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ORIGINAL ARTICLE

Bioassay guided screening, optimization and characterization of antioxidant compounds from high altitude wild edible plants of Ladakh

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Abstract Seven edible plants including three unexplored species of high altitude (Ladakh) region were screened for antioxidant activity by bioassay guided fractionation method. The objective of the study was to dereplicate the complex phytochemical matrix of a plant in reference to antioxidant activity in vitro. The screening result showed that ethylacetate fraction of *Nepeta longibracteata* possesses maximum antioxidant activity, comparable to that of green tea. It also exhibited significant protecting effect against oxidative stress induced by *t*-BHP in human RBCs. Phytochemical profiling of the most active fraction by nontargeted RP-HPLC–MS and MS/MS technique showed that rosmarinic acid and methylrosmarinate constituted nearly 51 % of the total metabolites apart from twelve other major chemotypes.

Keywords *Nepata longibracteata* · Antioxidant activity · Bioassay-guided fractionation · LC-ESI-QTOFMS · Rosmarinic acid · Methylrosmarinate

Introduction

In the recent decades, wild edible and medicinal plants used by ethnic communities of the developing nations have attracted wide response from many scientists across the globe. A number of studies on traditionally used food and medicinal plants have led to the discovery of many compounds including antioxidants and nutraceuticals (Tag et al. 2014). The Indian trans-Himalayan cold desert region of Ladakh represents a valuable source of large number of natural bio-resources beneficial for the armed forces as well as civilian population (Kumar et al. 2015). The adverse climatic conditions of this region leading to sustained energy deficit, malnutrition, vitamin and mineral deficiency and metabolic disorders in human population. However, the diverse flora and fauna growing in this region has the potential of providing additional physiological benefits and basic nutritional requirements to promote health benefits. High altitude stress generates oxidative/reductive stress with the generation of reactive oxygen and nitrogen species (RONS) in humans along with oxidative damage of lipids, proteins and DNA. As a part of our ongoing research to identify bioactive compounds from high altitude plants of Ladakh, we reported two flavonoid compounds from *Tanacetum gracile* possessing chemopreventive and antioxidant properties (Sinha et al. 2015). In the present study, we selected seven wild edible plants of Ladakh and Kashmir valley. The collection time and location of each plant is provided in Table 1. The literature search showed that *Perovskia abrotanoides* possesses anti-inflammatory, antimicrobial, antifungal and cytotoxic activity (Ashraf et al. 2014) while, *Echinacea purpurea* is used for common cold and flu (Yale and Liu 2004). *Rhodiola imbricata* is widely used as a nutraceutical supplement in the trans-Himalayan region and exhibits antihaemolytic potential by preventing radiation-induced membrane degeneration of human erythrocytes (Arora et al. 2008). *Lepidium latifolium* (perennial pepperweed) is one of the preferred phytofoods among locals. The

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Table 1 Accession details of plants and their origin

S. no.	Species	Family	Location	Altitude (m)	Voucher specimen no.	Time of collection
1	*Nepeta longibracteata Bentham*	Lamiaceae	South Polu	4500 m	22,081	July–August
2	*Waldheimia tomentosa Regel*	Asteraceae	Khardungla	4450 m	21,731	July–August
3	*Rhodiola imbricata Edgew*	Crassulaceae	Khardungla	5200 m	22,950	July–August
4	*Delphinium brunonianum Royle*	Ranunculaceae	Khardungla	4000 m	21,727	July–August
5	*Lepidium latifolium* L.	Brassicaceae	Leh	3500 m	22,107	July–August
6	*Echinacea purpurea* (L.) *Moench.*	Asteraceae	IIIM—Jammu	300 m	21,907	July–August
7	*Perovskia abrotanoides Karel*	Lamiaceae	Khardung—Nubra	3025 m	57,323	July–August

leaves, shoots and fruits of the plants are consumed as food (Kaur et al. 2013). Beside their traditional uses, very little scientific information was available for the rest three plants including, *Nepeta longibracteata, Waldheimia tomentosa* (Rinchen and Pant 2014) and *Delphinium brunonianum* (Gupta et al. 2013).

In the present study, air dried material of each plant (whole plant) was extracted and fractionated with organic and aqueous solvents of increasing polarity. The fractions and extracts, after removal of solvent were screened for antioxidant activity by DPPH based free radical scavenging assay and the most active fraction was identified. The total phenolic and flavonoid content was also determined for each fraction. During exposure to high altitude environment, a wide range of reactive nitrogen and oxygen species (RNOS) generating systems are activated in our body, which weakens the enzymatic and non-enzymatic antioxidant systems. Erythrocytes or red blood cells (RBCs) have widely been used as a model in studying oxidative stress. Oxidative damage in RBCs is induced by tertiarybutyl-hydrogen peroxide (*t*-BHP) exposure. It causes increased level of haemolysis and damage of poly unsaturated fatty acids, present in cell membrane (lipid peroxidation). Generally, these two parameters are studied to measure oxidative damage caused by an external/internal agent. In order to mimic RBC damage in vivo, the RBCs were treated with *t*-BHP and the most active fraction was examined to obtain percentage decrease in haemolysis and lipid peroxidation. Finally, the chemical profiling of the most active fraction was performed by liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry (LC-ESI-QTOFMS).

Materials and methods

Chemicals and reagents

Analytical grade solvents and chemicals were used for extraction and fractionation. HPLC grade acetonitrile and methanol (Merck, Darmstadt, Germany) and was used for HPLC and LC–MS analysis. 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tri(2-pyridyl)-*S*-triazine (TPTZ) and 2-thiobarbituric acid (TBA) were purchased from Sigma-Aldrich. Analytical grade quercetin and ascorbic acid (positive control) were purchased from Hi-Media Laboratories, Mumbai, India, while, luperox TBH70X (*t*-BHP) was purchased from Sigma-Aldrich. Bruker 500 MHz spectrometer was used for ^{1}H NMR and ^{13}C NMR spectra and chemical shift values were expressed in ppm (δ) units. HR-ESI–MS was performed on Bruker mass spectrometer coupled with Agilent 1100 LC.

Plant material

The plant materials were collected and authenticated by Dr. S. Kitchlu, Department of Botany, Indian Institute of Integrative Medicine (IIIM) and the voucher specimen is deposited at the herbarium of IIIM, Jammu.

Extraction and fractionation and phytochemical analysis

Air dried whole plant (100 g) material was crushed and macerated in methanol–water (9:1; 100 mL) for overnight followed by sonication in an ultrasonic bath at 35 °C for 30 min, twice and filtered. The residue was extracted with minimum volume of water. The concentrated organic fraction was fractionated with *n*-hexane, dichloromethane and ethylacetate successively. The solvent was removed in a rotary evaporator and dry weight was recorded. Each fraction was screened for antioxidant activity (Table 2).

Total phenolic content (TPC) was determined spectrophotometrically at 760 nm by modified Folin–Ciocalteau method as described by Celiktas et al. (2007) and was expressed as gallic acid equivalent (GAE) in mg per g (mg/g).

The total flavonoid content (TFC) was measured by spectrophotometric assay based on aluminium complex formation as described by Adnan and Fartosy (2011). Briefly, a solution containing $NaNO_2$, $AlCl_3$, NaOH along

Table 2 Net weight of the fractions, after complete removal of solvent

Plant	Fraction	Yield (%)
Nepeta longibracteata	*n*-hexane	0.95
	Dichloromethane	0.42
	Ethylacetate	0.32
	Aqueous residue	0.63
Waldheimia tomentosa	*n*-hexane	0.37
	Dichloromethane	0.62
	Ethylacetate	0.32
	Aqueous residue	0.51
Rhodiola imbricata	*n*-hexane	0.32
	Dichloromethane	0.45
	Ethylacetate	0.28
	Aqueous residue	1.23
Delphinium brunonianum	*n*-hexane	1.12
	Dichloromethane	0.87
	Ethylacetate	0.23
	Aqueous residue	1.34
Lepidium latifolium	*n*-hexane	1.11
	Dichloromethane	0.34
	Ethylacetate	0.67
	Aqueous residue	1.78
Echinacea purpurea	*n*-hexane	0.82
	Dichloromethane	0.76
	Ethylacetate	0.53
	Aqueous residue	1.82
Perovskia abrotanoides	*n*-hexane	0.66
	Dichloromethane	0.23
	Ethylacetate	0.5
	Aqueous residue	0.66

The concentrated organic extracts were successively fractionated with solvents of increasing polarity viz., *n*-hexane, dichloromethane and ethylacetate

with plant extract was mixed well and the absorbance was measured at 510 nm. The TFC was calculated in terms of quercetin equivalent (mg/g) by following the calibration curve.

Analysis of antioxidant activities

DPPH free radical scavenging assay

The DPPH free radical scavenging assay was performed according to Celiktas et al. (2007). Briefly, an aliquot of each sample was mixed with 1 mM DPPH solution followed by incubation for 30 min in dark. The absorbance of each sample was measured at 517 nm. The antioxidant activity was expressed in the concentration required to inhibit 50 % methanolic DPPH radical formation (IC_{50} in μg/mL) with ascorbic acid and quercetin as positive control.

Ferric reducing antioxidant power (FRAP) assay

FRAP assay was performed according to modified protocol of Benzie and Strain as adapted by Krishnaraju et al. (2009). The assay was based on the ability of a sample to reduce ferric to ferrous ions in the presence of 2,4,6-tri-(2-pyridyl)-*S*-triazine (TPTZ), forming an intense blue ferrous–TPTZ complex with absorption maxima at 593 nm. Effective concentration was defined as the concentration of the antioxidant sample having a ferric reducing ability, equivalent to that of 1 mM ferrous salt (EC_1).

Inhibition of haemolysis in RBCs

Venous blood samples were collected from healthy donors. The samples were processed by protocol as described by Battistelli et al. (2005). After the removal of plasma and buffy coat, the RBCs were washed thrice and resuspended in phosphate buffer saline (PBS) and was used for subsequent analysis. For haemolysis, modified protocol as described by Okoko and Ere (2012) was followed. The reaction mixture containing 200 μL of RBC suspension and 10 μL of the test solution was incubated for 30 min at 37 °C. Haemolysis was induced by addition of 100 μL of 100 μM of *t*-BHP followed by incubation at 37 °C for 3 h. Thereafter, 200 μL of the supernatant was diluted with 1.4 mL of PBS and were centrifuged and the absorbance was measured at 540 nm. The experiment without the test sample was considered for 100 % haemolysis and the results were expressed in percentage haemolysis inhibition.

Inhibition of lipid peroxidation

Lipid peroxidation against RBCs was measured by thiobarbituric acid reactive substances (TBARS) method (Maurya and Rizvi 2009). In brief, the processed RBCs were incubated with sample solutions for 3 h. Thereafter, the proteins were precipitated by the addition of 10 % Trichloro-acetic acid (TCA) followed by centrifugation at 3000 rpm for 5 min. To 1 mL of the supernatant, 0.67 % of TBA reagent was added and the reaction mixture was boiled for 20 min. A red colored adduct was formed which was quantified spectrophotometrically at 532 nm. The results were expressed in terms of percentage inhibition of lipid peroxidation.

Cell cytotoxicity assay

The cell cytotoxicity was performed as per the method described by Kakad and Dhembare (2014). Fibroblast cells obtained from chick embryo were cultured in DMEM medium supplemented with Fetal Bovine Serum (FBS) and gentamicin. The cells suspension (2 mL) was treated with

sample solution at IC_{50} concentration and twice the concentration of IC_{50} (DPPH assay). The microtitre plate was incubated aseptically in CO_2 incubator for 24 h at 37 °C. After incubation, cells were dis-aggregated using trypsin (0.25 %). Percent viability was calculated using MTT based assay.

Sample preparation, HPLC and LC/MS analyses

Ethylacetate fraction of *N. longibracteata* (NlE) was accurately weighed to 5 mg and dissolved in methanol. After centrifugation, the supernatant was concentrated and the residue was reconstituted with methanol.

HPLC analysis

The HPLC experiments were performed on Shimadzu LC system equipped with manual injector and SPD-M20A PDA detector. Solvent systems constituting water with 0.1 % formic acid (v/v) (buffer A) and methanol (buffer B) were used as mobile phase. An injection of 20 µL of aliquot at 1 mg/mL concentration was made on a Phenomenex Luna C-18 analytical column (5 µm, 4.6 × 250 mm) and gradient elution (10–100 % methanol, 25 min, 1 mL/min) was followed to produce a well resolved chromatogram. The retention time of two most intense peaks appearing at 9.15 min and 10.57 min were noted. Further chromatography on a semi-prep C-18 column (10 × 250 mm) was performed with the crude sample in isocratic mode with methanol–water (60:40) for 25 min with a flow rate of 5 mL/min to isolate the pure compounds (retention time 5.72 min and 6.03 min) for characterization.

Characterization of secondary metabolites

Rosmarinic acid (compound **2**): $C_{18}H_{16}O_8$; ESIMS (negative ion) m/z 359.07597 $[M-H]^-$ (calculated for 359.07656); ^{1}H NMR (CD_3OD, 500 MHz): δ 7.36 (1H, d, J = 16 Hz, H-7′), 7.08 (1H, d, J = 8.0 Hz, H-5), 6.95 (br s, H-2), 6.88 (br d, J = 8.0 Hz, H-6), 6.87 (d, J = 8.0 Hz, H-5′), 6.78 (br s, H-2′), 6.75 (br d, J = 8.0 Hz, H-6′), 6.14 (d, J = 16.0 Hz, H-8′), 5.28 (dd, J = 4.3 Hz, and J = 8.6 Hz, H-8), 3.18 (dd, J = 14 Hz and 8.6 Hz, H-7) and 3.07 (dd, J = 14 Hz and 4.3 Hz, H-7); ^{13}C NMR (CD_3OD, 125 MHz): δc172.3 (C-9), 167.2 (C-9′), 148.3 (C-3 and C-5′), 146.5 (C-4′), 145.4 (C-7′), 144.7 (C-4), 127.9 (C-7), 126.2 (C-1′), 121.9 (C-6), 120.5 (C-2′), 115.2 (C-5 and C-3′), 115.0 (C-8′), 112.9 (C-6′), 73.3 (C-8) and 37.5 (C-7).

Methylrosmarinate (compound **4**): $C_{19}H_{18}O_8$; ESIMS (negative ion): m/z 373.33339 $[M-H]^-$ (calculated for 373.33348); ^{1}H-NMR (CD_3OD):δ7.52 (1H, d, J = 15.5 Hz, H-7′), 7.04 (1H, d, J = 2.0 Hz, H-2′), 6.91 (H, dd, J = 8.5, 2.0 Hz, H-6′), 6.75 (1H, d, J = 8.5 Hz, H-5′), 6.71 (1H, d, J = 2.0 Hz, H-2), 6.64 (1H, d, J = 8.0 Hz, H-5), 6.61 (1H, dd, J = 8.0, 2.0 Hz, H-6), 6.27 (1H, d, J = 15.5 Hz, H-8′), 5.11 (1H, dd, J = 7.5, 5.0 Hz, H-8), 3.72 (3H, s, OCH_3), 3.06 (1H, dd, J = 14.5, 5.5 Hz, H-7a), 3.00 (1H, dd, J = 14.5, 5.5 Hz, H-7b); ^{13}C NMR (CD_3COCD_3, 125 MHz): δc 170.2 (C-9), 166.3 (C-9′), 148.2 (C-3 and C-3′), 146.2 (C-4′), 145.5 (C-7′), 144.6 (C-4), 143.6 (C-1), 129.2 (C-1′), 122.2 (C-6), 120.9 (C-6′), 116.8 (C-5 and C-5′), 115.6 (C-8′), 114.8 (C-2), 113.7 (C-2′), 73.3 (C-8), 51.6 (C-10), 31.2 (C-7).

LC/MS analysis

LC/MS analysis was performed with Kinetex 1.7u C-18 (100 × 2.1 mm) column on a ultimate 3000–Dionex HPLC system coupled with an electrospray mass spectrometer (QTOF, AB Sciex, Foster City, CA, USA). The LC effluent was introduced into the mass spectrometer in post-column splitting ratio of 5:1. High purity nitrogen (N_2) was used as a nebulising gas and ultra-high purity helium (He) as the collision gas. The ion source was operated in both positive and negative ion mode. The sample was scanned over a mass range of 50–1000 m/z with a voltage floating (ISVF) = 5500 V, curtain gas (CUR) = 25, ion source gas 1 (GS1) = 15, ion source gas 2 (GS2) = 25, interface heater temperature (IHT) = 120, column temperature = 40 and declustering potential (DP) = 80 V. Mass spectrometer was operated in a data dependent mode and top 15 m/z peaks were subjected to MS/MS fragmentation in each duty cycle. Peaks with +1 to +5 charge were selected for fragmentation with a threshold value of 120 cps (Basak et al. 2015).

Quantitative data analysis

The LC/MS system was controlled by Analyst TF 1.6 software. The TIC normalization was done using total sum area based module in an excel spread sheet using Marker View (ABSciex).

Identification of secondary metabolites

Each peak was searched against METLIN and MassBank database. Metabolite identification was performed by matching masses with a mass accuracy window of 30 ppm and RT window of ±2 min for METLIN database library. Few metabolites were also identified by comparing their m/z values in the total ion count (TIC) profile with the reported MS/MS spectra. The robustness of the identification was confirmed by matching the masses of the fragments from the MS–MS spectra for each of the metabolite.

All experiments were carried out in triplicates and the results were expressed as mean ± standard deviation (SD) values wherever applicable.

Results and discussions

Initial screening of the plantextracts/fractions for antioxidant activity was evaluated by DPPH radical scavenging and FRAP assay. The antioxidant potential of natural antioxidant, green tea (*Camellia sisensis*) evaluated by DPPH and FRAP assay exhibited IC_{50} value of 61.43 and 19.91 μg/mL respectively (Taheri et al. 2011). Also, NlE exhibited significantly higher radical scavenging activity compared to a phytococktail (Dhar et al. 2013), constituting sea buckthorn, apricot and roseroot, commercially used as natural antioxidant. For selection of potential antioxidant fractions amongst experimental plants, green tea extract (GTE) was considered as reference. Amongst seven plants, four plants including *N. longibracteata, P. abrotinoides*, *E. purpurea* and *D. brunonianum* were identified as potential antioxidants. In particular, ethylacetate fraction of *N. longibracteata* (NlE), *P. abrotinoides* and *E. purpurea* along with dichloromethane fraction of *D. brunonianum* showed maximum potency (Table 3). Fractionation of plant extracts with solvents of increasing polarity could effectively localize activity in a particular fraction. The following method has widely been used to divide complex phytochemical matrix of a plant extract and to confine biological activity in a particular fraction. Amongst these active fractions, NlE exhibiting IC_{50} value of 64.3 μg/mL, showed comparable activity to that of *C. sisensis* (Green tea, GTE). Further, the observation was established more emphatically by FRAP assay. At cellular level, antioxidant potential was measured by the degree of inhibition of *t*-BHP induced haemolysis and lipid peroxidation in reference to an established standard. The standard antioxidant compounds namely ascorbic acid and quercetin, respectively, exhibited 80.8 and 95.8 % inhibition while, NlE showed 78.3 % inhibition against haemolysis. Similar trend was also observed against *t*-BHP induced lipid peroxidation (Table 4). To correlate antioxidant activity of the fractions with chemical constituents present therein, total phenolic and flavonoid content was determined for each fraction separately. The total phenolic (116.7 GAE/g) and total flavonoid (223.4 QE/g) content was similar to GTE.

An extensive review on pharmacological property and chemical diversity of secondary metabolites of the genus *Nepeta* has been presented by Formisano et al. 2011. Therein, a large number of secondary metabolites including nepetalactones, monoterpenes (iridoids and their glucosides), diterpenes, triterpenes, phenolics and flavonoids have been reported. Mostly, flavonoids and phenolic compounds of *Nepeta* species were reported to have antioxidant activity. Additionally nepetalactones, a major constituent of Nepeta oil was reported to have both antioxidant and antibacterial activity.

Our investigation of the most active fraction NlE by LC–MS and MS/MS analysis identified 14 major compounds (Fig. 1) by comparing $[M-H]^-$ deprotonated molecule and its fragmentation in MS/MS spectra with available standards (Table 5). Best resolution was achieved in negative ion mode, hence, negative ion mode data was considered for analysis. Common fragmentation pathways based on CO_2 loss were observed for all phenolic acids with a characteristic deprotonated $[M-H]^-$ molecule and $[(M-H)-44]^-$ fragment. The ESI–MS spectra of a major compound (compound **2**) appearing at 4.57 min constituting 32.6 % of the total metabolites, showed deprotonated molecules at m/z 359.08. The MS/MS spectra produced ions at m/z 719.16 corresponding to $[2M-1]^-$, m/z 557.16 for $[M+C_9H_9O_5$ (dhanshenshu)$]^-$, m/z 395.05 for $[M+Cl]^-$ and m/z 197.06 for dhanshenshu $[C_9H_9O_5]^-$, exhibiting close resemblance with the fragmentation pattern of rosmarinic acid (RA) (Altintas et al. 2011). The appearance of higher molecular weight adducts is a common feature in LC–MS spectra of crude extracts. Another major metabolite (compound **4**), occupying 18.4 % of the total area, appeared at 5.01 min. It showed deprotonated molecule at m/z 373.08 and a fragment at m/z 359.07 in MS/MS spectra indicating a loss of m/z 14 (methyl moiety), characteristic of the deprotonated molecule of RA. The other significant fragments appeared at m/z 343.08, with a loss of m/z 30 (HCHO) and higher molecular adducts at m/z 747.19 $[2M-1]^-$, m/z 733.17 $[2M-14]^-$ and m/z 717.17 $[2M-30]^-$. The mass difference of m/z 14 between deprotonated molecules of 4 and 2 indicated that compound **4** could be the methyl ester derivative of RA. As, both the compounds were present in excess, they were isolated by semi-prep HPLC and were characterized by comparing ^1H and ^{13}C NMR data with the reported literature data and were unambiguously identified as rosmarinic acid and methylrosmarinate (Pereira et al. 2013).

The first minor compound (compound **1**) showed characteristic deprotonated $[M-H]^-$ molecule at m/z 169.08, corresponding to gallic acid ($C_7H_6O_5$). Compound **3**, appeared at 4.86 min exhibiting deprotonated molecule at m/z 199.1. The MS/MS fragment at m/z 153.09 $[(M-H)^- - (CO + H_2O)]^-$ could be due to the loss of a carboxyl group present at a terminal position. The molecular formula of $C_{10}H_{15}O_4$ corresponding to camalexin (4-isopropyl-*O*-pyrocatechuic acid) was suggested for the compound. Compound **5**, appearing at 5.29 min showed deprotonated moleculeat m/z 199.01 and a significant MS/MS fragment corresponding to the loss of CO_2 (m/z 44), indicating it to be an isomer of

Table 3 (a) Estimation of total phenolic and flavonoid content, (b) measurement of antioxidant activity of the fractions by DPPH and FRAP assay

Plant name	Fraction	Total phenolic and flavonoid content		Antioxidant activity	
		TPC[a]	TFC[b]	DPPH IC_{50}^{c}	FRAP[d]
N. longibracteata	*n*-hexane	33.34 ± 0.11	83.42 ± 0.22	139.33 ± 0.35	93.32 ± 0.03
	Dichloromethane	35.30 ± 0.23	39.96 ± 0.17	138.72 ± 0.09	56.14 ± 0.19
	Ethylacetate	116.67 ± 0.25	223.36 ± 0.08	64.31 ± 0.08	19.92 ± 0.11
	Aquous residue	66.69 ± 0.06	76.66 ± 0.02	151.36 ± 0.016	49.17 ± 0.16
W. tomentosa	*n*-hexane	33.33 ± 0.04	99.71 ± 0.58	130.75 ± 0.03	49.87 ± 0.21
	Dichloromethane	55.01 ± 0.02	66.69 ± 0.02	142.03 ± 0.08	101.24 ± 0.24
	Ethylacetate	40.02 ± 0.01	19.99 ± 0.02	153.75 ± 0.02	106.34 ± 0.25
	Aquous residue	48.33 ± 0.02	46.66 ± 0.01	>200	97.98 ± 0.25
R. imbricata	*n*-hexane	70.1 ± 0.01	54.47 ± 0.02	178.37 ± 0.21	121.53 ± 0.26
	Dichloromethane	133.27 ± 0.07	43.34 ± 0.04	133.41 ± 0.04	132.73 ± 0.19
	Ethylacetate	56.67 ± 0.03	67.34 ± 0.04	141.33 ± 0.04	92.13 ± 0.14
	Aquous residue	181.20 ± 0.01	22.63 ± 0.01	167.36 ± 0.06	92.18 ± 0.28
D. brunonianum	*n*-hexane	55.01 ± 0.01	19.97 ± 0.04	178.43 ± 0.23	55.12 ± 0.19
	Dichloromethane	66.65 ± 0.03	66.69 ± 0.02	123.4 ± 0.01	51.23 ± 0.16
	Ethylacetate	22.27 ± 0.13	46.66 ± 0.03	185.6 ± 0.30	97.12 ± 0.20
	Aquous residue	133.35 ± 0.03	22.65 ± 0.04	134.32 ± 0.02	91.18 ± 0.23
L. latifolia	*n*-hexane	42.33 ± 0.17	22.63 ± 0.17	148.56 ± 0.18	95.14 ± 0.14
	Dichloromethane	50.00 ± 0.04	83.33 ± 0.04	139.32 ± 0.03	89.85 ± 0.21
	Ethylacetate	68.34 ± 0.03	87.32 ± 0.02	132.66 ± 0.02	45.3 ± 0.18
	Aquous residue	81.67 ± 0.01	93.35 ± 0.02	136.58 ± 0.03	51.23 ± 0.19
E. purpurea	*n*-hexane	102.66 ± 0.03	22.64 ± 0.03	134.33 ± 0.02	91.28 ± 0.21
	Dichloromethane	67.32 ± 0.03	52.66 ± 0.02	146.34 ± 0.03	101.27 ± 0.28
	Ethylacetate	112.67 ± 0.15	178.82 ± 0.03	97.39 ± 0.03	28.71 ± 0.14
	Aquous residue	78.45 ± 0.18	76.78 ± 0.21	142.56 ± 0.17	78.35 ± 0.15
P. abrotanoides	*n*-hexane	46.67 ± 0.06	84.77 ± 0.06	134.69 ± 0.02	101.38 v 0.17
	Dichloromethane	42.78 ± 0.02	38.3 ± 0.01	148.79 ± 0.03	139.23 ± 0.24
	Ethylacetate	123.33 ± 0.02	226.69 ± 0.02	68.47 ± 0.51	21.03 ± 0.11
	Aquous residue	67.34 ± 0.25	78.37 ± 0.16	156.12 ± 0.16	105.26 ± 0.23
Camellia sinensis (L.) *Kuntze*	Ethylacetate	60.47 ± 0.50	240.27 ± 0.74	61.43 ± 1.16	19.91 ± 0.12
	Ascorbic acid			37.31 ± 0.14	18.91 ± 0.09
	Quercetin			28.41 ± 0.12	15.67 ± 0.08

[a] Total phenol content(TPC) expressed as mg GAE/g dry wt. of extract

[b] Total flavonoid content(TFC) expressed as mg QE/g dry wt. of extract

[c] DPPH radical scavenging activity expressed as IC_{50} in μg/mL extract required to scavenge 50 % of free radicals

[d] FRAP value expressed as EC_1 in μg/mL of extract having a ferric reducing ability equivalent to that of 1 mM Fe^{2+} salt

compound **3** (Müller et al. 2009). The sixth compound showed deprotonated molecule at m/z 285.04 and higher molecular adduct at m/z 571.08 corresponding to $[2M - 1]^-$. The MS/MS spectra of compound **6** exhibited fragments at m/z 213.0 and m/z 169.0 corresponding to the loss of m/z of 72 and m/z 116 (72 + 44) characteristics of kaempferol (Mišić et al. 2015) with molecular formula $C_{15}H_{10}O_6$. The next deprotonated molecule at m/z 479.14 and the higher molecular adduct at m/z 525.14 $[M+HCOOH]^-$, tentatively indicated the compound could be a gallic acid derivative. On the basis of the higher molecular adduct and deprotonated molecule, the eighth compound was identified as apigenin with molecular formula $C_{15}H_{10}O_5$ (Lin and Harnly 2010). The MS/MS fragments of the deprotonated molecule of compounds 9, 10, 12, 14 and 15 could not be matched directly with the available standard compounds. However, tentative molecular formulae were suggested on the basis of deprotonated molecules as shown in Table 5. Rest two compounds, appearing at 11.18 and 15.03 min exhibited deprotonated molecule at m/z 265.15 and m/z 375.27 corresponding to the molecular formula $C_{14}H_{18}O_5$ and

Table 4 Effect of the most active fraction in reducing haemolysis and lipid peroxidation against t-BHP induced RBCs

Fractions with antioxidant activity	Inhibition of *t*-BHP induced erythrocyte haemolysis (%)	Inhibition of *t*-BHP induced erythrocyte lipid peroxidation (%)
NlE[a]	78.32 ± 0.10	95.25 ± 0.19
PaE[b]	77.9 ± 0.08	94.3 ± 0.07
EpE[c]	73.4 ± 0.12	87.2 ± 0.09
DbD[d]	36.3 ± 0.07	51.4 ± 0.07
Camellia sinensis (L.) *Kuntze*	81.33 ± 0.21	95.73 ± 0.03
Ascorbic acid	80.85 ± 0.13	86.3 ± 0.11
Quercetin	95.8 ± 0.12	92.4 ± 0.15

% haemolysis/lipid peroxidation calculated by taking hemolysis/peroxidation caused by 100 μM *t*-BHP as 100 %

% of inhibition at 10 μg of extract/fraction/compound in a total volume of 1900 μL

[a] Ethylacetate fraction of *N. longibracteata*

[b] Ethylacetate fraction of *P. abrotanoides*

[c] Ethylacetate fraction of *E. purpurea*

[d] Dichloromethane fraction of *D. brunonianum*

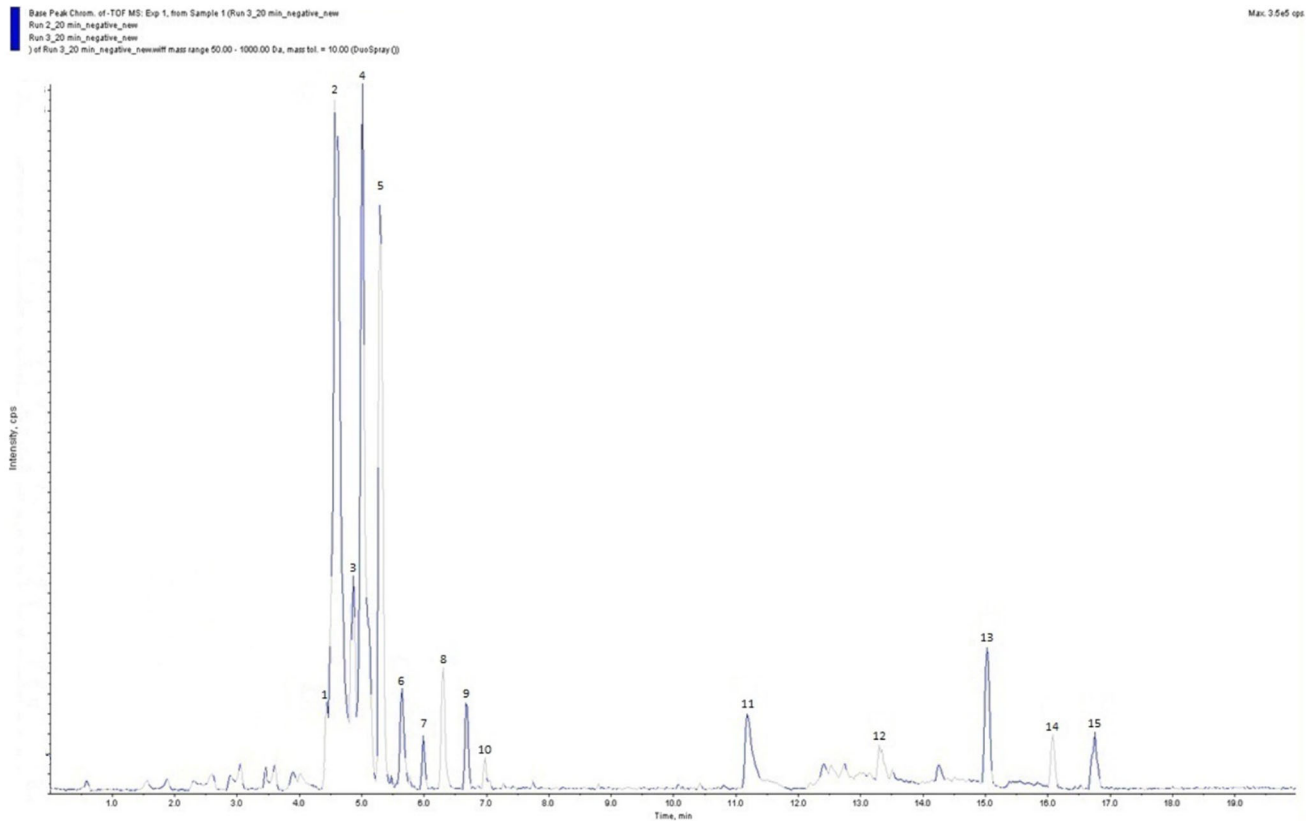

Fig. 1 LC-ESI–MS spectrum of ethylaceate fraction of *N. longibracteata* in negative ion mode

$C_{19}H_{20}O_8$ respectively (Liu et al. 2016). The majority of the compounds as per LC–MS analysis showed that they belong to phenolic or flavonoid class.

Cell cytotoxicity was measured with the fractions against fibroblast cells which were well within permissible limit.

Conclusion

In summary, fourteen compounds of the active antioxidant fraction were identified. Two major compounds constituted almost 51 % of the total chemical constituents and were established as rosmarinic acid and methylrosmarinate. Cell

Table 5 Identification of chemical constituents of ethylacetate fraction of *N. longibracteata* by LC-ESI-QTOFMS/MS in −ve ion mode

Peak	Retention time (min)	% Area	Identification[a]	ESI-MS m/z $[M-H]^{-b}$	(−) ESI-MS^2 m/z	Formula
1	4.42 (ill resolved)	5.29	Gallic acid	169.08	112.98 $[TFA-H]^-$	$C_7H_6O_5$
2	4.57	32.58	Rosmarinic acid	359.08	719.16 $[2M-H]^-$ 557.16 $[M+C_9H_9O_5$ (Dhanshenshu)$]^-$ 395.04 $[M+Cl^-]^-$ 197.06 $[C_9H_9O_5]^-$	$C_{18}H_{16}O_8$
3	4.87	ill resolved	Isopropyl-*O*-pyrocatecheic acid	199.1	187.1 $[M-12]^-$ 153.09 $[M-(HCOOH)]^-$	$C_{10}H_{15}O_4$
4	5.01	18.36	Methylrosmarinate	373.08	747.19 $[2M-1]^-$ 733.17 $[2M-CH_2]^-$ 717.17 $[2M-2\ CH_3]^-$ 343.08 $[M-CH_3]^-$	$C_{19}H_{18}O_8$
5	5.29	12.48	Isomer of compound **3**	199.09	155.11 $[M-CO_2]^-$	$C_{10}H_{16}O_4$
6	5.64	2.17	Kaempferol	285.03	571.08 $[2M-1]^-$ 169.00 $[C_7H_6O_5]^-$	$C_{15}H_{10}O_6$
7	5.99	0.74	Gallic acid derivative	479.14	525.14 $[M+(HCOOH)]^-$	$C_{23}H_{27}O_{11}$
8	6.3	2.53	Apigenin	269.04	539.10 $[2M-1]^-$ 329.06 $[M+59]^-$ 112.98 $[TFA-H]^-$	$C_{15}H_{10}O_5$
9	6.69	1.45	Adenosine	266.14	334.13	$C_{10}H_{13}N_5O_4$
10	6.97	0.54	Derivative of phenylpropionic acid glucoside	327.22	313.07 $[M-CH_2]^-$	$C_{19}H_{20}O_5$
11	11.18	3.57	Derivative of phenyl glucoside	265.15	283.26 $[M-H+H_2O]^-$ 168.99 $[M-96]^-$	$C_{14}H_{18}O_5$
12	13.3	2.59	Deprotonated TFA	325.18	112.98 $[TFA-H]^-$	$C_{16}H_{21}O_7$
13	15.03	3.38	Phenolic compound	375.27	376.27 $[M]^-$	$C_{19}H_{20}O_8$
14	16.08	1.04	4-(3,4-Dimethoxyphenyl benzoate), pinocamberine	255.23	323.22 $[M+68]^-$	$C_{15}H_{12}O_4$
15	16.74	1.38	4-[2,4-Dicarboxy-3-(4-carboxylatophenyl)phenyl]benzoate	403.31	723.59	$C_{22}H_{12}O_8$

[a] Identified using free chemical database, Chem Spider

[b] Exact mass of the parent ion

cytotoxicity measured at IC_{50} (DPPH assay) and twice the concentration of IC_{50} showed that cell cytotoxicity was well within the permissible limit i.e., 7.5 %. The results of lipid peroxidation suggested that the fraction scan be used as natural antioxidant for the treatment and prevention of diseases mediated by lipid peroxidation. Further, the bioassay guided fractionation method was successful to divide the complex matrix of the plant extract and to concentrate antioxidant compounds in one particular fraction. LC–MS profiling of the antioxidant fraction reveals a distinct pattern, which could be used to differentiate *N. longibracteata* from related species. The fraction can be used as an alternative source of natural antioxidants with consequential health promoting effects in the oxidative stress conditions.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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