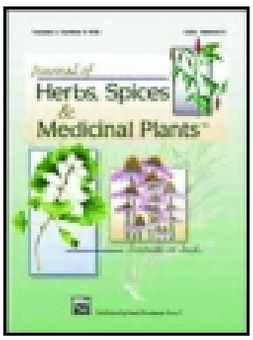

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Assessment of Radical Scavenging Activity and Estimation of EC50 Values of Various Extracts of Leaves and Roots from *Lobelia nicotianifolia* Roth. (Wild Tobacco)

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ABSTRACT

The antioxidant potential (% RSA and EC_{50}) of solvent extracts of leaves and roots of *Lobelia nicotianifolia* Roth. was assessed using five *in vitro* assays. The EC_{50} values of methanolic extract were comparable to that of ascorbic acid for nitric oxide (30.67 µg mL^{-1}) and hydrogen peroxide (81.01 µg mL^{-1}) radical assays. The % RSA and EC_{50} correlated with the total phenolic content and total flavonoid content. The characterization by liquid chromatography-high-resolution mass spectrometry showed the presence of five flavonoids, six phenolics, three carotenoids, one anthraquinone, coumarin, hydroxyquinone, and isoflavonoid. Embelin, gallic acid, and quercetin were quantified by high-performance liquid chromatography.

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KEYWORDS
LC-HRMS; HPLC

Introduction

Natural cellular metabolism of aerobic organisms generates free radicals that are neutralized by the cell antioxidant defense system. Free radicals react with lipids, proteins, carbohydrates, and DNA, leading to pathogenic complications.[1] Therefore, the scavenging of free radicals to reduce oxidative stress is a crucial phenomenon of a biological system, which can be achieved by supplementing exogenous antioxidants.[2] Antioxidants are categorized as natural and synthetic, and their supplementation can reduce morbidity risk.[3] Since long-term synthetic antioxidant use leads to toxicity and carcinogenicity, natural antioxidants, terpenes, and alkaloids, which are plant secondary metabolites (SMs), are receiving greater interest.[4]

Lobelia sp. (Campanulaceae) grown in tropical and warm temperate regions[5] is used in treating ophthalmic diseases and respiratory problems and as a diuretic, choleretic, antivenom, antibacterial, and anticancer agent.[6] A detailed investigation of *Lobelia* sp. revealed the presence of phenolics and piperidine alkaloids (lobeline), which exhibit antioxidant and anticancer

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activities.[6] *Lobelia nicotianifolia* Roth. (wild tobacco) is distributed in the Indo-Malaysian region and its ethnobotanical studies reported analgesic, antivenom, antimicrobial activities of *L. nicotianifolia* which may be attributed to the presence of lobeline, a pyridine alkaloid.[7]

The current study explored the antioxidant potential of *L. nicotianifolia* of leaves and roots. The leaves and roots of the plant were sequentially extracted using different organic solvents with increasing polarity. The extracts were analyzed for total phenolic content (TPC), total flavonoid content (TFC), and lobeline content (LC), and finally subjected to antioxidant activity [radical scavenging activity (% RSA) and half maximal effective concentration (EC_{50} value)]. Pearson correlation between SMs (TPC, TFC, and LC) and % RSA and EC_{50} was studied to determine the SMs responsible for antioxidant activity. The potent extract exhibiting higher activity was characterized through liquid chromatography-high resolution mass spectrometry (LC-HRMS), and selected phenolic compounds were quantified by high-performance liquid chromatography (HPLC).

Materials and Methods

Plant Material

L. nicotianifolia was collected from the Kas lake area (17°43′24" N, 73°48′47" E) of Satara district, India, and identified using *Flora of Maharashtra State*.[8] Herbarium specimen (NGCPR-1904) was deposited at Naoroji Godrej Center for Plant Research, Shirwal.

Chemicals

Organic solvents (HPLC grade), tannic acid, ascorbic acid, Folin–Ciocalteu phenol reagent, dimethyl sulfoxide (DMSO), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate, trichloroacetic acid, thiobarbituric acid, hydrogen peroxide, and phosphate buffer were obtained from Himedia, India. Aluminum chloride, Griess reagent, and different standards (lobeline, embelin, gallic acid, and quercetin) were obtained from Sigma, USA.

Preparation of Plant Part Extracts and Determination of Percent Yield

The leaves and roots of *L. nicotianifolia* were washed thoroughly under tap water to remove dust and dirt particles, and shade-dried at room temperature (RT). The dried plant material was packed in Soxhlet extractor (Borosil, India) and extracted sequentially (1:25 w/v) using petroleum ether, toluene, dichloromethane, chloroform, ethyl acetate, ethanol, methanol, and water with increasing polarity (Fig. 1). The extracts were then filtered (Whatman #1),

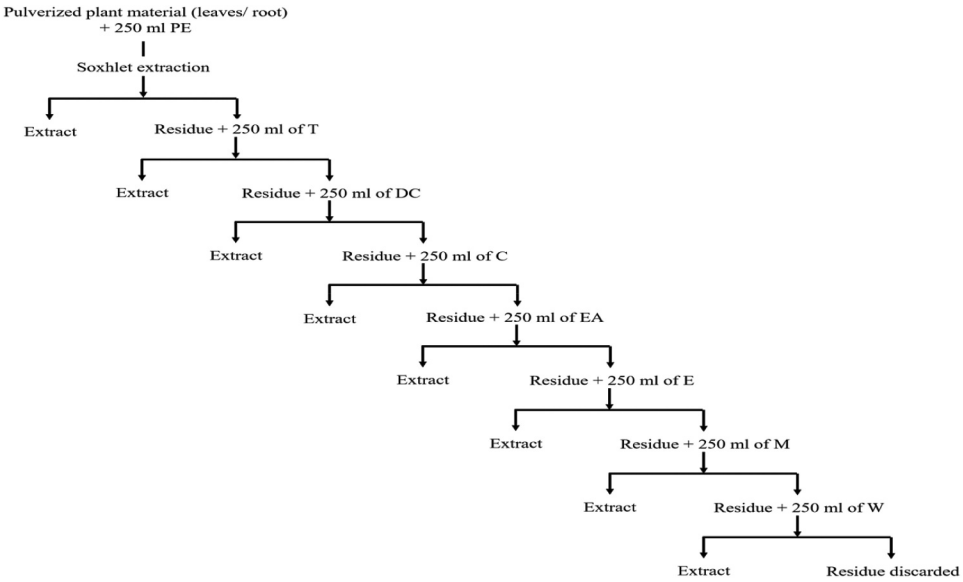

Figure 1. Schematic presentation of sequential extraction of SMs from leaves and roots of *Lobelia nicotianifolia*. PE, petroleum ether; ;T, toluene; DM, dichloromethane; C, chloroform; EA, ethyl acetate; E, ethanol; M, methanol; W, water. At every step, the residue was dried at 40°C prior to extraction with the next solvent.

concentrated on a rotary vacuum evaporator (PBU-6D Superfit rotary vacuum evaporator, India), weighed to determine their percent yields and stored at −20° C until further use. Percent yield of *L. nicotianifolia* extracts was calculated as

$$\text{Percent yield}\,(\%) = \frac{\text{A0}}{\text{A1}} \times 100$$

where A0 = weight of the extract, and A1 = the initial weight of the powdered plant material.

Determination of SMs

Determination of TPC

TPC in *L. nicotianifolia* extracts was determined using modified Folin–Ciocalteu method.[9] The extracts (50 µL, equivalent to 100 µg) were added to 200 µL of 2 N Folin–Ciocalteu and sodium carbonate (1 mL). This mix was incubated at 25°C for 30 min, and total phenolics were quantified by a spectrophotometer (Shimadzu UV-1900 UV-VIS) at 765 nm, using a standard calibration curve. TPC of the samples was expressed as mg of tannic acid equivalent (TAE) per g of the extract.

Determination of TFC

TFC of *L. nicotianifolia* extracts was assessed using ethanolic $AlCl_3$.[10] An equal volume of ethanolic $AlCl_3$ (2%) was added to the plant extracts and incubated at RT for 60 min. After incubation, flavonoid concentrations were estimated by spectrophotometer at 420 nm using a standard calibration curve. TFC was expressed as mg quercetin equivalent (QE) per g of the extract.

Determination of LC

Lobeline in different extracts was quantified using an Agilent's 1100 series quaternary HPLC system equipped with an autosampler, UV detector, and Agilent ChemStation. Chromatographic resolution was obtained with an isocratic mobile phase including 0.1% trifluoroacetic acid (TFA)–water–acetonitrile (70:30 v/v) on a Discovery RP Amide C16 column (4.6 × 150 mm, 5 µm) at a flow rate of 1 mL min^{-1} and λmax 249 nm at 25°C. The injection volume was 10 µL, and chromatographic run time was 15 min. Standard stock solutions of lobeline were prepared by dissolving 2 mg of lobeline in 5 mL 0.1% TFA and then diluted with 0.1% TFA to attain solutions at 62.5–1000 µg $mL^{-1}$and quantified using a standard curve.

In Vitro Antioxidant Activities

ABTS Radical Scavenging Activity

ABTS % RSA of *L. nicotianifolia* extracts were evaluated as described[11] with some modifications. The ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting equal volume of ABTS stock solution (7 mM) and potassium persulfate solution (2.45 mM) and store in the dark for 16 h at RT. The $ABTS^{\bullet+}$ solution was then diluted with methanol to obtain an absorbance of 0.700 at 734 nm. Different concentrations (100–500 µg mL^{-1}) of extracts were prepared using DMSO as the solvent. To this 3.950 mL $ABTS^{\bullet+}$ solution, 50 µL extract was added and incubated for 30 min at RT. The absorbance of the reaction mixture was recorded at 734 nm, and the % RSA of extracts was calculated as where Ac = absorbance of a negative control/blank ($ABTS^{\bullet+}$ solution + methanol), and As = absorbance of the test sample [$ABTS^{\bullet+}$ solution + extract/ascorbic acid (AA)]. AA was used as a positive control, and the antioxidant activity of the extracts was compared with that of standard lobeline.

DPPH Radical Scavenging Activity

DPPH % RSA of *L. nicotianifolia* extracts were estimated as described[12] with some modifications. Different concentrations (100–500 µg mL^{-1}) of the extracts were prepared using DMSO as a solvent, and the final volume of this solution was made up to 4 mL. This solution was further mixed with 1 mL methanolic DPPH (0.2 mM) and incubated for 20 min at RT and its

absorbance measured at 518 nm. The ability of the extracts to scavenge the DPPH radical was calculated as

where Ac = absorbance of a negative control/blank (DPPH solution without extract), and As = absorbance of the test sample (DPPH solution + extract/AA). AA was used as positive control, and the antioxidant activity of the extracts was compared with that of standard lobeline.

Hydroxyl Radical Scavenging Activity

Hydroxyl % RSA of *L. nicotianifolia* extracts was estimated[13] using a reaction mixture prepared, [14] and the final volume (800 µL) was adjusted with distilled water. To this mix, different volumes of extracts ranging from 0–100 µL were added with concentrations 100–500 µg mL^{-1} and incubated at 37°C for 30 min. The reaction was terminated by adding 2.8% trichloroacetic acid (0.5 mL) and 0.6% thiobarbituric acid (0.4 mL) and incubated in boiling water for 20 min. Absorbance was read at 532 nm, and hydroxyl % RSA was calculated as

Ac = absorbance of negative control/blank (reaction mixture solution), and As = absorbance of extract/AA (positive control) (solution of reaction mixture + extract/AA). AA was used as positive control, and antioxidant activity of the extracts was compared with standard lobeline.

Nitric Oxide Radical Scavenging Activity

Nitric oxide % RSA of *L. nicotianifolia* extracts were determined as described[15] with modifications. Griess reagent and buffer used in this assay were prepared as described.[14] Equal volumes of various concentrations (100–500 µg mL^{-1}) of the extracts and freshly prepared Griess reagent were mixed to obtain reaction mix. This reaction mix (150 µL) was transferred to the 96-well plate, and absorbance measured at 546 nm using a plate reader using the buffer without the extract as negative control and AA as the positive control. Nitric oxide % RSA of the extracts and AA was calculated as

where Ac = absorbance of negative control/blank (Griess reagent + buffer), and As = absorbance of extract/AA (positive control) (Griess reagent + buffer + extract/AA). The antioxidant activity of the extracts was compared with that of standard AA and lobeline.

H_2O_2 Radical Scavenging Activity

H_2O_2% RSA of the methanolic extract was determined as described.[16] A solution was prepared by mixing 100 µL of different concentrations of the extracts (100–500 µg mL^{-1}), 400 µL of the buffer and 0.6 mL H_2O_2 solution and mixed thoroughly. Absorbance was determined spectrophotometrically at 230 nm and the $H_2O_{2\%}$ RSA was calculated as

where Ac = absorbance of negative control/blank (solution of reaction mixture), and As = absorbance of extract/AA (positive control) (solution

of reaction mixture + extract/AA). AA was used as positive control, and the antioxidant activity of the extracts was compared with standard lobeline.

Estimation of EC_{50} Value

The EC_{50} of all extracts were calculated to determine the 50% inhibition of ABTS, DPPH, hydroxyl, nitric oxide, and H_2O_2 radicals, using AA and lobeline as standards.

Characterization of Extract Using LC-HRMS

SMs present in the methanolic leaf extracts of *L. nicotianifolia* were screened using an Agilent Binary (LC 1260) Triple Quad LC-HRMS mass spectrometer (Agilent Technologies, CA, USA). Agilent Zorbax Eclipse Plus C18, 2.1 × 50 mm 1.8 µm columns were used to obtain optimal separation with good peak shapes. The flow rate was maintained at 0.3 mL min^{-1} with a mobile phase of (A) water (0.1% formic acid) and (B) acetonitrile. The samples were acquired through a 30 min isocratic elution with 70% acetonitrile. Electrospray ionization (ESI) was used in positive (ESI^+) and negative (ESI^-) modes. Injection volume of the sample was 10 µL, and column temperature was maintained at 40°C. The Agilent 6540 Q-TOF MS system was equipped with a degasser, binary pump, cooled autosampler, column oven, and 6540 mass spectrometers. Gas temperature was maintained at 325°C, and the flow rate was 8 L min^{-1}. Sheath gas temperature was maintained at 295°C with a flow rate of 10 L min^{-1}, and nebulizer pressure was maintained at 25 psi for both negative and positive ion modes. Capillary voltage was 2500°C and 2000°C for positive and negative polarities, respectively. Mass range (m/z) was 80–2000 for both positive and negative modes, and the MS scan speed was 2 spectra s^{-1}. The centroid data type was acquired using MassHunter Workstation (vB.05.01) and the compounds identified by comparing them with available data in the literature and databases.

Quantification of Phenolic Compounds

Embelin, gallic acid, and quercitrin were analyzed using the Agilent 1100 HPLC system. Reverse phase chromatographic analyses were performed under gradient conditions using a LiChro CART Purospher STAR column (4.6 mm × 250 mm, 5 µm diameter particles). The mobile phase consisted of double distilled water (dDW) containing 1% formic acid (buffer A) and acetonitrile (buffer B), and different gradient programs was followed for the analysis. Methanolic leaf extracts of *L. nicotianifolia* were analyzed at 10 mg mL^{-1}. The flow rate was 1.0 mL min^{-1}, injection volume was 10 µL, and

analysis wavelength was 280 nm. Prior to their use, the buffers and extract were filtered through a 0.45 µm membrane filter, followed by degassing using an ultrasonic bath at RT for 10 min. Stock solutions of standard references were prepared in the HPLC mobile phase at 0.062–1.0 mg mL^{-1} concentration to obtain a calibration curve. These compounds in the extract were identified by comparing their retention time and UV absorption spectra with those of the standards Fig. 2). All chromatography operations were performed at ambient temperature and in triplicates.

Statistical Analysis

Data were presented as mean ± standard deviation of three readings. Data were subjected to one-way analysis of variance, followed by Duncan's multiple range test ($p \leq 0.05$), and the Pearson correlation coefficient of determination (R^2) was calculated (SPSS V 20).

Results

Percent Yield, TPC, TFC, LC, and Their Correlation

The percent yield obtained for leaves and roots extracts were in the following order: water (23.21, 14.12) > methanol (17.89, 11.15) > ethanol (14.35, 9.20) > ethyl acetate (7.32, 5.08) > chloroform (5.11, 4.71) > dichloromethane (5.21, 3.98) > toluene (3.31, 2.45) > petroleum ether (3.12, 2.64) (Table 1). Methanolic extracts of leaves showed higher TPC (15.74 mg TAE g^{-1} DW) and TFC (10.21 mg QE g^{-1} DW) compared to other solvent extracts (Table 1). A similar propensity was also observed in the roots of *L. nicotianifolia* extracted with polar solvents. Higher TPC and TFC in polar extracts also had higher polar phenolic compounds than nonpolar phenolic compounds (Table 1). The percent yield of leaf and roots was positively correlated with their phenolic and flavonoid contents (Table 2). Unlike TPC and TFC, higher LC was observed in mid polar extracts of both roots and leaves. Leaves and roots extracted with chloroform had higher LC, with, 4.78 µg mL^{-1} and 6.74 µg mL^{-1}, respectively (Fig. 2). There correlation was positive between TPC and TFC of leaves and root extracts and negative for lobeline (Table 2).

In Vitro Antioxidant Assays

In ABTS assay, methanolic leaf extracts at higher concentration (500 µg mL^{-1}) scavenged 82.98% ABTS radicals, whereas AA and lobeline scavenged 85.21% and 56.32% radicals, respectively (Fig. 3a). The ability of root extracts to scavenge the ABTS radicals was lower compared to the AA (Fig. 3b). The EC_{50} value of methanolic and water extracts of leaf (37.67 µg mL^{-1}) and roots

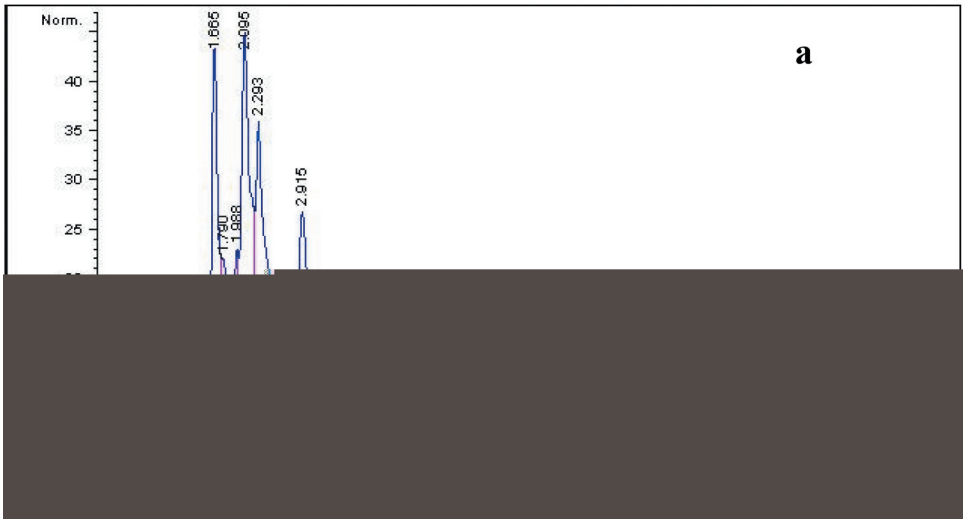

Figure 2. HPLC analysis of lobeline in chloroform root extract of *Lobelia nicotianifolia*.

(86.07 μg mL^{-1}) demonstrated the potential of leaves as good source of antioxidants (Tables 3 and 4). The methanolic extract of leaves at 200, 300, 400, or 500 μg mL^{-1} showed higher DPPH radicals scavenging activity compared to the AA and lobeline (Fig. 4a). Methanolic root extract had higher DPPH % RSA compared to all other root extracts, but lower than AA (Fig. 4b). The EC_{50} value of methanolic leaves extract was 35 μg mL^{-1} while that of AA was 36.61 μg mL^{-1} (Table 3). The EC_{50} value of root extract was 112.33 μg mL^{-1} (Table 4). In the present investigation, all the extracts showed concentration dependent antioxidant activity (Fig. 4a,b and Tables 3 and 4). Methanolic extract of leaf at 500 μg mL^{-1} scavenged the highest 88.92% of hydroxyl radicals compared to all the extracts tested (Fig. 5a,b). The EC_{50} value of methanolic leaves extract (42.33 μg mL^{-1}) was similar to AA (23 μg mL^{-1}); however, better with lobeline (139.33 μg mL^{-1}) (Table 3). Although the root extracts showed antioxidant activity, the EC_{50} values were lower than that of AA (Table 4). Methanolic extracts of leaf and root (500 μg mL^{-1}) scavenged 82.60% and 72.93% of nitric oxide radicals, respectively, whereas AA scavenged 82.49% of nitric oxide (Fig. 6a,b). Methanolic leaf extract effects were similar to that of AA. The EC_{50} values of leaf extracts ranged from 30.67 μg mL^{-1} to 158 μg mL^{-1}, which was similar to the EC_{50} value of AA (Tables 3 and 4). H_2O_2 RSA of methanolic leaf extracts of *L. nicotianifolia*, AA and lobeline was 81.01, 84.86, and 59.30% respectively. Methanolic root extract demonstrated higher $H_2O_{2\%}$ RSA (69.62) at higher concentration (500 μg mL^{-1}) (Fig. 7a,b). The EC_{50} values of methanolic leaf and root extracts were 25.36 μg mL^{-1} and 50.70 μg mL^{-1} respectively (Tables 3 and 4).

Table 1. The effect of extracting solvents on percent yield, total phenolic (TPC), total flavonoid (TF), and lobeline contents of leaves and roots of *Lobelia nicotianifolia*.

	Percent yield		TPC (mg TAE g^{-1} DW)		TFC (mg QE g^{-1} DW)		Lobeline (µg mL^{-1})	
Extracts	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots
PE	3.12	2.64	2.66 ± 0.24^{f}	1.36 ± 0.15^{f}	1.77 ± 0.05^{h}	1.04 ± 0.04^{g}	0.84 ± 0.14^{h}	1.68 ± 0.17^{f}
T	3.31	2.45	3.78 ± 0.10^{e}	2.29 ± 0.16^{e}	2.23 ± 0.12^{g}	2.05 ± 0.05^{f}	1.54 ± 0.15^{f}	2.10 ± 0.19^{e}
DC	5.21	3.98	4.79 ± 0.09^{d}	4.04 ± 0.06^{d}	3.26 ± 0.11^{f}	3.10 ± 0.08^{e}	3.52 ± 0.11^{b}	4.92 ± 0.18^{b}
C	5.11	4.78	10.19 ± 0.18^{c}	7.44 ± 0.06^{c}	6.91 ± 0.09^{e}	6.54 ± 0.16^{d}	4.78 ± 0.12^{a}	6.74 ± 0.18^{a}
EA	7.32	5.08	10.73 ± 0.80^{c}	7.77 ± 0.05^{c}	7.78 ± 0.12^{d}	7.07 ± 0.05^{c}	3.24 ± 0.16^{c}	4.30 ± 0.20^{c}
E	14.35	9.08	11.53 ± 0.56^{b}	9.82 ± 0.07^{b}	8.82 ± 0.14^{c}	7.60 ± 0.11^{b}	2.91 ± 0.12^{d}	4.04 ± 0.18^{c}
M	17.89	11.15	15.74 ± 0.51^{a}	10.45 ± 0.47^{a}	10.21 ± 0.11^{a}	7.88 ± 0.10^{a}	2.60 ± 0.17^{e}	3.16 ± 0.13^{d}
W	23.21	14.12	11.47 ± 0.14^{b}	9.92 ± 0.08^{b}	9.81 ± 0.12^{b}	8.04 ± 0.07^{a}	1.08 ± 0.13^{g}	2.06 ± 0.11^{e}

Values are the mean of three replicates ± SD. Mean values followed similar letters within a column are not different ($p \leq 0.05$).
PE, petroleum ether; T, toluene; DM, dichloromethane; C, chloroform; EA, ethyl acetate; E, ethanol; M, methanol; W, water.

Table 2. Correlation between percent yield and total phenolic, total flavonoid, and lobeline contents of leaves and roots of *Lobelia nicotianifolia*.

	PYL	PYR	TPCL	TPCR	TFCL	TFCR	LCL	LCR
PYL	1	0.996**	0.760*	0.828*	0.845**	0.772*	− 0.229	− 0.243
PYR	0.996**	1	0.788*	0.855**	0.870**	0.803*	− 0.164	− 0.172
TPCL	0.760*	0.788*	1	0.967**	0.973**	0.954**	0.313	0.249
TPCR	0.828*	0.855**	0.967**	1	0.993**	0.988**	0.295	0.258
TFCL	0.845**	0.870**	0.973**	0.993**	1	0.985**	0.223	0.186
TFCR	0.772*	0.803*	0.954**	0.988**	0.985**	1	0.346	0.316
LCL	− 0.229	− 0.164	0.313	0.295	0.223	0.346	1	0.985**
LCR	− 0.243	− 0.172	0.249	0.258	0.186	0.316	0.985**	1

**Correlation is significant at the 0.01 level (two-tailed), *Correlation is significant at the 0.05 level (two-tailed).
PYL, percent yield leaves; PYR, percent yield roots; TPCL, total phenolic content of leaves; TPCR, total phenolic content of roots; TFCL, total flavonoid content of leaves; TFCR, total flavonoid content of roots; LCL, lobeline content of leaves; LCR, lobeline content of roots.

Correlation of TPC, TFC, and LC with Antioxidant Activity

The results revealed a positive correlation between RSA and TPC and TFC and a negative correlation between RSA and LC content of leaves and roots of *L. nicotianifolia* (Table 5). TPC and TFC of the leaf extract had a positive correlation with the results of ABTS, DPPH, hydroxyl radicals, nitric oxide, and H_2O_2 radicals. These assays correlated with TPC and TFC of root extracts (Table 5), indicating that antioxidant activities attributed to a greater extent due to TPC and TFC rather than LC. The correlation between EC_{50} and TPC, TFC, and LC of leaves and root extracts was negative (Table 6).

Characterization and Quantification of Phenolic Compounds

Antioxidant activities of various extracts were compared with those of AA (positive control) and lobeline. Although these standard compounds are effective antioxidants, their antioxidant potential varied compared with methanolic leaf extract of *L. nicotianifolia*, hence the characterization of leaf extract is essential. The putative identification of these SMs was performed based on data such as protonated molecular ion m/z value, and mass error (Table 7). In this study, identified compounds included five flavonoids (quercitrin, epicatechin, hesperetin, xanthoxylin, and eriodictyol) (Series 1) and six phenolics (embelin, gallic acid, harderoporphyrin, cosmosiin [apigenin], α-tocopherol, and lecanoric acid) (Series 2). Moreover, three carotenoids (tunaxanthin E, myxoxanthophyll, and fucoxanthin) (Series 3), one anthraquinone (khayanthone) (Series 4), coumarin (osthol) (Series 5), hydroxyquinone (arbutin) (Series 6), and isoflavonoid (genistein) (Series 7). HPLC was performed to quantify commercially available phenolic compounds (embelin and gallic acid, and quercetin), which showed retention times of 2.601, 3.539, and 8.538 min, respectively (Fig. 8a). These three phenolic compounds were selected based on the putative identification through LC-HRMS (Table 7) and their antioxidant

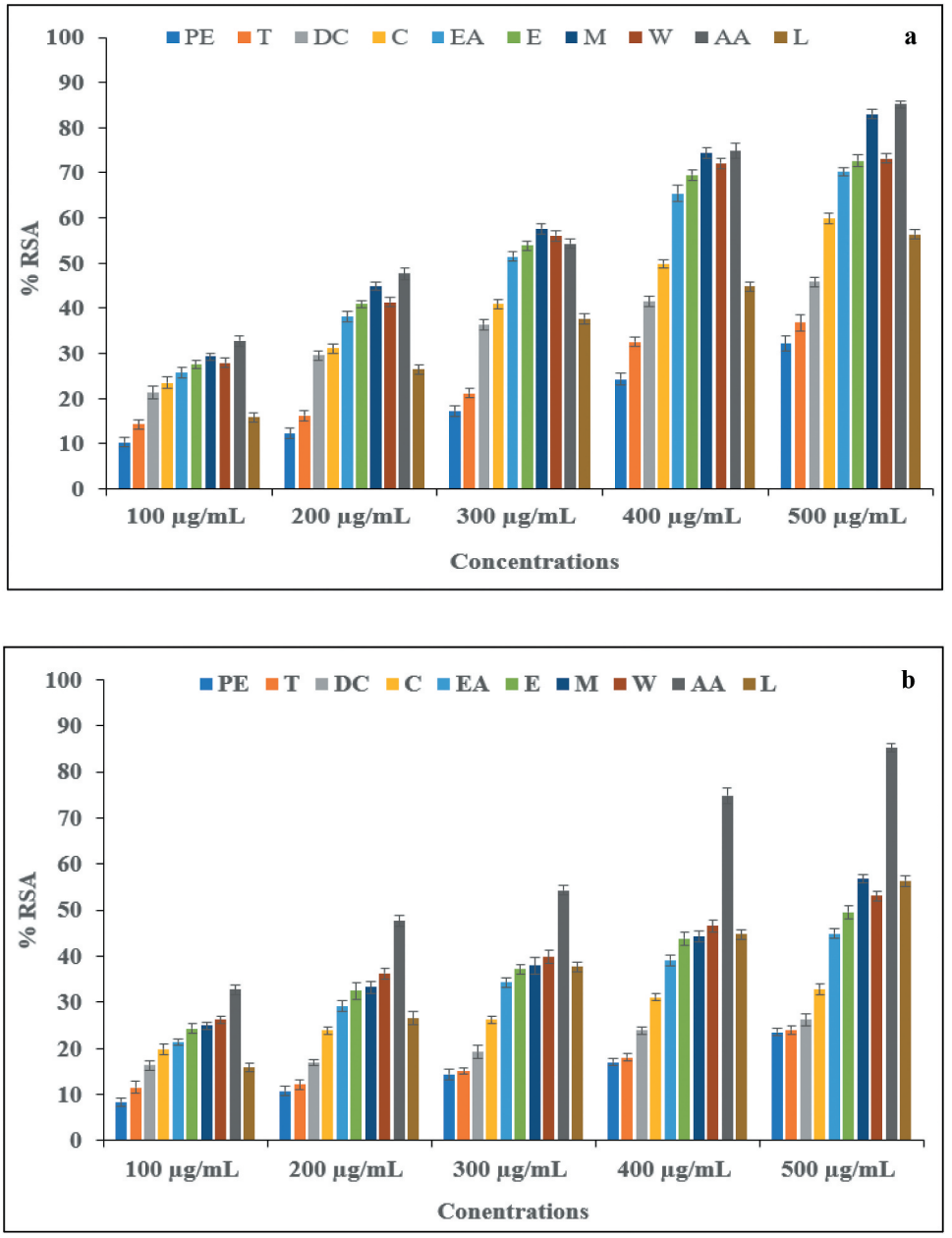

Figure 3. ABTS radical scavenging activity of *Lobelia nicotianifolia* leaf (a) and root (b) extracts. PE, petroleum ether; T, toluene; DM, dichloromethane; C, chloroform; EA, ethyl acetate; E, ethanol; M, methanol; W, water; AA, ascorbic acid; L, lobeline.

potential. Concentrations of embelin, gallic acid, and quercetin were evaluated on the basis of the calibration curves obtained (Fig. 8a–d).

Table 3. Estimation of EC_{50} (μg mL^{-1}) of solvent extracts of leaves *Lobelia nicotianifolia* for different antioxidant assays.

Extracts	ABTS radicals	DPPH radicals	Hydroxyl radicals	Nitric oxide radicals	H_2O_2 radicals
PE	260.00 ± 15.87 ^{h}	229.00 ± 15. 87 ^{f}	193.67 ± 14.01 ^{g}	158.00 ± 12.12 ^{g}	162.67 ± 13.32 ^{f}
T	223.33 ± 13.50 ^{g}	178.33 ± 17.90^{e}	178.67 ± 13.80^{fg}	131.33 ± 10.26 ^{f}	140.67 ± 11.50^{e}
DC	175.00 ± 14.73 ^{f}	141.33 ± 14.98^{d}	160.33 ± 16.77^{ef}	101.33 ± 12.34^{e}	123.00 ± 12.53^{de}
C	110.00 ± 12.00^{d}	110.00 ± 13.00 ^{c}	124.33 ± 11.24 ^{cd}	84.00 ± 11.79 ^{cd}	111.14 ± 11.75 ^{cd}
EA	82.67 ± 10.79 ^{cd}	99.00 ± 12.53 ^{c}	108.67 ± 12.66 ^{c}	77.33 ± 9.71 ^{c}	95.00 ± 12.77 ^{c}
E	49.67 ± 5.69^{b}	69.08 ± 10.25^{b}	80.33 ± 16.62^{b}	47.67 ± 3.06^{b}	66.22 ± 11.53^{b}
M	37.67 ± 4.93^{ab}	35.00 ± 8.72^{a}	42.33 ± 9.07^{a}	30.67 ± 3.79^{a}	25.36 ± 2.03^{a}
W	90.41 ± 11.06 ^{c}	92.33 ± 10.21 ^{c}	143.33 ± 20.60^{de}	109.67 ± 11.02^{e}	118.44 ± 13.58^{d}
AA	28.55 ± 1.10^{a}	36.61 ± 4.74^{a}	23.00 ± 2.00^{a}	20.33 ± 3.06^{a}	23.81 ± 5.15^{a}
L	131.67 ± 16.62^{e}	163.67 ± 16.92^{e}	139.33 ± 11.02^{df}	99.33 ± 4.93^{de}	124.33 ± 10.50^{de}

Values are the mean of three replicates ± SD. Mean values followed by similar letters within a column are not different ($p \leq 0.05$).

PE, petroleum ether; T, toluene; DM, dichloromethane; C, chloroform; EA, ethyl acetate; E, ethanol; M, methanol; W, water.

Table 4. Estimation of EC_{50} (μg mL^{-1}) of solvent extracts of roots of *Lobelia nicotianifolia* for different antioxidant assays.

Extracts	ABTS radicals	DPPH radicals	Hydroxyl radicals	Nitric oxide radicals	H_2O_2 radicals
PE	324.00 ± 22.65 ^{g}	276.33 ± 19.55^{e}	243.00 ± 19.08 ^{f}	306.00 ± 17.35 ^{g}	184.67 ± 16.50^{e}
T	295.33 ± 16.17 ^{f}	260.33 ± 13.61^{e}	213.00 ± 15.52^{e}	277.33 ± 12.01 ^{f}	162.67 ± 17.21^{de}
DC	270.67 ± 22.90 ^{f}	228.33 ± 11.02^{d}	194.67 ± 17.24^{e}	229.33 ± 15.31^{e}	151.67 ± 14.19^{d}
C	227.00 ± 17.52^{e}	180.00 ± 17.09 ^{c}	142.67 ± 12.06^{d}	171.00 ± 14.80^{d}	139.80 ± 12.18 ^{cd}
EA	142.33 ± 17.95^{d}	176.67 ± 12.06 ^{c}	131.67 ± 11.59 ^{cd}	136.33 ± 11.06 ^{c}	117.00 ± 20.30 ^{c}
E	137.67 ± 9.45 ^{cd}	131.67 ± 15.95^{b}	123.26 ± 13.93 ^{cd}	109.00 ± 15.10^{b}	88.22 ± 16.82^{b}
M	110.33 ± 12.50^{bc}	112.33 ± 14.50^{b}	85.33 ± 11.37^{b}	97.33 ± 10.69^{b}	50.70 ± 8.24^{a}
W	86.07 ± 6.46^{b}	174.00 ± 12.49 ^{c}	120.33 ± 10.02 ^{cd}	119.67 ± 16.77^{bc}	144.33 ± 12.90^{d}
AA	28.55 ± 1.10^{a}	36.61 ± 4.74^{a}	23.00 ± 2.00^{a}	20.33 ± 3.06^{a}	28.81 ± 5.15^{a}
L	131.67 ± 16.62^{d}	161.33 ± 14.22 ^{c}	139.33 ± 11.02 ^{c}	99.33 ± 4.93^{b}	124.33 ± 10.50^{d}

Values are the mean of three replicates ± SD. Mean values followed by similar letters within a column are not different ($p \leq 0.05$).

PE, petroleum ether; T, toluene; DM, dichloromethane; C, chloroform; EA, ethyl acetate; E, ethanol; M, methanol; W, water.

Discussion

Plant material extraction is one of the crucial steps in the recovery of SMs and the extraction process outcome largely depends on the chemical nature of SMs, extraction method, and polarity of solvent.[17] The percent yield for these solvents indicated the prevalence of a diverse range of polar, mid polar, and nonpolar SMs in the respective extracts based on their degrees of solubility.[18] Different quantities of TPC and TFC obtained using polar, mid polar, and nonpolar solvents were consistent with earlier reports[19] Among the extracts prepared in different solvents, higher LC was recorded in the chloroform extracts, which was in agreement with previous reports.[20] Positive correlation between TPC, TFC and percent yield is due to the difference in the quantity of phenolics and flavonoids of the extracts. A negative correlation was seen between percent yield and lobeline in leaf and root, which may be due to the solubility of lobeline in a solvent or the extraction processes.

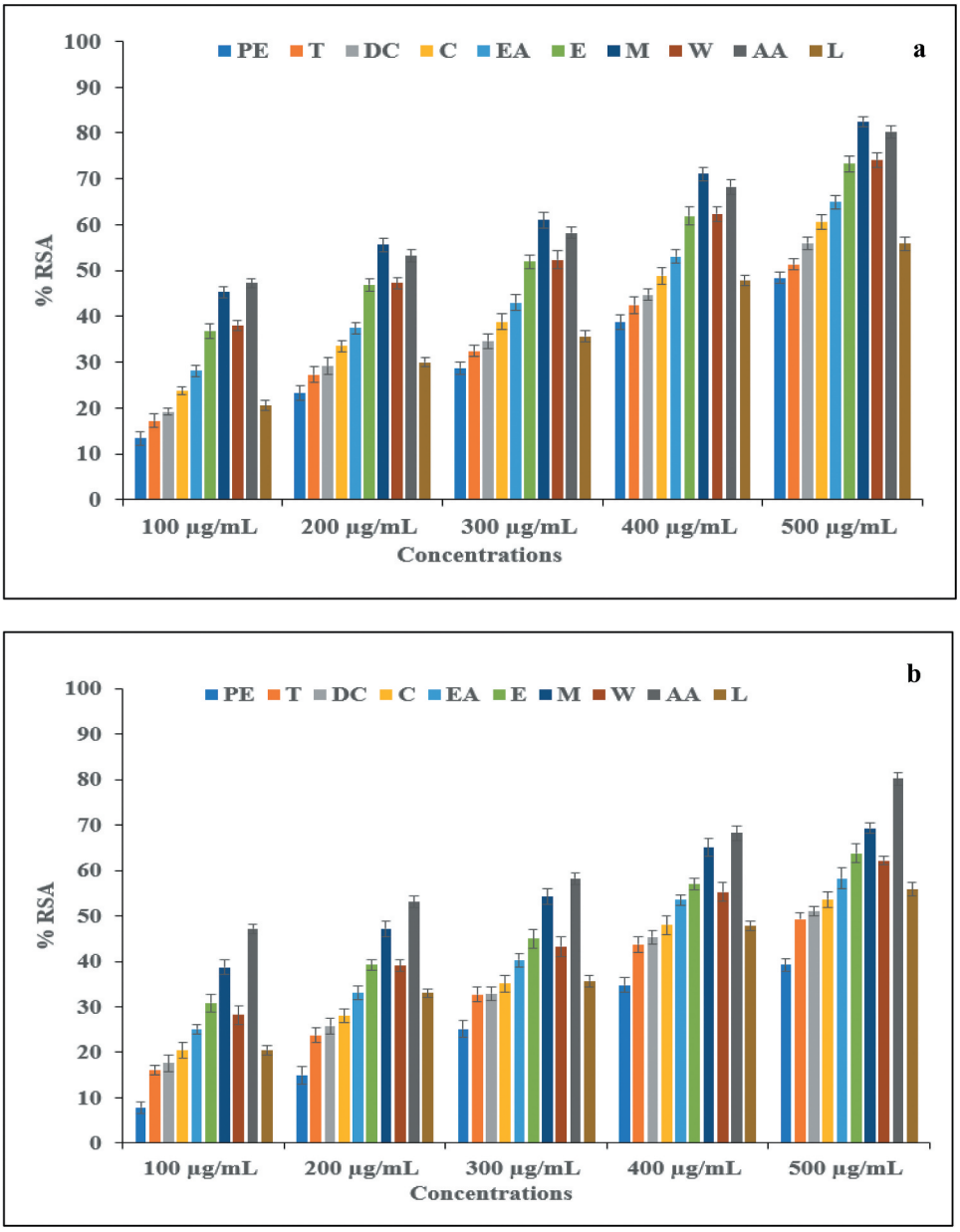

Figure 4. DPPH radical scavenging activity of *Lobelia nicotianifolia* leaf (a) and root (b) extracts. PE, petroleum ether; T, toluene; DM, dichloromethane; C, chloroform; EA, ethyl acetate; E, ethanol; M, methanol; W, water; AA, ascorbic acid; L, lobeline.

ABTS and DPPH are stable free radicals and these assays are based on the color change in the presence of antioxidants.[4,21] In ABTS assay blue green color of ABTS radical cation turned colorless indicating its potential as antioxidants. For DPPH the purple methanolic DPPH solution reduced to yellow color. For these assays change in color indicated the presence of antioxidant potential. For % RSA and EC_{50} values were higher for methanolic leaf extract,

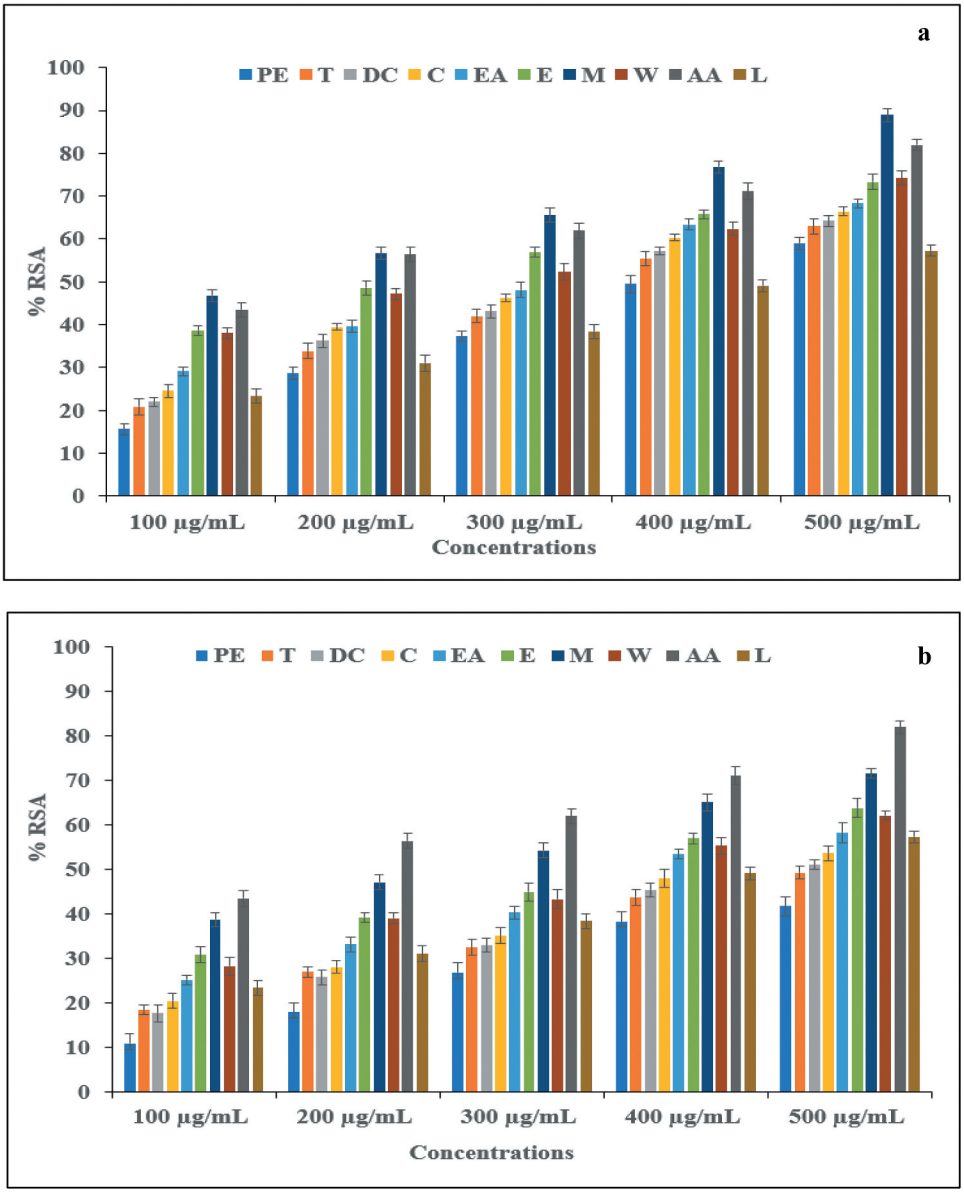

Figure 5. Hydroxyl radical scavenging activity of *Lobelia nicotianifolia* leaf (a) and root (b) extracts. PE, petroleum ether; T, toluene; DM, dichloromethane; C, chloroform; EA, ethyl acetate; E, ethanol; M, methanol; W, water; AA, ascorbic acid; L, lobeline.

which also had higher concentration of TPC and TFC compared to other studied extracts. It may be inferred that the antioxidant activity in ABTS and DPPH assays of leaf extracts related to the termination of oxidation reaction by reducing free radicals.[22] The phenolic compounds possess hydroxyl groups, which mainly contribute to the antioxidant activity by donating hydrogen atom.[16] Hydroxyl radicals are one of the most reactive oxygen

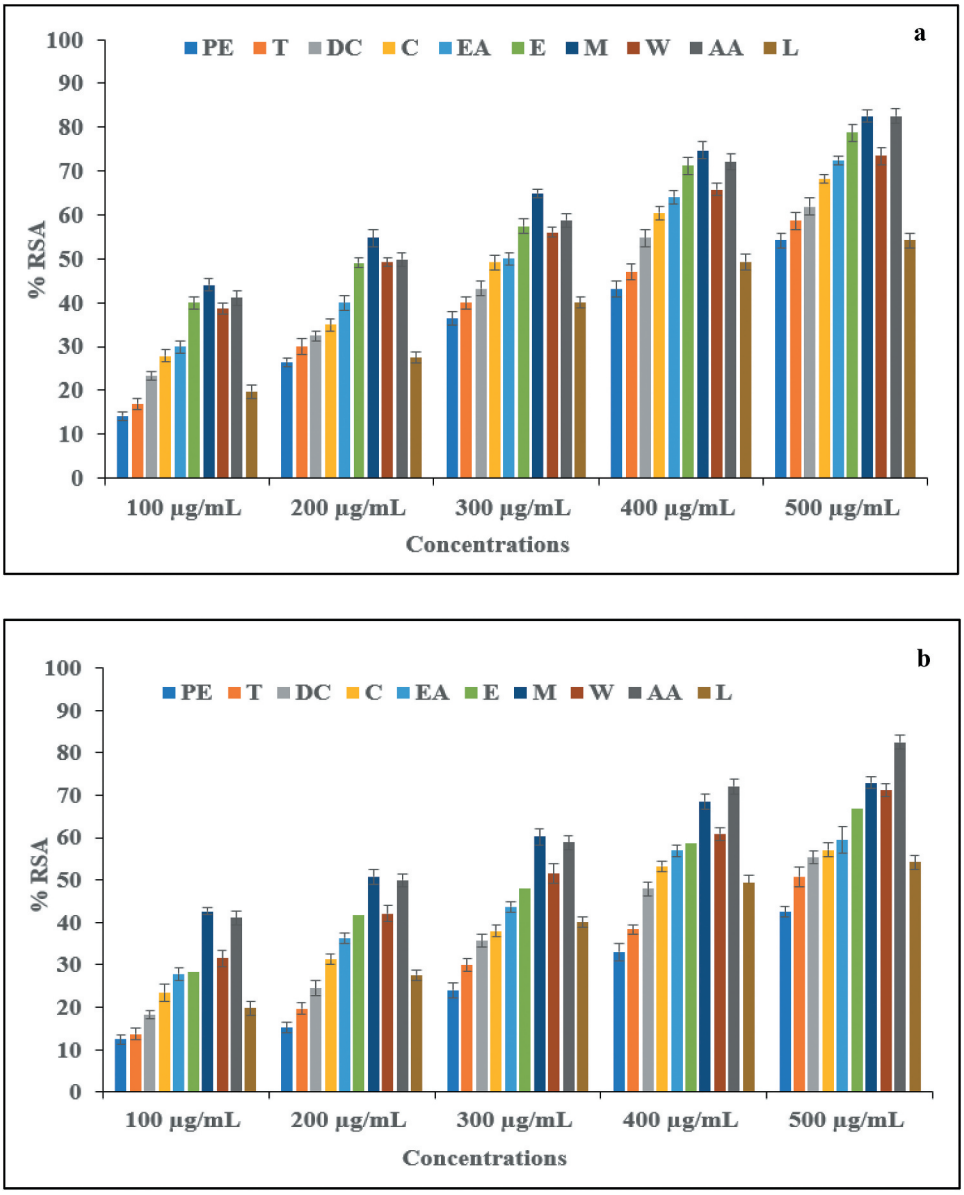

Figure 6. Nitric oxide radical scavenging activity of *Lobelia nicotianifolia* leaf (a) and root (b) extracts. PE, petroleum ether; T, toluene; DM, dichloromethane; C, chloroform; EA, ethyl acetate; E, ethanol; M, methanol; W, water; AA, ascorbic acid; L, lobeline.

species associated with severe damage to proteins by reducing the numbers of disulfide bonds.[21] Hydroxyl radicals are also involved in the lipid peroxidation, DNA breakdown, and cancer formation.[23] Scavenging of the hydroxyl radicals in this study is associated with the hydrogen donating ability of the phenolic compounds[20] as also noted in the present study. Nitric oxide radicals are formed during phagocytosis which are responsible for

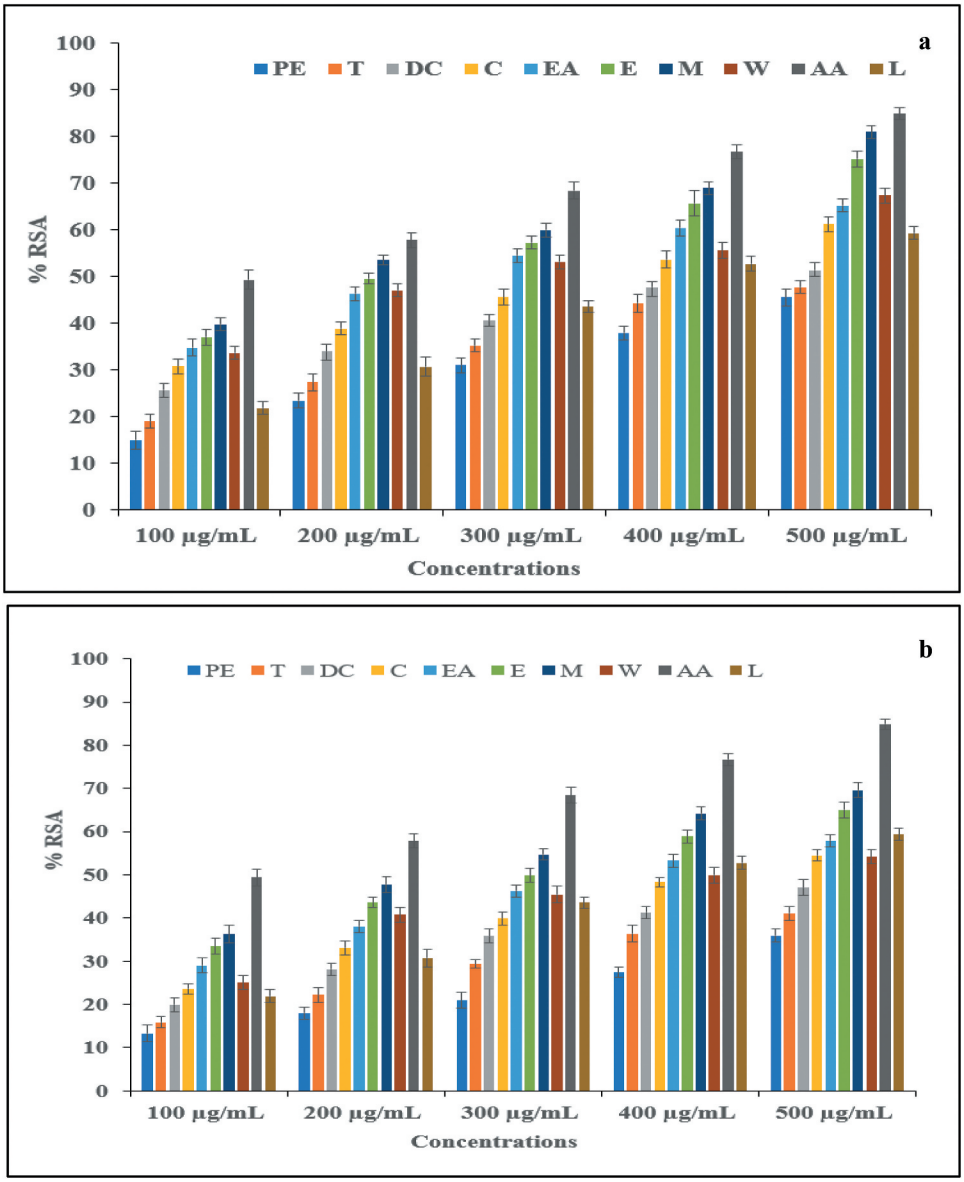

Figure 7. H_2O_2 radical scavenging activity of *Lobelia nicotianifolia* leaf (a) and root (b) extracts. PE, petroleum ether; T, toluene; DM, dichloromethane; C, chloroform; EA, ethyl acetate; E, ethanol; M, methanol; W, water; AA, ascorbic acid; L, lobeline.

inflammatory process.[20] It is a reactive free radical toxic to tissues and leading to injury and vascular collapse. Increased level of nitric oxide radicals is associated with non-communicable diseases such as diabetes, multiple sclerosis, arthritis, and ulcerative.[23,24] Since free radicals of nitric oxide are mainly related in the treatment of inflammation[24] the nitric oxide scavenging property of *L. nicotianifolia* can play a major role for the same. H_2O_2 is a weak

Table 5. Correlation between TPC, TFC, and LC of leaves and roots of *Lobelia nicotianifolia* with different antioxidant assays (% RSA).

	ABTS radicals		DPPH radicals		Hydroxyl radicals		Nitric oxide radicals		H_2O_2 radicals	
	Leaves	Root	Leaves	Root	Leaves	Root	Leaves	Root	Leaves	Root
TPCL	0.976**	0.931**	0.946**	0.920**	0.892**	0.929**	0.958**	0.895**	0.967**	0.945**
TPCR	0.982**	0.946**	0.951**	0.932**	0.827**	0.921**	0.957**	0.937**	0.953**	0.928**
TFCL	0.985**	0.965**	0.954**	0.921**	0.834**	0.918**	0.949**	0.929**	0.947**	0.905**
TFCR	0.974**	0.919**	0.907**	0.902**	0.760**	0.882**	0.931**	0.901**	0.918**	0.902**
LCL	0.285	0.028	0.129	0.235	0.085	0.190	0.288	0.144	0.250	0.415*
LCR	0.225	− 0.029	0.063	0.152	− 0.013	0.104	0.210	0.090	0.174	0.334

**Correlation is significant at the 0.01 level (two-tailed), *Correlation is significant at the 0.05 level (two-tailed). TPCL, total phenolic content of leaves; TPCR, total phenolic content of roots; TFCL, total flavonoid content of leaves; TFCR, total flavonoid content of roots; LCL, lobeline content of leaves; LCR, lobeline content of roots.

Table 6. Correlation between TPC, TFC, and LC of leaves and roots of *Lobelia nicotianifolia* with EC_{50}.

	ABTS radicals		DPPH radicals		Hydroxyl radicals		Nitric oxide radicals		H_2O_2 radicals	
	Leaves	Root	Leaves	Root	Leaves	Root	Leaves	Root	Leaves	Root
TPCL	−.950**	−.908**	−.943**	−.949**	−.900**	−.963**	−.855**	−.947**	−.874**	−.856**
TPCR	−.969**	−.946**	−.937**	−.943**	−.834**	−.955**	−.815**	−.978**	−.799**	−.775**
TFCL	−.951**	−.960**	−.917**	−.923**	−.819**	−.954**	−.780**	−.970**	−.781**	−.761**
TFCR	−.963**	−.942**	−.918**	−.916**	−.801**	−.948**	−.787**	−.976**	−.753**	−.729**
LCL	−.422*	−.090	−.411*	−.376	−.397	−.346	−.542**	−.336	−.350	−.307
LCR	−.360	−.051	−.344	−.313	−.295	−.287	−.444*	−.287	−.236	−.199

**Correlation is significant at the 0.01 level (two-tailed), *Correlation is significant at the 0.05 level (two-tailed). TPCL, total phenolic content of leaves; TPCR, total phenolic content of roots; TFCL, total flavonoid content of leaves; TFCR, total flavonoid content of roots; LCL, lobeline content of leaves; LCR, lobeline content of roots.

oxidizing agent, which easily enters into the body through air, food, and contact of plants and microorganisms.[25] It has the ability to cross cell membranes, which further reacts with ions Fe^{2+} and Cu^{2+} to generate hydroxyl radicals.[26] H_2O_2 RSA is associated with phenolic contents which have ability to donate electrons and convert H_2O_2 to water.[27] *L. nicotianifolia* methanolic leaf extracts can give productive results where H_2O_2 radicals damage proteins leading to unfolding and further triggering abnormal spatial configurations engaged in cancer formation[23], which can arrest further damage.

The antioxidant studies showed a positive correlation between % RSA and EC_{50} of assays with TPC and TFC. Similar correlations of TPC and TFC with antioxidant activity for different plant species have been reported .[25,28] In this investigation, higher TPC and TFC contributed to a higher antioxidant potential in different extracts, which was in agreement with previous report.[29] Negative correlation was observed between EC_{50} with LC of leaves only for DPPH and nitric oxide assays. The lowest EC_{50} values of antioxidant assays is associated with the highest antioxidant activity, hence the correlation was negative. The higher the concentrations of TPC and TFC, lower was the quantity required in scavenging of free radicals.

Comparing the % RSA and EC_{50} of extracts with standard AA, lobeline in crude extracts had higher antioxidant activity, which is consistent earlier reports.[30] Other studies[31] found nonsignificant RSA for lobeline

Table 7. Identification of phenolic compounds by LC-HRMS in methanolic leaf extracts of *Lobelia nicotianifolia*.

	Observed			Database		
SMs	m/z	RT	Mass	Formula	Mass	Mass error (ppm)
Series 1 Flavonoids						
Quercitrin	449.1079	7.37	448.1005	$C_{21}H_{20}O_{11}$	448.1006	0.06
Epicatechin monogallate	443.0975	8.73	442.0904	$C_{22}H_{18}O_{10}$	442.09	−0.91
Hesperetin	285.076	8.81	302.0793	$C_{16}H_{14}O_6$	302.079	−0.7
Xanthoxylin	197.0812	6.15	196.074	$C_{10}H_{12}O_4$	196.0736	−2.36
Eriodictyol	271.0605	9.25	288.0639	$C_{15}H_{12}O_6$	288.0634	−1.63
Series 2 Phenolics						
Embelin	277.1788	11.35	294.182	$C_{17}H_{26}O_4$	294.1831	2.67
Gallic acid	169.0137	0.75	170.021	$C_7H_6O_5$	170.0215	2.8
Harderoporphyrin	1239.5169	18.38	608.2636	$C_{35}H_{36}N_4O_6$	608.2635	−0.25
Cosmosiin	433.1134	7.39	432.1061	$C_{21}H_{20}O_{10}$	432.1056	−1.12
Alpha-Tocopherol	413.3766	28.92	430.3799	$C_{29}H_{50}O_2$	430.3811	2.85
Lecanoric acid	301.0713	9.48	318.0745	$C_{16}H_{14}O_7$	318.074	−1.82
Series 3 Carotenoids						
Tunaxanthin E	551.4244	20.82	568.4277	$C_{40}H_{56}O_2$	568.428	0.63
Myxol glycoside	764.5106	14.92	746.4764	$C_{46}H_{66}O_8$	746.4758	−0.88
Fucoxanthinol	599.4101	13.55	616.4133	$C_{40}H_{56}O_5$	616.4128	−0.88
Series-4 Anthraquinone						
Khayanthone	553.2799	20.31	570.2833	$C_{32}H_{42}O_9$	570.2829	−0.7
Series-5 Coumarin						
Osthol	244.134	7.88	244.1108	$C_{15}H_{16}O_3$	244.1099	−3.48
Series-6 Hydroxyquinone						
Arbutin	562.214	7.49	272.0906	$C_{12}H_{16}O_7$	272.0896	−3.49
Series-7 Isoflavonoids						
Genistein, 8-methyl	305.0221	1.91	284.0696	$C_{16}H_{12}O_5$	284.0685	−4.12

compared with quercetin (flavonoid), which is in agreement with the results of the present study. In the present investigation, it may be that the antioxidant activity of methanolic leaf extract was associated with the presence of several SMs that can donate more hydrogen hence the screening of this extracts was essential. In order to identify the possible SMs contributing to the strong antioxidant activity of methanolic leaf extract, LC-HRMS was performed as it is a powerful technique for identifying unknown chemical constituents in plant extracts through efficient separation capabilities of HPLC and exact structural characterization by mass spectrum.[32] This analysis revealed the presence of flavonoids, phenolics, carotenoids, anthraquinones, coumarins, hydroxyquinones, and isoflavonoids (Table 7). The highest antioxidant activity was detected for methanolic extracts, followed by other mid polar and nonpolar leaf extracts (Figs. 3–8), which may be attributed to variations in phenolics and flavonoids in terms of their numbers and concentrations, which supported previous observations[33–35] for other plant species. Phenolic compounds were quantified in polar, mid polar, and nonpolar (methanol, ethyl acetate, and toluene) leaf extracts, which showed highest, optimum and lowest antioxidant activity, respectively. The chromatogram of polar and nonpolar (methanol and toluene) leaf extracts presented nine peaks, whereas seven peaks were observed for

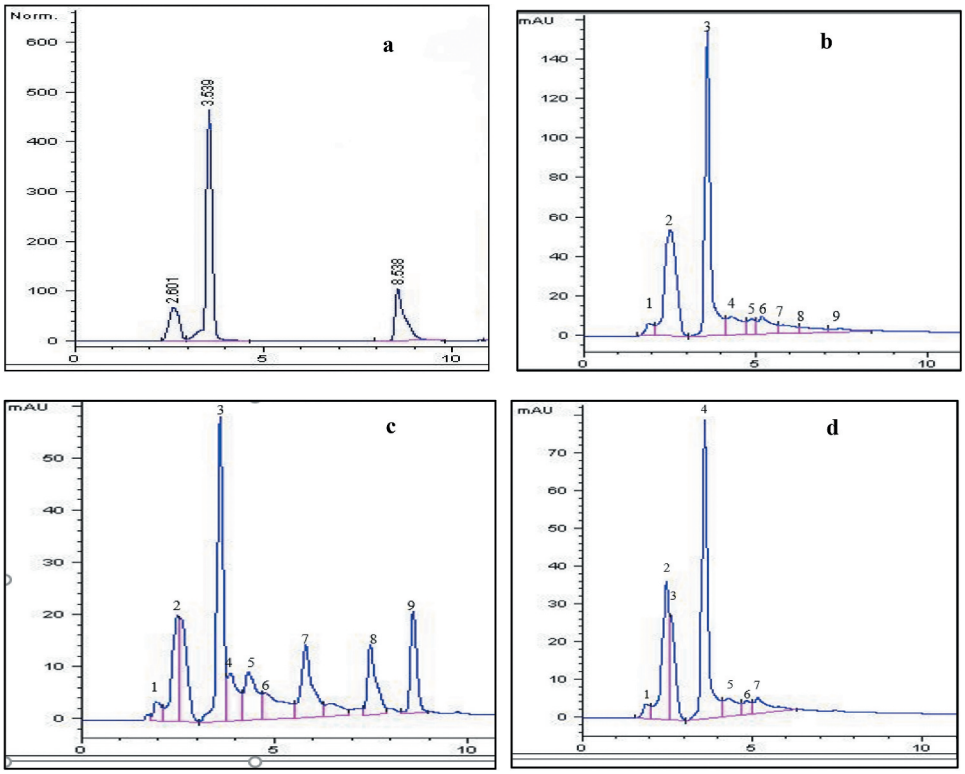

Figure 8. HPLC analysis of embelin, gallic acid, and quercetin in leaf extracts (methanol, ethyl acetate, and toluene) of *Lobelia nicotianifolia*. HPLC chromatograms of (a) standard embelin (RT = 2.601), gallic acid (RT = 3.539), and quercetin (RT = 8.538); (b) methanolic leaf extract; (c) toluene leaf extract; and (d) ethyl acetate leaf extract.

mid polar (ethyl acetate) extract. The retention time of these peaks was very similar to that of the peaks for the standard compounds, which confirmed the presence of embelin, gallic acid, and quercetin in the extracts. Higher embelin (16.36 μg g^{-1} DW), gallic acid (53.47 μg g^{-1} DW), and quercetin (18.93 μg g^{-1} DW) were recorded in methanolic leaf extracts than in ethyl acetate and toluene leaf extracts. This study revealed the presence of embelin and gallic acid in the ethyl acetate extract, whereas the toluene extract showed the presence of only gallic acid, which suggested that the polarity of extracting solvents influences the extractability and solubility of phenolics and flavonoids.[33]

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Disclosure Statement

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