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Effect of extraction solvents/techniques on polyphenolic contents and antioxidant potential of the aerial parts of *Nepeta leucophylla* and the analysis of their phytoconstituents using RP-HPLC-DAD and GC-MS

Ajay sharma[1*], Damanjit Singh Cannoo[1]

[1]Department of Chemistry, Sant Longowal Institute of Engineering and Technology, Longowal 148106 (Punjab) India

Email: sharmaajay9981@gmail.com, djs6311@gmail, **Fax:** 01672-280072, **Mob:** 91-9779733277

Abstract

In the present study, evaluation of antioxidant potential, percentage yield phytochemical and polyphenolic composition of methanol, chloroform and hexane extracts obtained from the aerial parts (leaves and flowers) of *Nepeta leucophylla* using different extraction techniques (ultrasound, Soxhlet, and maceration) was carried out. The antioxidant potential of different extract was measured using DPPH (2,2-diphenyl-1-picrylhydrazyl radical scavenging), NOS (nitric oxide scavenging), FRAP (ferric reducing antioxidant power) and TAC (total antioxidant capacity) assays. The results of percentage yield, TPC (total polyphenolic content), TFC (total flavonoids content), FRAP, TAC, percentage inhibition of DPPH and NO scavenging for different extracts varied from 3.66 to 17.66%, 0.98±0.47 to 141.9±3.86 mg GAE/g DPE, 6.21±0.49 to 394.48±15.45 mg RE/g DPE, 4.64±0.71 to 196.38±0.31 mg Fe (II) E /g DPE, 10.98±1.36 to 74.72±1.1 mg AAE/g DPE, 8.93±2.09 to 92.57±0.11% and 6.13±0.41 to 64.03±2.29%, respectively. RP-HPLC-DAD and GC-MS analysis of bioactive extracts was also carried out to determine their chemical composition.

Keywords: *Nepeta leucophylla*, antioxidant potential, polyphenolic compounds, RP-HPLC-DAD, and GC-MS.

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1. Introduction

The bioactive secondary metabolites of plant origin are extensively used in food, nutraceutical, cosmetic and pharmaceutical industries due to their wide range of biological potential *viz.* antioxidant, antimicrobial, anti-inflammatory, antifungal, etc.[1] Nowadays, the production of free radicals, especially reactive oxygen species have increased in living beings due to a stressful lifestyle and various kinds of pollution, which can lead to the oxidative stress conditions.[2] The oxidative stress conditions may lead to the origin of various kinds of diseases such as cancer, atherosclerosis, hypertension, diabetes, neurological disorders, acute respiratory distress syndrome and asthma.[3] The various studies have shown that the antioxidants of plant origin are able to prevent or delay the damage caused by oxidative stress conditions.[4] Further, the plants act as renewable source of natural antioxidants, which are ecofriendly in nature.[5] Due to this, the interest has largely grown in plant based natural antioxidants such as polyphenols, flavonoids, tannins, terpenoids, carotenoids, coumarins etc.

Nepeta, a genus of about 300 species belongs to the mint (Lamiaceae) family and *Nepeta leucophylla* is a member of this genus. The species of this genus are cosmopolitan in distribution and are mainly found in North Africa, Central and Southwest Asia, Central and North America, Central and Southern Europe. In India, 41 species are reported from Western *Himalayan* region.[6,7] The phytochemical studies have revealed the presence of various classes of bioactive secondary metabolites viz. terpenoids, glycosides, steroids, flavonoids and phenolic acids, which contribute to its biological potential. [6,7] In folk medicine, many species of this genus have been used as antispasmodic, diaphoretic, diuretic, expectorant, febrifuge, laxative, sedative and for tooth trouble, liver and kidney disease.[8] The recent pharmacological investigations have shown that the genus *Nepeta* also exhibit antibacterial, anticancer, antialzheimer, antioxidant, anti-inflammatory, antiviral, disinfectant and fungicidal potential.[9,7]

Traditionally, the leaves' paste of *N. leucophylla* is used to cure malarial fever.[10] Essential oil and the extracts obtained from different parts of this plant are known to possess antibacterial and antifungal activity.[11] Previous phytochemical analysis of *N. leucophylla* showed the presence of iridodial β-monoenol acetate, dihydroiridodial diacetate and iridodial dienol diacetate in the essential and coleon U 12-methyl ether in chloroform extract obtained from the roots.[12,13,11]

However, to the best of our knowledge, in the literature no reports demonstrating the polyphenolic profile and antioxidant potential of aerial parts of *N. leucophylla* is found. Therefore, the aim of present study is to analyze the effect of different extraction techniques (MM-maceration method, UAEM-ultrasound assisted extraction method and SEM-soxhlet extraction method) and solvents (hexane, chloroform and methanol) on the antioxidant potential, phytochemical and the polyphenolic composition of the aerial parts (leaves and flowers) of *N. leucophylla*. In addition, the phytochemical composition

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and polyphenolic profile of bioactive extracts is also investigated using GC-MS (Gas Chromatography Mass Spectrometry) and RP-HPLC-DAD (Reverse phase-high performance liquid chromatography- diode array detector).

2. Material and Methods

2.1. Chemicals and reagents

The various HPLC standards were purchased from Thermo Fisher Scientific (Acros Organics) India Pvt. Ltd, Mumbai, Loba Chemie, India Pvt. Ltd, Mumbai, Otto Chemie, India Pvt. Ltd, Mumbai and Sigma Aldrich, India Pvt. Ltd, Mumbai. Acetonitril and methanol (HPLC grade) were purchased from Merck, India Pvt. Ltd, Mumbai.DPPH and glacial acetic acid (HPLC grade) were procured from Otto Chemie, India Pvt. Ltd, Mumbai. All other chemicals, solvents and reagents used were of analytical grade and obtained from Thermo Fisher Scientific (Acros Organics), Fluka, Otto Chemie, Loba Chemie, Sd-Fine Chemicals India Pvt. Ltd. Throughout the experimental work, the deionized water obtained from Millipore Direct Q 3 with pump, instrument was used.

2.2. Plant material

The aerial parts of *Nepeta leucophylla* were collected from Manimhesh hills (Hadsar, latitude 32°15'-32.26° N and longitude 76°19'-76.32° E) in Chamba district of Himachal Pradesh (India) at an altitude >2000 m in the month of October, 2013. The voucher specimen (PUN58876) collected was identified (by Dr. M.I.S. Saggoo, Department of Botany, Punjabi University, Patiala) and deposited in the Herbarium of Punjabi University, Patiala. The shade dried (at room temperature: 24-32 ˚C) plant material (30 days) was powdered and stored in air tight polyethylene bags at low temperature (<4 ˚C) for further use.

2.3. Extraction of plant material

2.3.1. Ultrasound assisted extraction method

The UAEM was performed using a CPX 500 model of Cole Parmer, USA (500 W, 20 kHz) having a standard Horn probe with threaded end and replaceable tip (¾", 125mm L × 19 mm Dia.). The dried aerial parts (20 g) of *N. leucophylla* were extracted with solvents of different polarity. The same plant material was extracted three times with 150, 75 and 75 mL of solvent for 30, 15 and 15 min, respectively. The extraction was carried out at 55±1 ˚C with the controlled temperature sensitive probe. Every time, the liquid extract was filtered twice using Whatman filter paper no. 1. Finally, it was evaporated to dryness with the help of rotary vacuum evaporator at 45 ˚C. The percentage yield of the extracts was noted and these were stored at low temperature for further studies.

2.3.2. Soxhlet extraction method

N. leucophylla dried aerial parts (80 g in methanol, 85 g in chloroform and hexane) were extracted for 36 h at the boiling point of solvent (300 mL) used. After the extraction, same steps were followed as described above to obtain the dried plant extract.

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2.3.3. Maceration method

In this method, the plant material (20 g) was extracted three times in a row using 150, 75 and 75 mL of solvent at room temperature (30±3 ˚C) in the dark. In each case, the extraction time was 32 h. After this, a similar procedure was followed as illustrated in the previous methods to get the final material.

2.4. Total polyphenolic content

The quantitative determination of TPC was carried out with the help of Folin-Ciocalteu reagent according to the method described by Stoilova et al.[14] 1 mL of different extracts (1 mg/mL) or standard solution (30-150 mg/L) was mixed with 10 mL of deionized water and then 1 mL Folin-Ciocalteu phenol reagent was added. After 5 min, 20% sodium carbonate solution (2 mL) was added to the mixture. The mixture was incubated for 60 min in the dark and the absorbance was measured at 750 nm on UV-Visible spectrophotometer 1800 (Shimadzu). The gallic acid was used as a standard. The results were expressed in mg of gallic acid equivalents (GAE) /1 g of dry plant extract (DPE). Each sample was analyzed in triplicate.

2.5. Total flavonoids content

The total flavonoids content in various extracts was evaluated quantitatively with the help of aluminum chloride method described by Zhishen et al.[15] 1 mL of different extracts (1 mg/ml) or standard solutions (60-300 mg/L) was mixed with 4 mL of deionized water. Then, 5% (w/v) sodium nitrite solution (0.3 mL) was added followed by 5 min incubation. Successively, 10% (w/v) aluminum chloride (0.3 mL) and 1 M sodium hydroxide (2 mL) was added to the mixture and the total volume was made 10 mL with deionized water. Finally, the absorbance was noted at 510 nm on UV-Visible spectrophotometer 1800 (Shimadzu). The TFC was expressed in mg of rutin trihydrate equivalents (RE) /1 g of DPE. Again, each sample was analyzed in triplicate.

2.6. Antioxidant potential

2.6.1. DPPH free radical scavenging assay

The DPPH free radical scavenging potential was determined according to the procedure used by Uddin et al.[16] 0.2 mL of different extracts (1 mg/mL) was added to 0.004% methanolic solution of DPPH (3 mL). The different samples were incubated for 30 min in the dark at room temperature and the absorbance was noted at 517 nm on UV-Visible spectrophotometer 1800 (Shimadzu). The percentage inhibition (I%) of DPPH free radical was evaluated using the following equation:

$I\%\ [\text{DPPH free radical}] = [(A_C - A_S) / A_C] \times 100$

A_C - the absorbance of the control and A_S - the absorbance of the samples/standard solutions. The ascorbic acid and quercetin dihydtrate were used as standards. The percentage inhibition of each sample was evaluated in triplicate.

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2.6.2. NO scavenging assay

The nitric oxide scavenging potential was measured by the method described by Hazra et al.[17] The different extracts (1 mg/mL) were mixed with 10 mM sodium nitroprusside solution (0.8 mL) prepared in phosphate buffered saline (pH 7.4). Then, the samples were incubated for 150 min at room temperature. Subsequently, sulfanilamide (1 mL, 0.33% in 20% glacial acetic acid) was added and again the resulting solution was incubated for 5 min. In the latter, 0.1% (w/v) solution of napthylethylenediamine dihydrochloride (1 mL) was added and the reaction mixture was further incubated for 30 min at room temperature. Finally, the pink colored chromophore generated was measured at 540 nm on UV-Visible spectrophotometer. The quercetin dihydtrate and ascorbic acid were used as positive control. The percentage inhibition (I%) of NO free radical was evaluated using the following equation:

$I\% [NO^{\cdot}] = [(Ac – As) / Ac] \times 100$

A_C - the absorbance of the control and A_S - the absorbance of the samples/standard solutions. The percentage inhibition of each sample was evaluated in triplicate.

2.6.3. FRAP assay

The ferric ion reducing antioxidant power of different extracts and standard was measured according to the method used by Benzie and Strain.[18] 0.2 mL of different extracts (0.5 mg/mL) and standards (60-300 mg/L) were mixed with 2.8 mL of FRAP solution [300 mM acetate buffer (25 mL), 10 mM 2,4,6-tripyridyl-*s*-triazine in 40 mM HCl (2.5 mL) and 20 mM $FeCl_3 \cdot 6H_2O$ (2.5 mL)]. Ferrous sulphate was used as standard. The absorbance was measured at 593 nm on UV-Visible spectrophotometer after the incubation of solutions for 30 min in the dark. The results were expressed as mg of ferrous II equivalent (Fe (II) E) /1g of DPE. Each experiment was carried out in triplicate.

2.6.4. TAC assay

The total antioxidant capacity was evaluated according to phosphomolybdenum reducing method used by Prieto et al.[19] In this assay 0.3 mL of different extract (1 mg/mL) or standard (60-300 mg/L) was taken in a test tube. After this, the reagent solution (3 mL; 0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) was added to each test tube. All the test tubes were incubated at 95 ^{0}C for 90 min. Upon cooling to room temperature, the absorbance of the solutions was noted at 695 nm on UV-Visible spectrophotometer. The results were represented in mg of ascorbic acid equivalent (AAE) /1 g of DPE. Again, each experiment was carried out in triplicate.

2.7. RP-HPLC-DAD analysis of polyphenolic compounds

The RP-HPLC-DAD study of methanol extracts of *N. leucophylla* aerial parts was carried out using the method of Wu et al.[20] with some modifications. The study was performed with the HPLC system (Waters, USA) equipped with a binary pump (1525), a

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photo diode array detector (2998; range 210 to 400 nm), an auto-sampler (2707) and inline degasser (AF). The chromatographic separation was carried out on Sunfire C-18 (Waters, USA) reversed phase column (125 × 4.6 mm length, 5 µm particle size). The standards and samples were dissolved in methanol (HPLC grade) and then filtered by syringe filter (0.22 µm, Millipore Millex GV, hydrophilic PVDF). The solvents used for elution were also filtered prior to HPLC injection using Millipore solvent filtration assembly (durapore GVWP 0.22 µm, 47 mm filter). The injection volume (20 µL) with a flow rate of 1 mL/min was used to elute the samples and standards employing gradient solvent program for 60 min at room temperature. 2% Acetic acid (v/v) in water and 1% acetic acid in water: acetonitrile (1:1, v/v) constitute the solvents A and B, respectively. The used elution gradient was: A:B (85:15) for 0 to 1 min, A:B (65:35) for 1 to 30 min, A:B (25:75) for 30 to 40 min, A:B (10:90) for 40 to 45 min, A:B (0:100) for 45 to 52 min, A:B (80:20) for 52 to 56 min and finally A:B (95: 05) for 56 to 60 min, respectively. Fourteen polyphenolic standards *viz.* benzoic acid, caffeic acid, catechin hydrate, chlorogenic acid, trans-cinnamic acid, ρ-coumaric acid, ferulic acid, gallic acid, p-hydroxybenzoic acid, myricetin, quercetin dihydtrate, rutin trihydrate, syringic acid and vanillic acid were used. The samples were analyzed in triplicate. The polyphenolics were identified by comparing the retention times and UV-Visible chromatogram of samples with the commercial standards, whereas the quantification was carried out using calibration curves of standards plotted after linear regression of the concentration and the peak area. The results were represented as ug/g of DPE. The values of limits of detection (LOD), limits of quantification (LOQ) and average recovery ranged between 0.15-0.69 µg/g, 0.46-2.1 µg/g and 75-86%, respectively. The threshold limit was 10% for recovery study, while the linearity demanded R^2 > 0.9916. The method proved to be very sensitive as revealed by the values of LOD, LOQ and average recovery.

2.8. GC-MS analysis

The GC-MS analysis of different bioactive methanol extracts (1 µL) obtained by SEM, UAEM and MM were carried out on Shimadzu (GC-2010 Plus) GC system tied with Mass spectroscopy (GC-MS-QP 2010 Ultra). The capillary column RTx-5Sil MS (30 m × 0.25 mm × 0.25 mm, Restesk USA) was used during analysis. Helium, at a flow rate of 1 mL/min was used as the carrier gas and the split ration was 1:5. The temperature of injection port was 280 °C. The oven temperature was programmed as: the Initial temperature was 100 °C (hold 1 min), then increased at the rate of 4 °C/min to 250 °C (hold 5 min) and finally increased further at the rate of 5 °C/min to 280 °C (hold 30 min). Mass spectra were recorded with electron ionization (EI) mode at 70 eV and the spectral range was 40-700 m/z. The interface and ion source temperature were 280 and 200 °C, respectively. The identification of various constituents was carried out only by a comparison of their retention time and mass spectral data with that of retention time and mass spectral database of Wiley8 and NIST11 library. The compounds were quantified on the basis of the area under the peak and results were presented in percentage. The values of average recovery, LOD and LOQ varied from

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85-96%, 0.002–0.14 µg/g and 0.03–0.30 µg/g, respectively. The values of average recovery, LOD and LOQ displayed that the method proved to be very sensitive. Each sample was analyzed in triplicate.

2.9. Statistical analysis

The results are expressed as the mean ± standard deviation (SD). The statistical comparisons were evaluated with the help of one-way analysis of variance (ANOVA) followed by Duncan's multiple range test ($p < 0.05$). The results were calculated using MS excel and Statistica 7 software.

3. Results and Discussion

3.1. Percentage yield

The percentage yield of various extracts ranged from 3.66 to 17.66% (w/w – yield of dry extract in g /total powdered dry material taken in g × 100) (Table 1). The methanol extracts showed the highest percentage yield followed by chloroform and hexane extracts, which may be attributed to the polar nature of methanol that facilitates the solubilisation of secondary metabolites based on the fact 'like dissolves like'.[21] This candidly exhibits the direct proportionality between the percentage yield and polarity of solvent. Further with polar solvents, the percentage yield had a marginal difference between SEM and UAEM, whereas in MM, it was significantly lower. Similar results were also reported previously, where various polar extracts obtained by SEM from *Potentilla atrosanguinea* aerial parts[21] and *Prunus laurocerasus* leaves and fruit[22] showed a maximum percentage yield. The higher yields in SEM may be ascribed to the exhaustive extraction (36 h) of plant material with repeated washings by warm solvent and to the acoustic cavitations in UAEM.[23,24] Apart from this, high temperature may be another factor that boosts up the percentage yield by increasing the diffusion coefficients and the solubility of secondary metabolites, reducing the solvent viscosity and improving mass transfer due to more penetration of solvent into the plant cell in SEM and UAEM.[25,26]

3.2. Determination of total polyphenolic and total flavonoids content

The plant based polyphenolic compounds are known to be good antioxidants which act as radical scavengers or metal chelators.[27] Due to this, the quality of different extracts is assessed in terms of TPC and TFC. The TPC and TFC of various extracts varied from 0.98±0.47 to 141.9±3.86 mg GAE/g DPE and 6.21±0.49 to 394.48±15.45 mg RE/g DPE (Table 2), respectively. The methanol extracts showed the maximum TPC and TFC followed by chloroform and hexane extracts. Highly polar methanol (as compared to chloroform and hexane) probably facilitates the solubilisation of polyphenolic compounds to a larger extent. Alike results were also reported previously by Prasad et *al.*[28], *Shahriar et al.*[29] *and Iqbal et al.*[30] *in Ipomoea aquatica Leaves, Terminalia arjuna bark and Artemisia annua leaves,* respectively. Methanol and chloroform extracts obtained by SEM exhibited the highest value of TPC and TFC followed by UAEM and MM, whereas the hexane extract obtained by SEM also showed the

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same order only for TFC. However, for TPC the order in hexane extract was UAEM > SEM > MM. The exhaustive extraction of the secondary metabolites due to repeated and continuous washing of the plant material in SEM explains its high TPC and TFC content as compared to other extraction methods. Previous reports on the extracts obtained by SEM from aerial parts of *Potentilla atrosanguinea*.[21] and *Osbeckia parvifolia*[31] also provided similar results. Further, high TPC and TFC contents in the extracts obtained by UAEM over MM may be attributed to the effect of ultrasound waves (acoustic cavitations) and high temperature as explained earlier in case of percentage yield. The values of TPC and TFC varied significantly depending upon the nature of extraction solvents and methods as shown by the values of Duncan's multiple range tests (Table 2). There was also a fairly good correlation between the polarity of the solvent and TPC (R^2=0.82) / TFC (R^2=0.64).

3.3. Antioxidant potential

The natural antioxidants work according to different mechanisms like prevention of chain initiation reaction, decomposition of peroxides, radical scavenging, reductive capacity, continued hydrogen abstraction and binding to transition-metal ion catalysts.[32] So, it is very difficult to assign a complete profile of antioxidant potential of any plant extract by a single antioxidant assay. Hence, it is always better to assess the same by employing a number of antioxidant assays. The results of antioxidant activity through different assays are given in Table 2.

3.3.1. Radical scavenging activity

The radical scavenging activity of DPPH free radical varied from 8.93±2.09 to 92.57±0.11%, whereas that of NO free radical ranged between 6.13±0.41 to 64.03±2.29%. Among the solvents used, the highest percentage scavenging activity was observed in the methanol extracts followed by chloroform and hexane extracts. The results showed that the polarity of solvent significantly affects their radical scavenging activity. The higher radical scavenging activity of methanol over chloroform and hexane is ascribed to its high TPC and TFC, which in turn is related to the polar nature of methanol as explained earlier. Identical results were also reported previously for *Ipomoea aquatic* leaves[28] and *Terminalia arjuna* bark.[29] Amongst different extraction methods, the extracts (methanol and chloroform) obtained by SEM showed the highest DPPH and NO radical scavenging activity followed by UAEM and MM. This trend was consonant with the TPC and TFC as obtained in different methodologies, which was further supported by higher correlation coefficient between DPPH/NO radical scavenging activity and TPC (r = 0.97 and 0.95) / TFC (r = 0.93 and 0.96) (Table 4). But, in a hexane extract UAEM had an edge over SEM and MM. The ascorbic acid /quercetin used as positive controls, showed the percentage inhibition 58.42±0.16/89.52±0.89% for DPPH assay and 48.85±0.93/75.48±0.33% for NOS assay, which were comparable to the inhibitions brought about by methanol extract (92.57±0.11 and 64.03±2.29%). A close comparison between SEM, UAEM and MM through statistical analysis revealed a significant difference in their scavenging efficiencies (Table 2). The present results of DPPH free radical scavenging assay were

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in line with the previous studies, where the methanol extract obtained from *Prunus laurocerasus* leaves and fruits by SEM showed high DPPH scavenging potential as compared to UAEM and MM.[22] Similar results were also found for NO scavenging activity by Senevirathne et al.[34] and Aly et al.[35] in case of *Ecklonia cava* and *N. cataria's* aerial parts.

3.3.2. FRAP assay

The reduction of 2,4,6-tripyridyl-*s*-triazine - Fe III complex to 2,4,6-tripyridyl-*s*-triazine - Fe II complex in the presence of natural antioxidants form the basis of FRAP assay. The FRAP values of various extracts ranged between 4.64±0.71 to 196.38±0.31 mg Fe (II) E/g DPE. From the different solvents used, the highest FRAP values were observed in the case of methanol extracts followed by chloroform and hexane extracts. A similar trend was also observed in the extracts obtained from *Artimisia annua* leaves.[30] But, in case of extraction methods, the superior FRAP values were noted in the extracts isolated by SEM followed by UAEM and MM. Further, the results of FRAP values showed high correlation with that of TPC (r = 0.99) and TFC (r = 0.99), therefore the high FRAP values of various extracts were attributed to their higher TPC and TFC. Identical results were also previously reported by Kalia et al.[21] for *Potentilla atrosanguinea's* aerial parts. Further, Murugan and Parimelazhagan[28] reported that the methanol extract obtained from the whole plant of *Osbeckia parvifolia* by SEM as compared to ethanol, ethyl acetate and hexane extracts showed the highest FRAP values followed by MM. The positive control (ascorbic acid) had a much higher FRAP value (385.3±2.76 mg Fe (II) E/g of ascorbic acid) as compared to all the extracts used in the present study. It is evident from the present results that FRAP values strongly depend on extraction methods and the type of solvents used (Table 2).

3.3.3. Total antioxidant capacity

The natural antioxidant reduced Mo (VI) to Mo (V) in acidic medium with the formation of the green complex - phosphate/Mo (V) that read at 695nm. The results of present study varied from 10.98±1.36 to 74.72±1.1 mg AAE/g DPE. Independent of the extraction method used, methanol extracts showed higher TAC values followed by chloroform and hexane extracts. Similar results were reported previously, where the various extracts obtained from the bark of *Terminalia arjuna* followed the same trend.[29] Amongst the extraction methods, the highest TAC values were shown by the different extracts obtained with SEM followed by UAEM and MM. The high TAC values of extracts obtained by SEM were due to their higher total polyphenolic and flavonoids content, which was also supported by the high correlation between TAC values and TPC (r =0.98) and TFC (r =0.98). Similar results were also reported previously in the case of *Potentilla atrosanguinea* aerial parts, where methanol extract obtained by SEM had higher TAC value as compared to ethanol, ethyl acetate and hexane extracts.[21] The presents results were in good agreement with the previous finding of Murugan and Parimelazhagan[31] in case of whole plant of *Osbeckia parvifolia*, where the various extracts obtained by SEM showed higher TAC values as compared to the same extracts obtained by MM. The TAC value of quercetin (positive control) was 488.95±7.95 mg AAE/g of quercetin, which was much higher than all the extracts

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examined in the present study. The present analysis established that the results of TPC, TFC and different antioxidant assays were affected considerably by the extraction methods and the polarity of solvents used. These findings were also strongly supported by the results of one way analysis of variance (ANOVA) ($p < 0.05$) of various studied extracts followed by Duncan's multiple range test (Table 2).

From the present results of antioxidant potential with different assays, it was found that the antioxidant potential of different extracts obtained by UAEM was significantly higher than the corresponding extracts obtained by MM. This may be attributed to the effects of ultrasound waves and high extraction temperature in case of UAEM, which helps in the extraction of biologically active compounds in large quantity. Ultrasound waves and high temperature facilitate the solubility of antioxidant compounds and improved mass transfer of these compounds due to more penetration of solvent into the plant matrix.[23-26]

3.4. RP-HPLC–DAD analysis of polyphenolic composition

The polyphenolic constituents of most bioactive extracts (methanol) of *N. leucophyllas'* aerial parts were characterized using RP-HPLC–DAD. Out of the fourteen polyphenolic standards studied, only seven were identified in each methanol extract. The statistical analysis showed a significant difference in the concentration of seven identified polyphenolic constituents for a single methanol extract. However, for different extraction methods, the values of an individual polyphenolic varied marginally (Table 3, Fig. 1). No polyphenolic component was detected in the chloroform and hexane extracts obtained by different extraction methods. The overall concentration of reported polyphenolic constituents followed the order - SEM > UAEM > MM, whereas the individual constituent concentration varied as myricetin > chlorogenic acid > rutin trihydrate > caffeic acid > catechin hydrate > syringic acid > vanillic acid. These results were in good agreement with that of TPC and TFC, which follows the same order - SEM > UAEM > MM. The reason for the higher over all concentration of different polyphenolic constituent in SEM over UAEM and MM was same as explained earlier in case of TPC and TFC. Polyphenolics like chlorogenic acid, caffeic acid, rutin and vanillic acid were also reported previously in the other *Nepeta* species.[6,9] Till date to the best of our knowledge, this is the first study in which polyphenolic compounds obtained from *N. Leucophylla* aerial parts have been evaluated.

3.5. GC-MS analysis

The GC-MS analysis of most bioactive methanol extracts obtained by SEM, UAEM and MM showed the presence of twenty six, twenty five and twenty six constituents, respectively. These extracts showed the presence of steroids, phenolics, fatty acids and their esters as the key classes of secondary metabolites. On the other hand, various chloroform and hexane extracts showed the presence of long chain hydrocarbons and fatty acids as the major classes of secondary metabolites (data provided in supplementary information). Fig. 2 represents the GC-MS chromatograms of different methanol extracts obtained by SEM, UAEM and MM, while the list of major constituents detected (>2.5%) in the analyzed extracts is represented in Table 4.

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The statistical analysis of table 4 showed that the concentration of various constituents varied significantly within a methodology. Also, the methodologies employed immensely influence the percentage of identified constituents. The polarity of the solvent used significantly affects the composition of non-polar volatile phytochemicals. In the methanol extracts, steroids, phenolics, fatty acids and their esters were found to be the key constituents (Table 4), whereas in chloroform and hexane extracts, long chain hydrocarbons were present as major constituents along with a small amount of fatty acids and phenolics (data provided in supplementary information). The compounds like Abieta-9 (11), 8 (14), 12-trien-12-ol, Stigmast-5-en-3-ol, and Vitamin E are well known for their antioxidant activity. So, these compounds along with polyphenolics (major ones), also contribute towards the total antioxidant activity of various extracts obtained from aerial parts of *N. leucophyllya*.

The GC-MS analysis of acetone, methanol and ethanol extracts isolated from the leaves of *Aloe vera*[36] and leaves of *Cinnamomum iners*[37] also showed the presence of fatty acid and steroids as major classes of secondary metabolites. These compounds were known to show a broad range of applications in various nutraceutical, food and pharmaceutical industries.[37] Thus, the aerial parts of *N. leucophylla* serve as a potent source for the isolation of above mentioned bioactive constituents.

3.6. Correlations

The correlations between the results of the TPC, TFC, FRAP, TAC, DPPH and NO scavenging activity of different extracts obtained from *N. leucophylla* aerial parts using different extraction methods are given in Table 5. The values of correlation coefficients (r) for the various assays used in the present study ranged between 0.92 to 0.99 which demonstrated that there was a high correlation among these assays. The highest correlation was reported in FRAP values (r = 0.99) with TPC and TFC followed by TAC values (r = 0.98) with TPC and TFC, whereas the lowest correlation was reported between DPPH percentage inhibition values (r = 0.92) and NOS percentage inhibition values. As in TPC and FRAP a similar mechanism (single electron transfer) operates, therefore, the results of these assays correlated significantly. Similar results have also been reported.[38]

The high correlation between the results of polyphenolic content (TPC, TFC) of various extracts obtained by different extraction methods and results of various antioxidants assays confirmed that the polyphenolic constituents were the major components responsible for the antioxidant potential of different extracts obtained from aerial parts of *N leucophylla*. The higher antioxidant potential of polyphenolic compounds might be due to the ability to scavenge different free radicals, to act as metal chelators, to decompose peroxides, to quench reactive oxygen and nitrogen species.[24] The earlier available data also support the present results, where polyhenolic compounds present in the plant extracts were the key constituent responsible for their antioxidant potential.[30]

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4. Conclusions

So, the present investigations demonstrated that the solvent type and the extraction techniques used significantly affected the percentage yield, phytochemical composition, TPC, TFC and antioxidant potential of different extracts isolated from the aerial parts (leaves and flowers) of *N. leucophylla*. With a high TPC and TFC, methanol was the most efficient solvent for the extraction of polyphenolics because of its protic and polar character which enables effective solvation of phenol and carboxyl containing species. The high correlation between polyphenolic content (TPC, TFC) and antioxidant potential (FRAP, TAC, DPPH and NO scavenging) of methanol extracts revealed that the polyphenolic compounds were mainly responsible for the high antioxidant potential of various methanol extracts. SEM in case of extraction methods provided the best results related to percentage yield, phytochemical composition, DPPH scavenging activity, NO scavenging activity, TAC, FRAP, TPC and TFC. This may be ascribed to exhaustive extraction of plant material in SEM due to repeated and continuous washing of the plant material with warm solvent till the complete extraction of secondary metabolites. The RP-HPLC-DAD analysis of the methanol extracts revealed the presence of seven out of the fourteen polyphenolic constituents analyzed. The GC-MS analysis of methanol, chloroform and hexane extracts showed the presence of fatty acid/esters, phenolics, steroids and long chain hydrocarbons as the key classes of secondary metabolites. Finally, it is concluded that the methanol and SEM are the best selections for the isolation of bioactive phytochemicals from aerial parts of *N. leucophylla*. The high antioxidant activity and polyphenolic content of *N. leucophylla* aerial parts clearly highlight their potential as a potent source of natural antioxidants that may promote good health, reduce the risk of degenerative diseases and replace synthetic antioxidants for their use in food industries.

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Figure Captions

Fig. 1. RP-HPLC-DAD chromatogram of methanol extract obtained by (A) SEM (B) UAEM (C) MM

Fig. 2. GC-MS chromatogram of different bioactive methanol extracts obtained by (A) SEM (B) UAEM (C) MM

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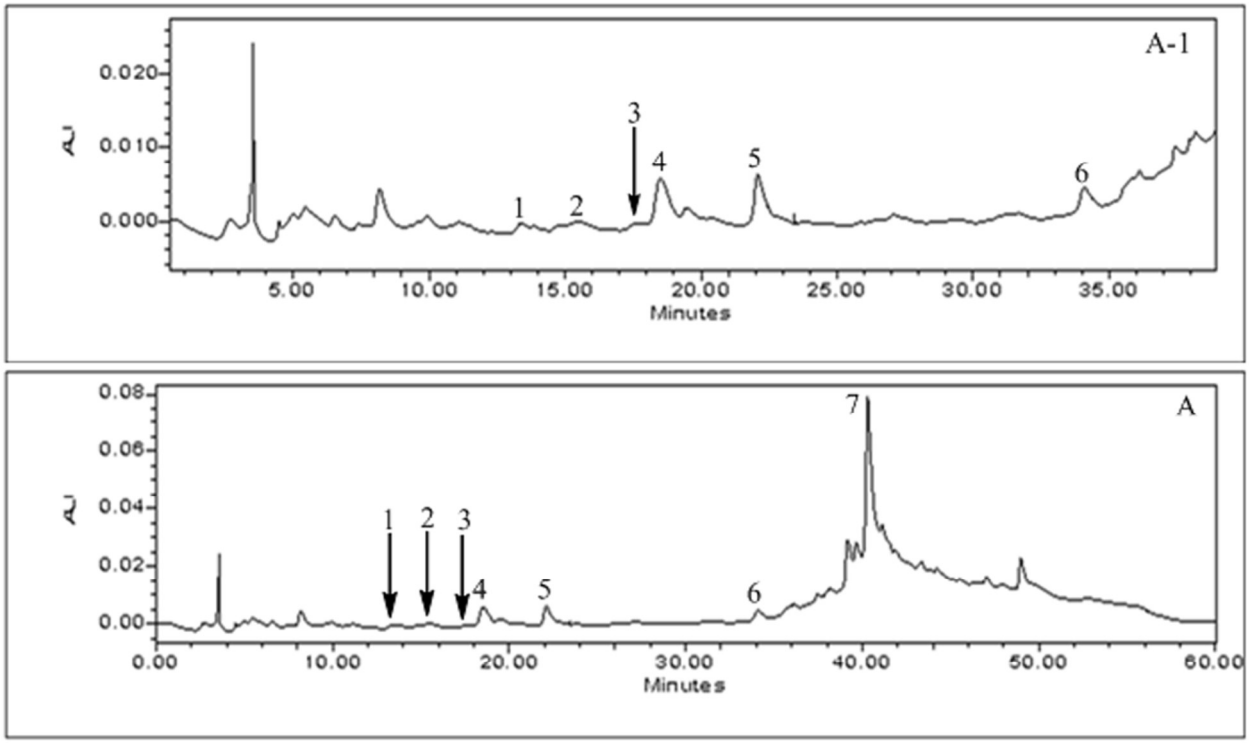
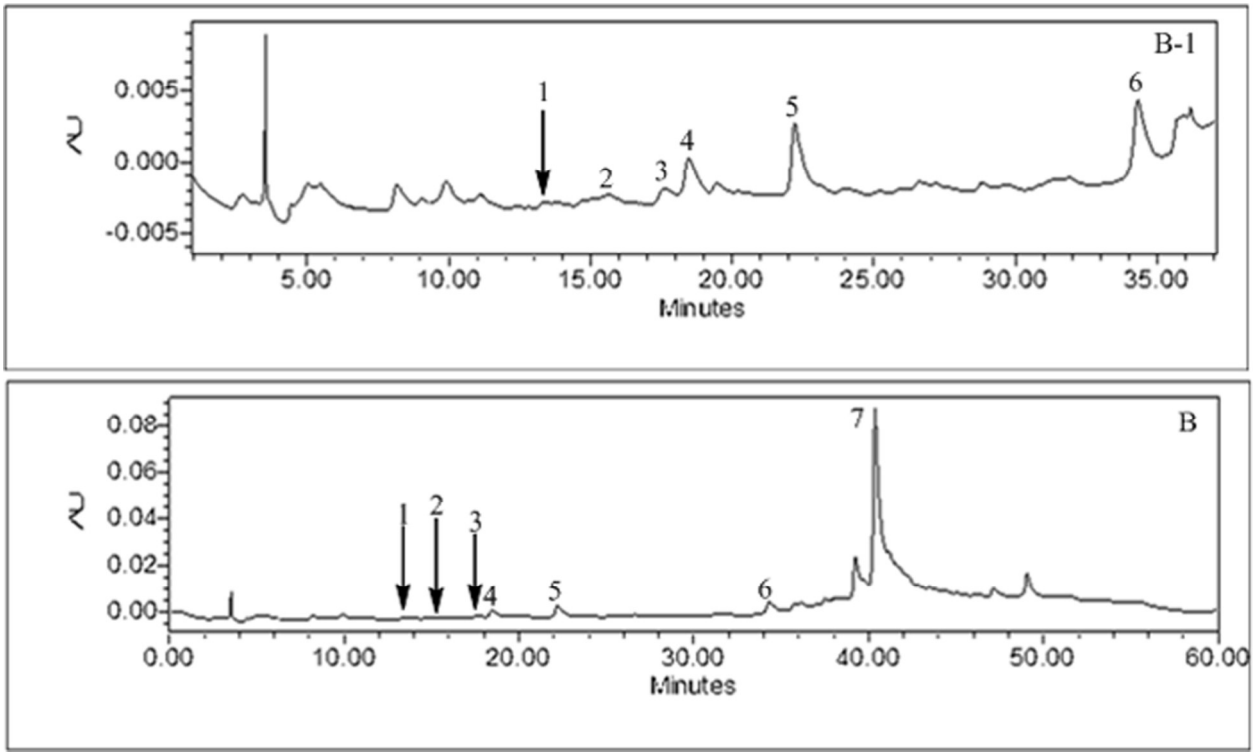
Fig. 1.



(A)



(B)

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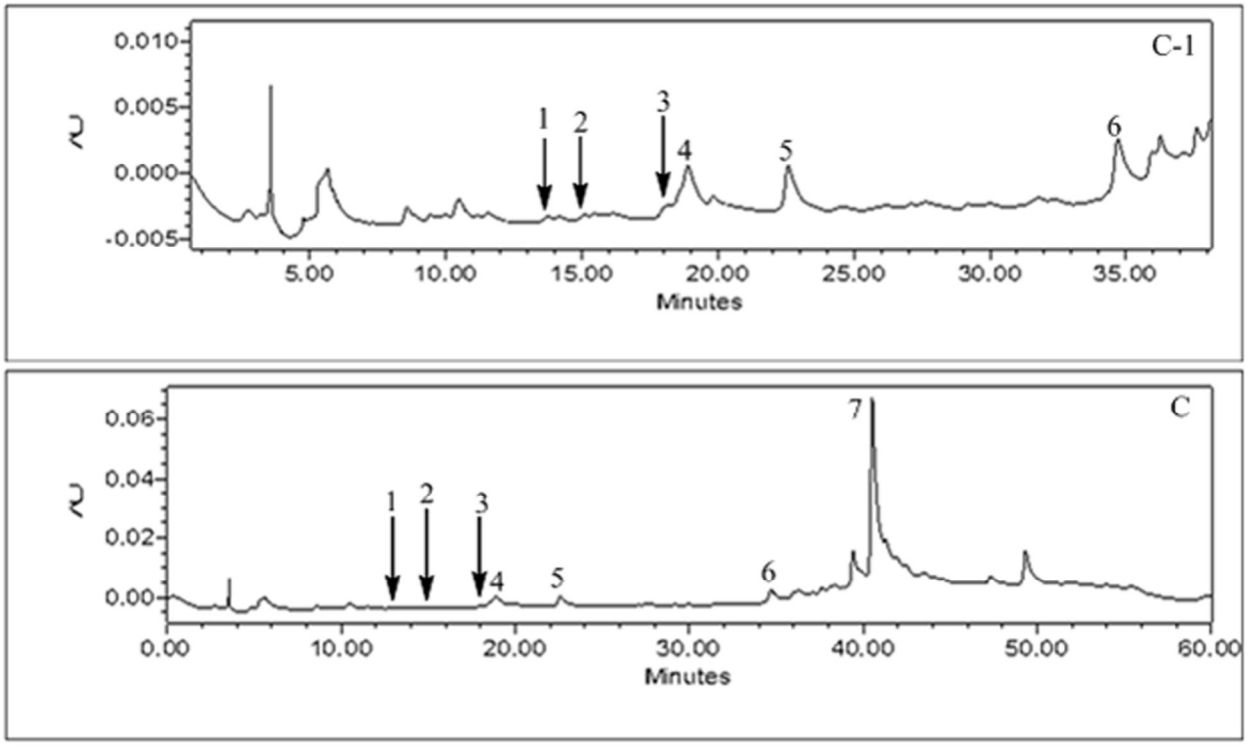

(C)

Fig. 1.

RP-HPLC chromatogram of methanol extract obtained by (A) SEM, A-1 zoom of A (B) UAEM, B-1 zoom of B (C) MM, C-1 zoom of C. Targeted compounds are labeled with numbers: Catechin Hydrate (1), Chlorogenic acid (2), Caffeic Acid (3), Syringic acid (4), Vanillic acid (5), Rutin trihydrate (6), Myricetin (7).

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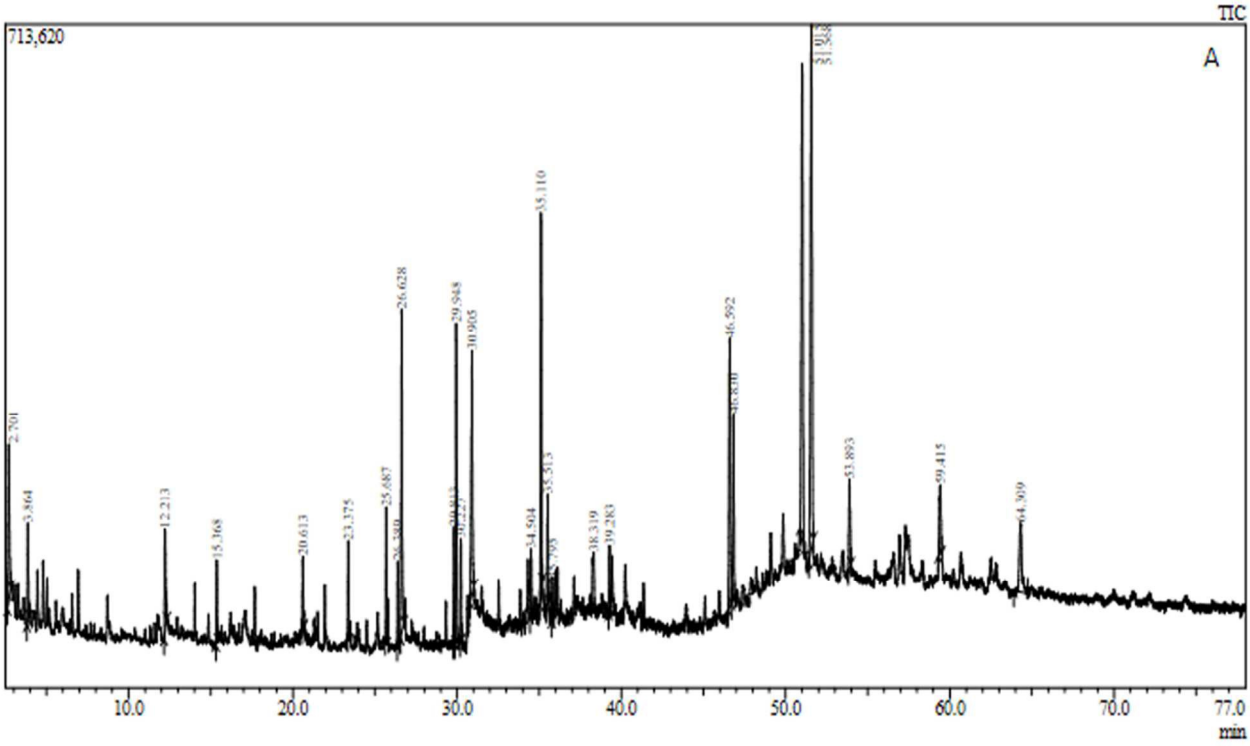
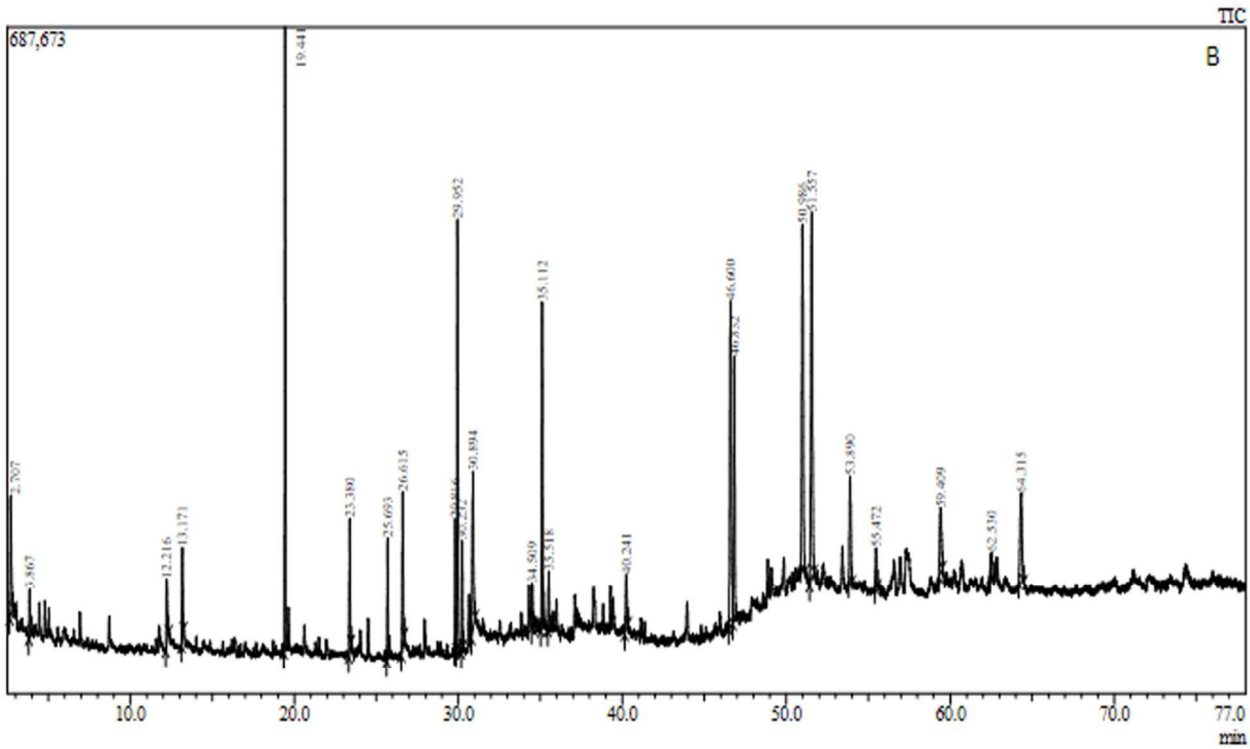
Fig. 2.



(A)



(B)

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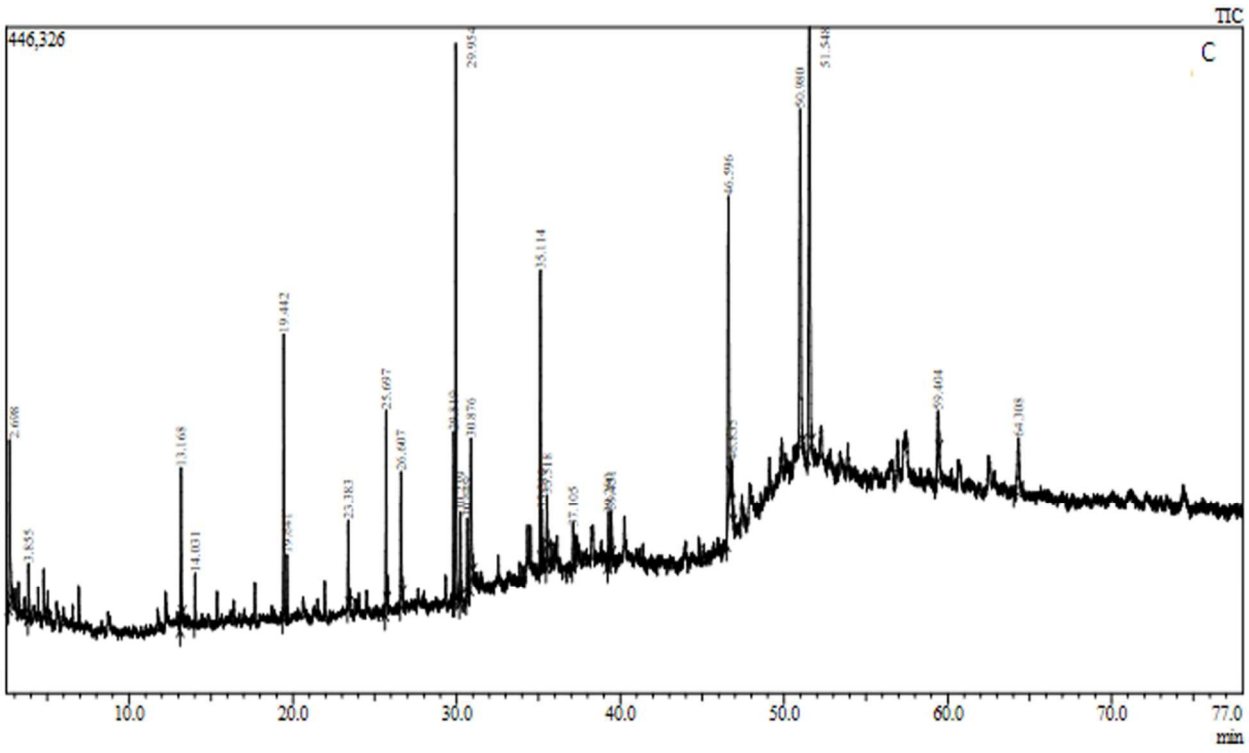

(C)

Fig. 2.

GC-MS chromatogram of different bioactive methanol extracts obtained by (A) SEM (B) UAEM (C) MM

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Table 1

The yield/ percentage yield of various extracts isolated from the aerial parts of *N. leucophylla*.

Name of Extract	Yield (g) / Percentage Yield (%) (w/w)		
	MM	UAEM	SEM
Methanol	2.172/10.86	3.513/17.57	14.13/17.66
Chloroform	1.868/9.34	2.758/13.79	11.83/13.92
Hexane	0.731/3.66	0.835/4.18	3.15/3.7

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Table 2

Results of various antioxidants assays, TPC and TFC of different extracts obtained from the aerial parts of *N. leucophylla*

EM	SU	DPPH	NOS	FRAP	TAC	TPC	TFC
SEM	Meth	92.57±0.11^{a}	64.03±2.29^{a}	196.38±0.31^{a}	74.72±1.1^{a}	141.9±3.86^{a}	394.48±15.45^{a}
	Chl	19.65±3.68^{d}	29.45±0.46^{c}	38.56±0.22^{d}	19.0±0.94^{c}	35.96±1.08^{d}	90.92±14.69^{d}
	Hex	10.02±0.9^{fg}	11.1±0.2^{e}	11.29±1.31^{f}	17.57±0.59^{cd}	1.66±0.46^{f}	17.38±2.62^{f}
UAEM	Meth	76.52±1.08^{b}	41.45±1.16^{b}	107.11±2.94^{b}	40.0±1.24^{b}	83.90±1.63^{b}	165.1±3.48^{b}
	Chl	12.87±1.85^{ef}	19.73±0.27^{d}	26.15±0.09^{e}	15.58±0.34^{e}	9.46±2.22^{e}	37.7±11.23^{e}
	Hex	11.2±0.97^{efg}	18.28±0.46^{d}	10.28±0.13^{f}	13.2±1.26^{f}	7.11±1.84^{e}	15.69±2.63^{f}
MM	Meth	50.66±2.66^{c}	28.57±0.12^{c}	79.34±3.25^{c}	39.42±0.6^{b}	77.16±0.58^{c}	136.76±5.24^{c}
	Chl	14.41±1.20^{e}	8.44±0.2^{f}	10.86±0.62^{f}	16.31±0.44d^{e}	8.37±1.23^{e}	18.21±0.86^{f}
	Hex	8.93±2.09^{g}	6.13±0.41^{g}	4.64±0.71^{g}	10.98±1.36^{g}	0.98±0.47^{f}	6.21±0.49^{f}
Ascorbic acid		58.42±0.16	48.85±0.93	385.3±2.76	-	-	-
Quercetin		89.52±0.89	75.48±0.33	-	488.95±7.95	-	-

EM - extraction methods, SU - solvent used, Meth - methanol, Chl – chloroform, Hex – hexane. The results of DPPH assay were expressed as % Inhibition, NOS as % Inhibition, FRAP as mg Fe (II) E /g DPE, TAC as mg AAE/g DPE, TPC as mg GAE/g DPE and that of TFC as mg RE/g DPE.

The values having different superscript letters within a column were significantly different ($p < 0.05$).

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Table 3

Results of polyphenolic content (μg/g of DPE) of methanol extracts of aerial parts of *N. leucophylla* analyzed by RP-HPLC-DAD.

EM	CH	CLA	CA	SA	VA	RT	MY
SEM	107.11 ± 1.47^{eA}	324.82 ± 1.44^{bA}	226.38 ± 0.17^{dB}	65.14 ± 1.36^{fAB}	63.35 ± 1.70^{FA}	292.98 ± 1.65^{cB}	667.75 ± 4.05^{aA}
UAEM	89.6 ± 0.64^{eB}	310.88 ± 0.43^{bB}	228.03 ± 0.31^{dA}	61.68 ± 0.66^{fB}	61.64 ± 0.1^{fA}	309.30 ± 1.97^{cA}	666.22 ± 5.81^{aA}
MM	88.94 ± 1.35^{eB}	310.26 ± 0.07^{bB}	228.25 ± 1.17^{dA}	66.41 ± 1.99^{fA}	58.13 ± 0.1^{gB}	289.97 ± 0.87^{cB}	555.52 ± 4.06^{aB}

The values having different superscript (small alphabet) letters within a row were significantly different ($p < 0.05$).
The values having different superscript (capital alphabet) letters within a column were significantly different ($p < 0.05$).
EM- Extraction method, CH- Catechin hydrate, CLA- Chlorogenic acid, CA- Caffeic acid, SA- Syringic acid, VA- Vanillic acid, RT- Rutin trihydrate, MY- Myricetin

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Table 4

Main constituents (>2.50%) detected by GC-MS analysis of most bioactive methanol extracts obtained by different extraction methods

Name of compound	RT±SD			Peak Area (%)±SD		
	SEM	UAEM	MM	SEM	UAEM	MM
1,2,3-Propanetriol	2.707±0.017	2.707±0.006	2.701±0.003	3.65±0.28^{d}	2.55±0.7^{d}	3.32±0.62^{fg}
1-Dodecanol	-	13.172±0.007	13.166±0.005	-	Tr	3.09±0.16^{fg}
2-Propenoic acid, Tetradecyl ester	-	19.443±0.004	19.439±0.004	-	9.31±1.35^{b}	5.26±0.42^{de}
Hexadecanoic acid, methyl ester	25.692±0.008	25.687±0.017	25.692±0.005	Tr	Tr	3.82±0.22^{fg}
Hexadecanoic acid	26.631±0.019	26.613±0.014	26.599±0.003	6.20±2.47^{cd}	3.41±1.90^{d}	2.73±0.12^{g}
Linoleic acid, methyl ester	29.816±0.006	29.818±0.007	29.815±0.005	Tr	Tr	3.19±0.10^{fg}
Linolenic acid, methyl ester	29.949±0.004	29.952±0.003	29.950±0.004	5.75±0.42^{cd}	7.96±0.51^{bc}	12.51±1.43$_{b}$
Linolenic acid	30.903±0.025	30.891±0.003	30.858±0.016	6.62±2.90^{c}	5.54±2.44^{c}	2.66±1.43$_{g}$
Abieta-9(11),8(14),12-trien-12-ol	35.116±0.008	35.114±0.003	35.113±0.007	6.22±0.53^{cd}	5.9±0.37^{c}	6.37±0.18^{d}
UI	46.601±0.014	46.600±0.002	46.599±0.007	7.22±1.24^{c}	9.99±0.64^{b}	9.11±0.46^{c}
Squalene	46.836±0.007	46.837±0.006	46.844±0.023	4.36±0.14^{cd}	6.52±0.46^{c}	Tr
UI	51.011±0.017	50.989±0.009	50.977±0.003	14.07±2.07^{b}	9.97±0.75^{b}	12.04±1.24^{b}
UI	51.610±0.054	51.558±0.011	51.544±0.003	16.52±2.13^{a}	12.77±1.36^{a}	16.00±2.05^{a}
Vitamin E	53.899±0.009	53.898±0.009	-	2.51±0.25^{d}	3.06±0.24^{d}	-
Stigmast-5-en-3-ol, (3.beta.,24S)	59.420±0.017	59.405±0.015	59.404±0.003	2.66±0.72^{d}	3.04±0.56^{d}	4.64±1.68^{ef}
Long chain hydrocarbon	64.332±0.021	64.320±0,005	64.304±0.008	3.34±0.44^{d}	3.09±0.92^{d}	2.87±0.23^{fg}

RT- retention time, SD- standard deviation, UI- unidentified, - = not detected, Tr- amount present < 2.5%
The values having different superscript letters within a column were significantly different ($p < 0.05$).

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Table 5

Correlation among the different assay viz. TPC, TFC, DPPH scavenging, NOS, FRAP and TAC.

	TPC	TFC	DPPH	NOS	FRAP	TAC
TPC	1.00					
TFC	0.97	1.00				
DPPH	0.97	0.93	1.00			
NOS	0.95	0.96	0.92	1.00		
FRAP	0.99	0.99	0.97	0.96	1.00	
TAC	0.98	0.98	0.95	0.93	0.98	1.00

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