

calculated using the formula as described for DPPH assay and the values are presented as means of triplicate analyses.

2.4.4. FRAP (Ferric Reducing Antioxidant Power) Assay. The FRAP assay was determined by the method of Benzie and Strain [30] with minor modifications. It depends on the ability of the sample to reduce the ferric tripyridyltriazine (Fe (III)-TPTZ) complex to ferrous tripyridyltriazine (Fe (II)-TPTZ) at low pH. Fe (II)-TPTZ has an intensive blue color which can be read at 593 nm. The stock solutions consist of 300 mM acetate buffer (pH 3.6), 10 mM 2, 4, 6 tripyridyl S triazine (TPTZ) in 40 mM of HCl, and 20 mM ferric chloride solution. The fresh working solution was prepared by mixing 25 mL of acetate buffer, 2.5 mL of TPTZ, and 2.5 mL of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and the temperature was maintained to 37 °C before use. The various concentrations of extract, compound, and BHT (10–50 $\mu\text{g}/\text{mL}$) were allowed to react with 2 mL of the FRAP solution for 30 min in the dark condition. The absorbance was recorded at 593 nm. The results are expressed in μM Fe (II)/g and were estimated using aqueous $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (20–100 μM) as standard for calibration.

2.4.5. Nitric Oxide Radical Scavenging Assay. At physiological pH, nitric oxide generated from sodium nitroprusside in aqueous solution interacts with oxygen to produce nitrite ions, which were measured by the Griess reaction [31]. Briefly, 3 mL of the reaction mixture containing 10 mM sodium nitroprusside and the test samples (10–50 $\mu\text{g}/\text{mL}$) in phosphate buffer were incubated for 150 min at 25 °C. After incubation, 0.5 mL of the reaction mixture was mixed with 1 mL of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for complete diazotization. Then, 1 mL of naphthyl ethylene diamine dihydrochloride (0.1% w/v) was added and the mixture was allowed to stand for 30 min at 25 °C. A pink colored chromophore is generated and the absorbance was measured spectrophotometrically at 540 nm against a blank sample. The nitric oxide radical scavenging activity of the extract and compound is reported as % inhibition and was calculated using the formula as described for DPPH assay.

2.4.6. Lipid Peroxidation Assay. Lipid peroxidation (LPO) assay was performed according to the protocol described by Damien et al. [32] to measure the lipid peroxide formed, using egg yolk homogenates as lipid-rich media. Briefly, 0.5 mL of egg homogenate (10% v/v prepared in 1.15% w/v KCl) was added to 0.1 mL of each test samples (10–50 $\mu\text{g}/\text{mL}$) taken in a test tube and made up to 1 mL with double distilled water. Thereafter, 0.05 mL of FeSO_4 (0.07 M) was added to induce lipid peroxidation, and the mixture was incubated for 30 min at room temperature. Then, 1.5 mL of 3.5 M acetic acid (pH adjusted to 3.5) was added, followed by 1.5 mL of TBA (0.06 M) in sodium dodecyl sulphate (0.04 M). The resulting mixture was vortex and heated at 95 °C for 1 hr. After cooling, 5 mL of butanol was added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm and the above procedure was

followed for the control by using 0.1 mL of SDS instead of the test sample. The percentage inhibition was calculated according to the following formula:

$$\text{Percentage inhibition of lipid peroxidation} = \left(1 - \frac{E}{C}\right) \times 100, \quad (2)$$

where E is the absorbance of the test sample and C is the absorbance of the fully oxidized control.

2.5. Statistical Analysis. The experiments were carried out in triplicate and data were expressed as means \pm standard deviation (SD). All statistical analyses were performed using graph pad prism (version 5.0; Graph Pad software Inc. San Diego, CA, USA). The IC_{50} value represented the concentration of the test samples that caused 50% inhibition. P values <0.05 were considered as significant.

3. Results and Discussion

3.1. Isolation and Structure Determination of Antioxidant Compound. The methanol extract and potential antioxidant fractions of *L. nodiflora* were initially screened by spectrophotometric DPPH assay and the results are shown in Table 1. The DPPH assay revealed that the methanol extract had significant scavenging effects with increasing concentrations in the range of 10–50 $\mu\text{g/mL}$. Moreover, the scavenging effect of methanol extract was significantly similar to that of standard BHT. At 50 $\mu\text{g/mL}$, methanol extract and BHT exhibited 79.35% and 86.42% inhibition and the IC_{50} values (the concentration with scavenging activity of 50%) were 24 and 19 $\mu\text{g/mL}$, respectively. The result obtained herein was lower than the reported scavenging activity of methanol extract of *L. nodiflora* (12.03 $\mu\text{g/mL}$) [33]. Among the fractions, the DPPH* was significantly scavenged by ethyl acetate fraction (EAF) in a dose-dependent manner, with an IC_{50} value of 26 $\mu\text{g/mL}$, followed by WF and BF, with IC_{50} values of 66 and 83 $\mu\text{g/mL}$ (Table 1). Hence, the EAF was subjected to repeated bioassay-guided fractionation on silica gel column chromatography using petroleum ether: EtOAc gradient elution system. The further purification of active fractions obtained from silica gel column chromatography finally yielded a bioactive antioxidant compound from the petroleum ether-ethyl acetate (85:15 v/v) mixture. The extraction procedure for the isolation of active compound was schematically shown in Figure 1. Figure 2(a) shows the HPLC profile of the chemical constituents present in the 90% methanol extract of *L. nodiflora*. The HPLC chromatogram of purified compound exposed the presence of a peak, with a retention time of 7.2 min, eluted isocratically with the mobile phase of methanol-0.5% phosphoric acid in water (60:40, v/v) (Figure 2(b)).

The compound was obtained as pale white amorphous powder (yield: 117 mg, 0.68%). The FTIR peaks determined the bonds relevant to alcoholic O–H stretching (3267 cm^{-1}), =C–H stretching (3010 cm^{-1}), –C–H stretching (2975 cm^{-1}), –C=O stretching (1605 cm^{-1}), –C=C stretching (1506 cm^{-1}),

TABLE 1: DPPH radical scavenging activity of the crude extract and fractions of aerial parts of *Lippia nodiflora* L.

Sample	DPPH (IC_{50}) ($\mu\text{g/mL}$)
CME	24.66 \pm 1.1
EAF	26.06 \pm 0.95
BF	83.62 \pm 0.45
WF	66.39 \pm 0.52
BHT ^a	19.12 \pm 0.57

^aBHT: Standard antioxidant; data represented as means \pm SD ($n = 3$).

–C–H bending (1154 cm^{-1}), C–O stretching (1315 cm^{-1}), =C–H bending (1030 cm^{-1}), and O–H bending (993 cm^{-1}). ¹H NMR (CDCl_3 , 400 MHz): δ (ppm) 3.91 (3H, s, 4'-OMe), 3.89 (3H, s, 7-OMe), 3.87 (3H, s, 3'-OMe), 6.49 (s, 1H, OH), 6.64 (d, $J = 6.63$ Hz, 1H, H6), 6.898 (s, 1H, H8), 6.99 (d, $J = 6.3$ Hz, 1H, H2'), 7.52 (d, $J = 6.3$ Hz, 1H, H5'), 8.178 (s, 1H, H6'); ¹³C NMR (CDCl_3 , 100 MHz): δ (ppm) 171.61 (C-4), 144.30 (C-4'), 125.69 (C-1'), 162.87 (C-2), 161.77 (C-3') 156.82 (C-5), 130.87 (C-6'), 114.28 (C-5'), 137.29 (C-9), 103.92 (C-2'), 157.74 (C-7), 98.92 (C-6), 98.19 (C-8), 111.47 (C-3), 113.58 (C-10), 54.51 (7-OMe), 54.76 (3'-OMe), 54.88 (4'-OMe) (Figures S₁ and S₂ of the Supplementary Information available online at <http://dx.doi.org/10.1155/2014/549836>). The compound exhibits a molecular weight of 329 (ESI-MS; m/z 351.084 [$M + \text{Na}$]⁺) with an elemental formula of $\text{C}_{18}\text{H}_{16}\text{O}_6$ (Figure 3). From these spectral interpretations, the isolated compound has been characterized as 2-(3, 4-dimethoxyphenyl)-5-hydroxy-7-methoxy-4H-chromen-4-one (5-hydroxy-3', 4', 7-trimethoxyflavone) (Figure 4), which agreed with the data reported [34].

3.2. Antioxidant Activities of Methanol Extract and Isolated Compound from Ethyl Acetate Fraction (EAF). Flavonoids, a major group of polyphenols, are considered to be the active principles in diverse medicinal plants and have been reported to possess numerous pharmacological properties. The most essential biological activity of flavonoids is mainly due to their antioxidant property by acting as radical scavengers, hydrogen donors, reducing agents, and peroxidation inhibitors [35]. Previous studies reported that the pharmacological effects of *L. nodiflora*, such as antioxidant, diuretic, anti-inflammatory, and antimicrobial activities, were recognized due to the presence of the phenol and flavonoid compounds [33, 36]. In the present study, the isolated compound was identified as flavone and its antioxidant activities were examined by using various *in vitro* antioxidant models.

3.2.1. DPPH Radical Scavenging Effect. The substances are considered to be antioxidants, when they are capable of reducing the stable DPPH radical (purple) to the nonradical form DPPH-H (yellow), and thus they act as radical scavengers due to their hydrogen donating abilities [37]. The results of DPPH scavenging activity of all test samples are presented in Figure 5(a). The scavenging activity of methanolic extract, compound, and BHT increased with an increase in sample concentration (10–50 $\mu\text{g/mL}$). The

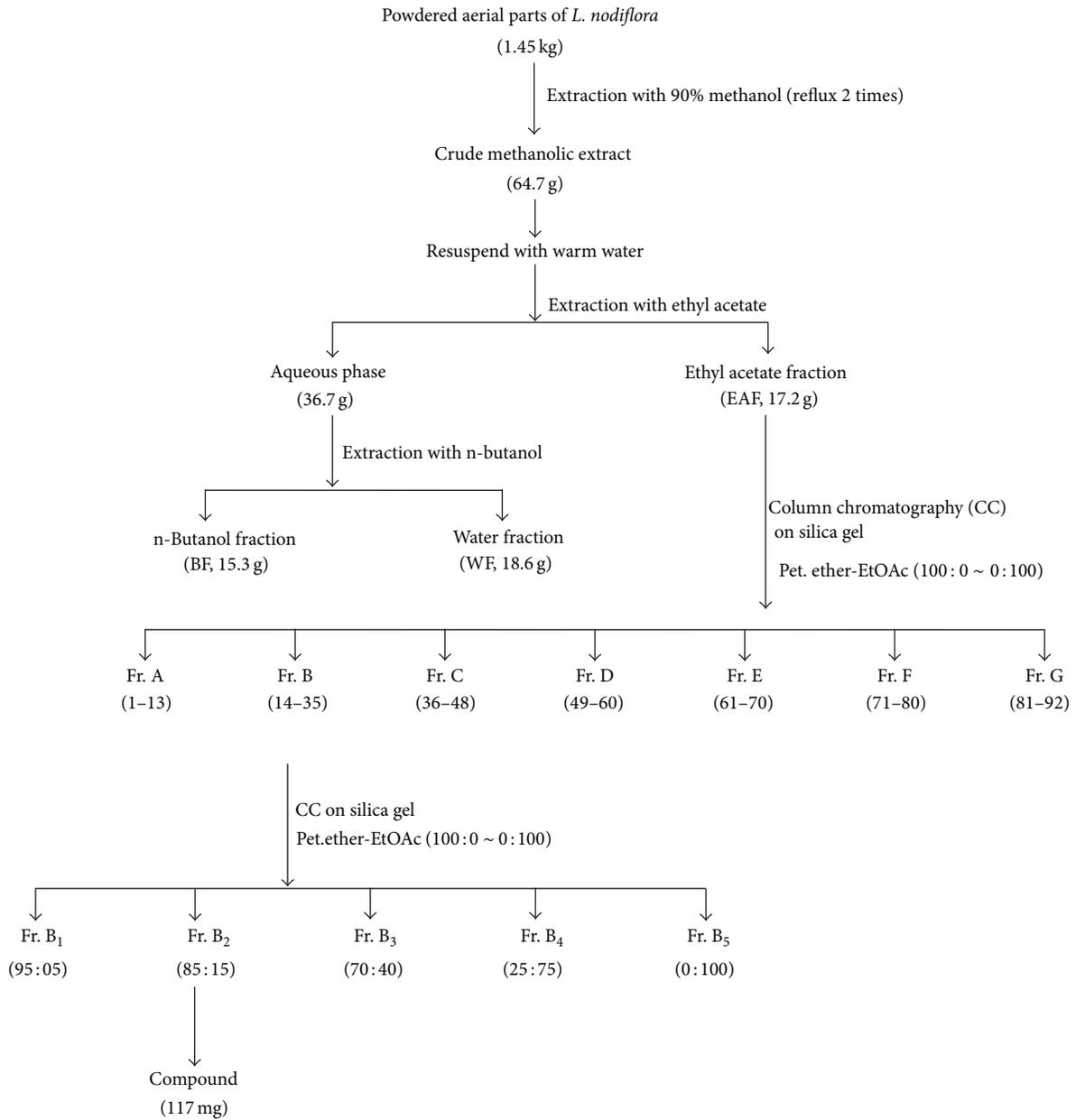


FIGURE 1: Extraction scheme for the isolation of antioxidant compound from aerial parts of *Lippia nodiflora* L.

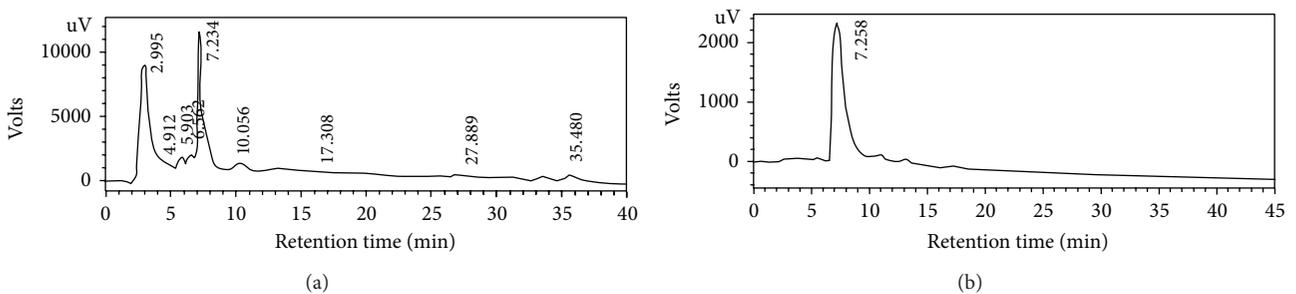


FIGURE 2: (a) HPLC chromatogram of whole methanol extract of *L. nodiflora*. (b) HPLC chromatogram of isolated compound (5-hydroxy-3', 4', 7-trimethoxyflavone) at 280 nm. The HPLC profile shows the purity of the isolated compound from aerial parts of *L. nodiflora*.

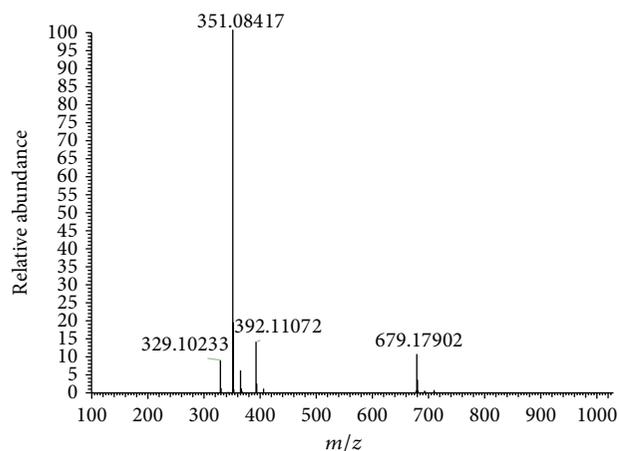


FIGURE 3: Molecular mass of isolated compound from aerial parts of *L. nodiflora*.

highest DPPH scavenging activity for extract, compound, and BHT was found to be 79.35%, 72.66%, and 86.09%, respectively, at 50 $\mu\text{g/mL}$. It should be noted that the scavenging activity of compound was found to be close to the extract. The IC_{50} values of scavenging activity on DPPH radical of extract and BHT are given in Table 1, whereas, for compound, it is found to be 27 $\mu\text{g/mL}$. From these obtained data, the extract and compound were considered as an effective free-radical inhibitor as well as the primary antioxidants, which may limit free-radical damage that takes place in the body.

3.2.2. Superoxide Radical Scavenging Effect. The formation of reactive oxygen species such as hydroxyl radical, hydrogen peroxide, and singlet oxygen in living system was mainly due to the participation of superoxide anion radicals, either directly or widely through enzyme or metal catalyzed progression [38]. It was therefore anticipated to evaluate the relative interceptive capacity of the extract and compound to scavenge the superoxide radical. From the data presented in Figure 5(b), it was noted that the extract, compound, and BHT showed the highest radical scavenging activities (71.07%–84.64%) at 50 $\mu\text{g/mL}$ and the scavenging activity increased with increasing concentration of the samples. The scavenging ability of compound on superoxide radicals was found to be moderate compared to methanolic extract. However, the scavenging activities of extract (83.09%) were found to be very closer to that of BHT (84.64%), which is considered to be a strong superoxide radical scavenger. The IC_{50} value of plant extract, compound, and BHT was found to be 32, 38, and 26 $\mu\text{g/mL}$, respectively.

3.2.3. Hydroxyl Radical Scavenging Effect. Hydroxyl radical, an extremely known reactive oxygen species, was competent to attack and spoil almost every molecule in the living cells [11]. They were also capable of stimulating lipid peroxidation process rapidly by attacking the fatty acid side chains of the membrane phospholipids [38]. The scavenging activities of methanolic extract, compound, and BHT on hydroxyl radical

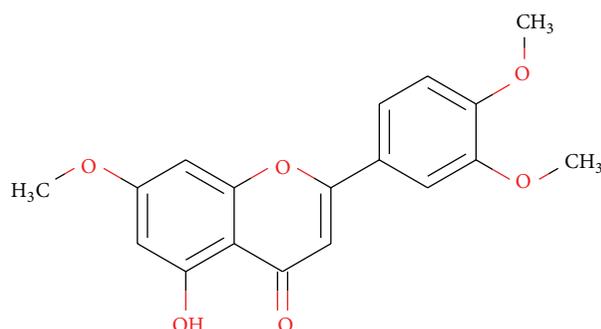


FIGURE 4: Structure of isolated compound (5-hydroxy-3', 4', 7-trimethoxyflavone) from ethyl acetate (EAF) fraction of aerial parts of *L. nodiflora*.

inhibition are shown in Figure 6. All the examined samples showed significant hydroxyl radical scavenging activity at 50 $\mu\text{g/mL}$ concentration and the scavenging activity for extract, compound, and BHT was 68.38%, 60.90%, and 74.16%, respectively. The methanolic extract of *L. nodiflora* ($\text{IC}_{50} = 36 \mu\text{g/mL}$) was more powerful than the compound ($\text{IC}_{50} = 43 \mu\text{g/mL}$). The positive control, BHT, was extremely effectual on hydroxyl radical scavenging, with an IC_{50} value of 32 $\mu\text{g/mL}$. This observed capacity of the extract and compounds to scavenge $\cdot\text{OH}$ radical pointed out that the tested samples could considerably inhibit lipid peroxidation, since $\cdot\text{OH}$ radicals are extremely distressed during peroxidation.

3.2.4. FRAP (Ferric Reducing Antioxidant Power) Assay. The ferric reducing/antioxidant power (FRAP assay) is widely used in the assessment of the antioxidant component in dietary polyphenols [39]. The reducing properties are usually related to the presence of compounds which exert their action by breaking the free-radical chain by donating a hydrogen atom [40]. The results of reductive potential of plant extract and compound relative to BHT, a well-known antioxidant data, are shown in Table 2. The reducing ability of the extract was in the range of 23.46–71.14 $\mu\text{M Fe (II)/g}$. The FRAP values for the methanol extract were significantly higher than that of compound and BHT, while the compound revealed the lowest FRAP values (11.18–51.70 $\mu\text{M Fe (II)/g}$). At 50 $\mu\text{g/mL}$, the FRAP value of *L. nodiflora* extract was found to be 71.14 compared to compound and BHT with FRAP value of 51.70 and 63.18, respectively (Table 2). This result implies that the isolated compound did not show reliable reducing power, when compared to its DPPH and superoxide radical scavenging abilities.

3.2.5. Nitric Oxide Radical Scavenging Assay. Nitric oxide radicals ($\text{NO}\cdot$) play a vital role in inducing inflammatory response and their toxic effects increase only when they react with superoxide radicals that damage biomolecules like proteins, lipids, and nucleic acids [41]. The suppression of NO radicals release may be attributed to a direct ($\text{NO}\cdot$) scavenging effect as both extract and compound decreased the amount of nitrite generated from the decomposition of sodium nitroprusside through *in vitro* studies as shown in

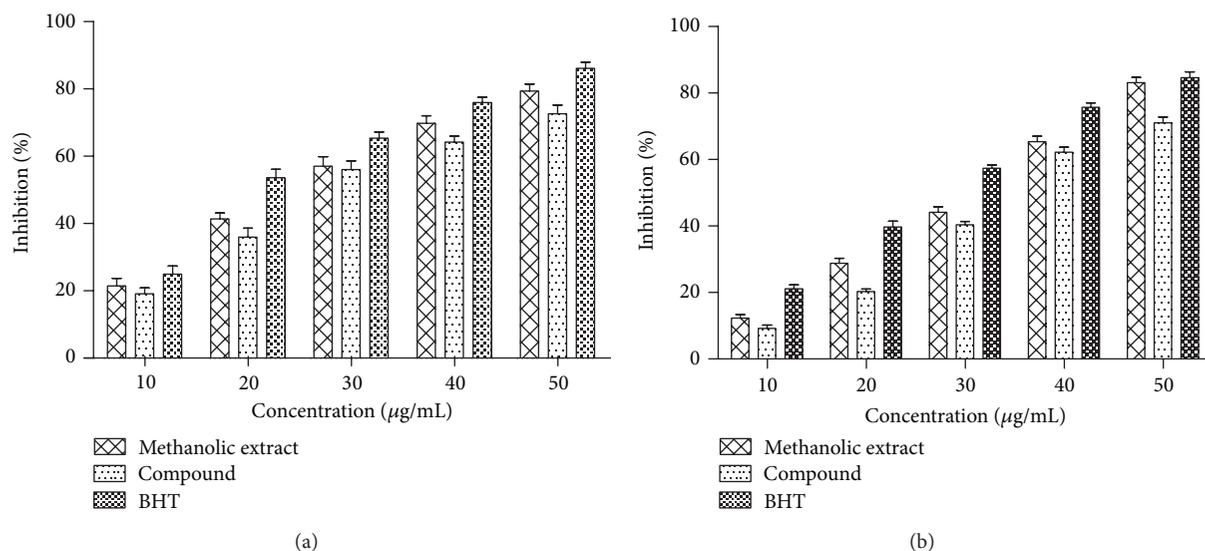


FIGURE 5: (a) DPPH radical scavenging and (b) superoxide radical scavenging activities of extract, compound, and BHT at different concentrations. Values are mean ± SD (n = 3).

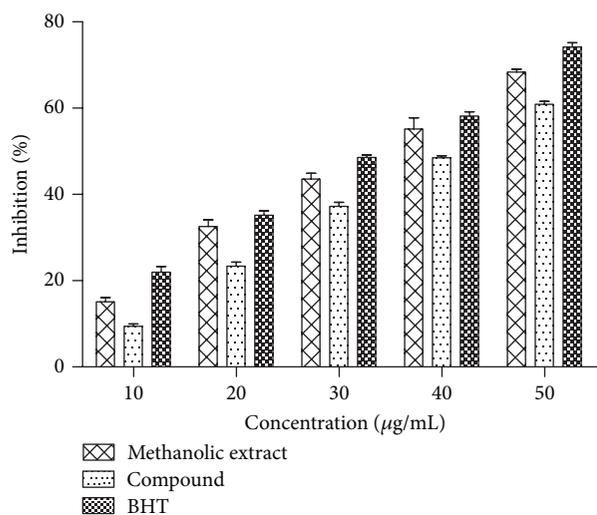


FIGURE 6: Hydroxyl radical scavenging effect of extract, compound, and BHT at different concentrations. Values are mean ± SD (n = 3).

Figure 7(a). The extract and compound at 50 µg/mL exhibited 48.6% and 40% inhibition which was comparable to the standard BHT, which exhibited 49.8% inhibition at 40 µg/mL. However, both extract (IC₅₀ = 54 µg/mL) and compound (IC₅₀ = 67 µg/mL) can inhibit NO radicals but lesser when compared to standard BHT (IC₅₀ = 41 µg/mL). The NO radical-scavenging activities of the extract and compound also followed a concentration-dependent pattern. The results suggest that the isolated flavone compound of *L. nodiflora* might be an effective and novel therapeutic agent for scavenging and regulating the pathological conditions caused by excessive generation of NO and its oxidation product, peroxynitrite.

TABLE 2: Antioxidant potentials of extract, compound, and BHT at different concentrations examined by FRAP assay.

Sample	Test concentration (µg/mL)	FRAP value (µM Fe(II)/g) ^a
Methanol extract	10	23.4 ± 0.54
	20	27.5 ± 1.24
	30	52.5 ± 0.69
	40	63.9 ± 0.70
	50	71.1 ± 1.69
Compound	10	11.1 ± 1.00
	20	23.6 ± 0.57
	30	35.1 ± 0.63
	40	42.7 ± 0.58
	50	51.7 ± 0.83
BHT	10	20.7 ± 0.70
	20	30.0 ± 0.72
	30	48.5 ± 0.77
	40	54.7 ± 0.54
	50	63.1 ± 0.20

^aThe values are the average of three independent experiments.

3.2.6. Inhibitory Activity towards Lipid Peroxidation. The disturbances in the membrane assembly lead to changes in fluidity and permeability, modifications of ion transport, and inhibition of metabolic processes, altogether revealed to be the collective consequence of reactive oxygen species formed during lipid peroxidation [42]. The extract and compound were capable of preventing MDA formation in a concentration-dependent manner (Figure 7(b)). At 50 µg/mL, methanolic extract and compound possessed 62.44% and 54.95% inhibition, while BHT exhibited 68.64%

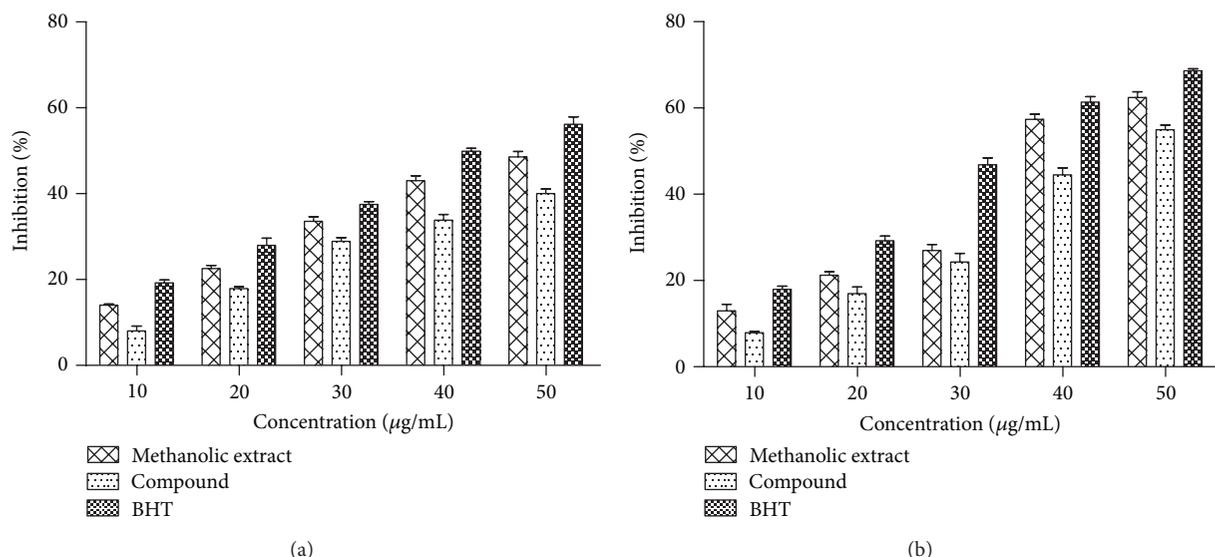


FIGURE 7: (a) Nitric oxide radical scavenging and (b) lipid peroxidation inhibition activities of extract, compound, and BHT at different concentrations. Values are mean \pm SD ($n = 3$).

inhibition at the same concentration. The IC_{50} values for extract and compound were found to be 39 and 46 $\mu\text{g/mL}$. There is no significant difference in the IC_{50} values of extract and compound ($P < 0.05$). However, IC_{50} values of extract were in good agreement with BHT ($IC_{50} = 34 \mu\text{g/mL}$). The presence of hydroxyl and electron-donating methoxy group in the compound (5-hydroxy-3', 4', 7-trimethoxyflavone) may be accountable for the antioxidant activity in all the experimental assays.

Based on *in vitro* antioxidant results of the present work, the methanol extract and isolated compound from ethyl acetate fraction of *L. nodiflora* were believed to be an electron donor, capable of counteracting free radicals. This is the first study to give an account on the antioxidant and free-radical scavenging activity of 5-hydroxy-3', 4', 7-trimethoxyflavone from *L. nodiflora*. The results of the present work also propose that the numerous pharmacological properties exerted by *L. nodiflora* may be partly due to the presence of antioxidant flavone compound.

4. Conclusion

The present study was projected to assess the antioxidant and free-radical scavenging activities of extract and fractions from aerial parts of *L. nodiflora* by using *in vitro* antioxidant models. The ethyl acetate fraction (EAF) exhibited highest free-radical scavenging activity, among the fractions. A bioassay-guided fractionation and purification of EAF resulted in the identification of the flavone compound, namely, 5-hydroxy-3', 4', 7-trimethoxyflavone. The measurement of antioxidant activity of the flavone compound, by using various *in vitro* antioxidant models, proved it to be a potent antioxidant compound. These results signify that methanol extract, ethyl acetate fractions, and isolated compound exhibited interesting antioxidant properties and

afford an essential basis for the use of *L. nodiflora* in the treatment of oxidative damages. Furthermore, these findings hold great perception in the development of alternative antioxidant agents, and still further work is warranted to sort out and characterize the active principles from other fractions, in order to establish their therapeutic efficacy and mechanism of action.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

This work was financially supported by Department of Science and Technology (DST WOS-A), New Delhi (Grant no. SR/WOS-A/LS-237/2010). The authors thank Dr. K. Kulanjiappar, Electro Organic Division, Central Electrochemical Research Institute, Karaikudi, for helping in NMR interpretation of the isolated compound.

References

- [1] B. N. Singh, B. R. Singh, R. L. Singh et al., "Oxidative DNA damage protective activity, antioxidant and anti-quorum sensing potentials of *Moringa oleifera*," *Food and Chemical Toxicology*, vol. 47, no. 6, pp. 1109–1116, 2009.
- [2] S. Shukla, A. Mehta, J. John, S. Singh, P. Mehta, and S. P. Vyas, "Antioxidant activity and total phenolic content of ethanolic extract of *Caesalpinia bonducella* seeds," *Food and Chemical Toxicology*, vol. 47, no. 8, pp. 1848–1851, 2009.
- [3] I. I. Koleva, H. A. G. Niederländer, and T. A. Van Beek, "An on-line HPLC method for detection of radical scavenging compounds in complex mixtures," *Analytical Chemistry*, vol. 72, no. 10, pp. 2323–2328, 2000.

- [4] J. Whysner, C. X. Wang, E. Zang, M. J. Iatropoulos, and G. M. Williams, "Dose response of promotion by butylated hydroxyanisole in chemically initiated tumours of the rat forestomach," *Food and Chemical Toxicology*, vol. 32, no. 3, pp. 215–222, 1994.
- [5] B. A. Silva, F. Ferreres, J. O. Malva, and A. C. P. Dias, "Phytochemical and antioxidant characterization of *Hypericum perforatum* alcoholic extracts," *Food Chemistry*, vol. 90, no. 1-2, pp. 157–167, 2005.
- [6] A. R. Verma, M. Vijayakumar, C. S. Mathela, and C. V. Rao, "In Vitro and in vivo antioxidant properties of different fractions of *Moringa oleifera* leaves," *Food and Chemical Toxicology*, vol. 47, no. 9, pp. 2196–2201, 2009.
- [7] K. Saito, M. Kohno, F. Yoshizaki, and Y. Niwano, "Extensive screening for edible herbal extracts with potent scavenging activity against superoxide anions," *Plant Foods for Human Nutrition*, vol. 63, no. 2, pp. 65–70, 2008.
- [8] J. Delgado Adámez, E. Gamero Samino, E. Valdés Sánchez, and D. González-Gómez, "In Vitro estimation of the antibacterial activity and antioxidant capacity of aqueous extracts from grape-seeds (*Vitis vinifera* L.)," *Food Control*, vol. 24, no. 1-2, pp. 136–141, 2012.
- [9] S. Sreelatha and P. R. Padma, "Antioxidant activity and total phenolic content of *Moringa oleifera* leaves in two stages of maturity," *Plant Foods for Human Nutrition*, vol. 64, no. 4, pp. 303–311, 2009.
- [10] E. S. Abdel-Hameed, "Total phenolic contents and free radical scavenging activity of certain Egyptian *Ficus* species leaf samples," *Food Chemistry*, vol. 114, no. 4, pp. 1271–1277, 2009.
- [11] B. Halliwell, "Dietary polyphenols: good, bad, or indifferent for your health?" *Cardiovascular Research*, vol. 73, no. 2, pp. 341–347, 2007.
- [12] V. López, S. Akerreta, E. Casanova, J. M. García-Mina, R. Y. Cavero, and M. I. Calvo, "In Vitro antioxidant and anti-rhizopus activities of lamiales herbal extracts," *Plant Foods for Human Nutrition*, vol. 62, no. 4, pp. 151–155, 2007.
- [13] D. O. Rios, L. M. G. Antunes, and M. D. L. P. Bianchi, "Bixin and lycopene modulation of free radical generation induced by cisplatin-DNA interaction," *Food Chemistry*, vol. 113, no. 4, pp. 1113–1118, 2009.
- [14] M. Yusuf, J. Begum, M. N. Hoque, and J. U. Chowdhury, *Medicinal Plants of Bangladesh*, BCSIR Laboratories, Chittagong, Bangladesh, 2009, Revised and Enlarged.
- [15] A. M. Forestieri, M. T. Monforte, S. Ragusa, A. Trovato, and L. Iauk, "Antiinflammatory, analgesic and antipyretic activity in rodents of plant extracts used in African medicine," *Phytotherapy Research*, vol. 10, no. 2, pp. 100–106, 1996.
- [16] G. Balakrishnan, L. Janakarajan, A. Balakrishnan, and B. S. Lakshmi, "Molecular basis of the anti-inflammatory property exhibited by cyclo-pentano phenanthrenol isolated from *Lippia nodiflora*," *Immunological Investigations*, vol. 39, no. 7, pp. 713–739, 2010.
- [17] R. Balamurugan and S. Ignacimuthu, "Antidiabetic and hypolipidemic effect of methanol extract of *Lippia nodiflora* L. in streptozotocin induced diabetic rats," *Asian Pacific Journal of Tropical Biomedicine*, vol. 1, supplement 1, pp. S30–S36, 2011.
- [18] L. Yen, M. C. Wang, C. J. Liang, H. H. Ko, and C. W. Lee, "Melanogenesis inhibitor(s) from *Phylla nodiflora* extract," *Evidence Based Complementary and Alternative Medicine*, vol. 2012, Article ID 867494, 9 pages, 2012.
- [19] A. M. Abbasi, M. A. Khan, M. Ahmad, M. Zafar, S. Jahan, and S. Sultana, "Ethnopharmacological application of medicinal plants to cure skin diseases and in folk cosmetics among the tribal communities of North-West Frontier Province, Pakistan," *Journal of Ethnopharmacology*, vol. 128, no. 2, pp. 322–335, 2010.
- [20] K. Basu, P. Chakraborti, and P. K. Sanyal, "Nodifloretin- a new flavone from *Lippia nodiflora*," *Journal of Indian Chemical Society*, vol. 46, no. 4, pp. 271–272, 1969.
- [21] F. A. Tomás-Barberán, J. B. Harborne, and R. Self, "Twelve 6-oxygenated flavone sulphates from *Lippia nodiflora* and *L. canescens*," *Phytochemistry*, vol. 26, no. 8, pp. 2281–2284, 1987.
- [22] G. R. Nair, P. Ramesh, and S. Nagarajan, "New flavones glycosides from *Lippia nodiflora*," *Indian Journal of Chemistry*, vol. 2, pp. 1316–1317, 1973.
- [23] S. D. Elakovich and K. L. Stevens, "Volatile constituents of *Lippia nodiflora*," *Journal of Natural Products*, vol. 48, no. 3, pp. 504–506, 1985.
- [24] V. Ravikanth, P. Ramesh, P. V. Diwan, and Y. Venkateswarlu, "Halleridone and Hallerone from *Phylla nodiflora* as taxonomic markers," *Biochemical Systematics and Ecology*, vol. 28, no. 9, pp. 905–906, 2000.
- [25] B. S. Siddiqui, F. Ahmed, S. K. Ali, S. Perwaiz, and S. Begum, "Steroidal constituents from the aerial parts of *Lippia nodiflora* Linn.," *Natural Product Research*, vol. 23, no. 5, pp. 436–441, 2009.
- [26] R. Balamurugan, V. Duraipandiyar, and S. Ignacimuthu, "Antidiabetic activity of γ -sitosterol isolated from *Lippia nodiflora* L. in streptozotocin induced diabetic rats," *European Journal of Pharmacology*, vol. 667, no. 1–3, pp. 410–418, 2011.
- [27] T. Yamaguchi, H. Takamura, T. Matoba, and J. Terao, "HPLC method for evaluation of the free radical-scavenging activity of foods by using 1,1-diphenyl-2-picrylhydrazyl," *Bioscience, Biotechnology and Biochemistry*, vol. 62, no. 6, pp. 1201–1204, 1998.
- [28] M. Nishikimi, N. Appaji Rao, and K. Yagi, "The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen," *Biochemical and Biophysical Research Communications*, vol. 46, no. 2, pp. 849–854, 1972.
- [29] I. Hinneburg, H. J. Damien Dorman, and R. Hiltunen, "Antioxidant activities of extracts from selected culinary herbs and spices," *Food Chemistry*, vol. 97, no. 1, pp. 122–129, 2006.
- [30] I. F. F. Benzie and J. J. Strain, "The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: the FRAP assay," *Analytical Biochemistry*, vol. 239, no. 1, pp. 70–76, 1996.
- [31] L. C. Green, D. A. Wagner, and J. Glogowski, "Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids," *Analytical Biochemistry*, vol. 126, no. 1, pp. 131–138, 1982.
- [32] H. J. Damien Dorman, S. G. Deans, R. C. Noble, and P. Surai, "Evaluation in vitro of plant essential oils as natural antioxidants," *Journal of Essential Oil Research*, vol. 7, no. 6, pp. 645–651, 1995.
- [33] D. Ashokkumar, V. Thamilselvan, G. P. Senthilkumar, U. K. Mazumder, and M. Gupta, "Antioxidant and free radical scavenging effects of *Lippia nodiflora*," *Pharmaceutical Biology*, vol. 46, no. 10-11, pp. 762–771, 2008.
- [34] T. Ersöz, S. I. Harput, I. Saracoğlu, I. Calis, and Y. Ogihara, "Phenolic compounds from *Scutellaria pontica*," *Turkish Journal of Chemistry*, vol. 26, no. 4, pp. 581–588, 2002.
- [35] T. T. Mai, N. Fumie, and N. Van Chuyen, "Antioxidant activities and hypolipidemic effects of an aqueous extract from flower buds of *Cleistocalyx operculatus* (Roxb.) Merr. and Perry," *Journal of Food Biochemistry*, vol. 33, no. 6, pp. 790–807, 2009.

- [36] S. Shukla, R. Patel, and R. Kukkar, "Study of phytochemical and diuretic potential of methanol and aqueous extracts of aerial parts of *Phyla nodiflora* Linn.," *International Journal of Pharmacy and Pharmaceutical Sciences*, vol. 1, no. 1, pp. 85–91, 2009.
- [37] R. P. Singh, K. N. Chidambara Murthy, and G. K. Jayaprakasha, "Studies on the antioxidant activity of pomegranate (*Punica granatum*) peel and seed extracts using *in vitro* models," *Journal of Agricultural and Food Chemistry*, vol. 50, no. 1, pp. 81–86, 2002.
- [38] M. Valko, D. Leibfritz, J. Moncol, M. T. D. Cronin, M. Mazur, and J. Telser, "Free radicals and antioxidants in normal physiological functions and human disease," *International Journal of Biochemistry and Cell Biology*, vol. 39, no. 1, pp. 44–84, 2007.
- [39] A. Luximon-Ramma, T. Bahorun, M. A. Soobrattee, and O. I. Aruoma, "Antioxidant activities of phenolic, proanthocyanidin, and flavonoid components in extracts of *Cassia fistula*," *Journal of Agricultural and Food Chemistry*, vol. 50, no. 18, pp. 5042–5047, 2002.
- [40] P. D. Duh, P. C. Du, and G. C. Yen, "Action of methanolic extract of mung bean hulls as inhibitors of lipid peroxidation and non-lipid oxidative damage," *Food and Chemical Toxicology*, vol. 37, no. 11, pp. 1055–1061, 1999.
- [41] S. Moncada, R. M. J. Palmer, and E. A. Higgs, "Nitric oxide: physiology, pathophysiology, and pharmacology," *Pharmacological Reviews*, vol. 43, no. 2, pp. 109–142, 1991.
- [42] S. Nigam and T. Schewe, "Phospholipase A2s and lipid peroxidation," *Biochimica et Biophysica Acta-Molecular and Cell Biology of Lipids*, vol. 1488, no. 1-2, pp. 167–181, 2000.



Hindawi

Submit your manuscripts at
<http://www.hindawi.com>

