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Original Research Article

Assessment of Total Phenolics and Antioxidant Activity from Different Solvent Extracts of *Maytenus senegalensis* (Lam) Excell: Purification and Partial Characterization of Antioxidant

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A b s t r a c t

Antioxidants have been investigated to exhibit as free radical scavengers and inhibit the oxidation reaction. Therefore, they have been utilized as therapeutic candidates for prevention of various disorders generated by oxidative stress. Here we report assessment of amount of total phenolics content, antioxidant activity and isolation and partial characterization of antioxidant from *Maytenus senegalensis* (Lam) Excell. The amount of total phenolics were observed in the order of leaves methanol extract (15.8mg/ml) > leaves acetone extract (2.9mg/ml) > leaves ethyl acetate extract (2.3mg/ml) > leaves chloroform extract (0.9mg/ml) > leaves petroleum ether extract (0.5 mg/ml) and seeds methanol extract (15mg/ml) > seeds ethyl acetate extract (1.9 mg/ml) > seeds acetone extract (1.5mg/ml) > seeds chloroform extract (0.5mg/ml) > seeds petroleum extract (0.25mg/ml). Ethyl extract of leaves exhibited the highest antioxidant potential while methanol extract of seeds exhibited the lowest antioxidant potential. Antioxidant efficacies of non-polar and polar extracts were in the order of leaves ethyl acetate extract (IC_{50} 14.58µg/ml) > leaves methanol extract (IC_{50} 21.6µg/ml) > leaves acetone extract (IC_{50} 35.41µg/ml) and seeds ethyl acetate extract (IC_{50} 33.33µg/ml) > seeds methanol extract (IC_{50} 37.50µg/ml) > leaves acetone extract (IC_{50} 40µg/ml). The antioxidant potential of characterized compound 1^{st} (IC_{50} 15.12µg/ml) was slightly lower than ascorbic acid (IC_{50} 9.33µg/ml). FTIR data confirmed that the isolated compound (1^{st}) was phenolic/aromatic type. The elemental data showed that the compound (1^{st}) contains carbon (0.136%), hydrogen (0.128%), oxygen (0.286%) and nitrogen (1.245%) elements. From this study it is concluded that the *M. senegalensis* extract may be used as probable antioxidative agent.

K e y w o r d s

Antioxidant activity
Elemental analysis
Maytenus senegalensis
Phytochemicals
Total phenolic content

Introduction

Medicinal plant constituents are used as therapeutic candidates to control of cellular and metabolic diseases such as obesity, diabetes, cancer and inflammatory diseases (Shirin and Zahra, 2013). Damage of transient chemical species is occurs due to the elevation the in the level of reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radical and super oxide anion. The imbalance in the ROS generating and scavenging systems in the body causes damage of tissue and cellular organs (Uttaram et al., 2009). In some cases, damage to or collapse of free radical scavenging system of the living organs leads to variety of disorders including Alzheimer's disease, atherosclerosis, cancers, hypertension, diabetes mellitus, damage of central nervous system and inflammatory diseases (Harman, 1956; Mantle et al., 2000).

Many synthetic compounds have been used as free radical scavenger for many years but failure in clinical trials is occurred due to toxicity and therefore, search for a natural source of potential antioxidant has gained considerable interest (Valentao et al., 2002). Extensive researches have been carried out in treating plant polyphenols (Flavonoids, Tannins and Phenolic acids) as promising antioxidants or scavengers of free radicals (Nilgun et al., 2007; Terao and Piskula, 1997). It is evidenced that natural antioxidants are effective in the prevention of ROS generating disorders and could be used in the place of synthetic antioxidants to avoid toxicity and other side effects (Branen, 1986). Antioxidants are beneficial for prevention of oxidation in food items enhancing the shelf life, and preventing loss of nutritive constituents as well as formation of harmful substances (Grice, 1986).

The polar extract of *M. senegalensis* leaves has been already investigated to exhibit antioxidant activity (Wichi, 1998). But the antioxidant activities in non-polar solvents and other parts like seeds have not been studied. Apart from antioxidant activity, this plant exhibits as anti-inflammatory and antibacterial activities (Silva et al., 2011). Ethanol extract of *M. senegalensis* stems has been demonstrated to cytotoxic effects against carcinoma in cell cultures and leukemia in mice (Tin et al., 1971). Methanol and water extracts of *M. senegalensis* exhibit moderate inhibitory effects against HIV-1 protease (Otake et al., 1995). In the present study, we have evaluated the antioxidant activity and the amount of phenolic compounds from different solvent extracts (petroleum ether, chloroform, acetone, ethyl acetate and methanol) of leaves and seeds of *M. senegalensis* by using DPPH and Folin-Ciocalteau reagents. We have also isolated some antioxidants from ethyl acetate extract of *M. senegalensis* leaves by activity based fractionation and have tried characterization by using FTIR.

Materials and methods

Materials

DPPH (2,2-diphenyl-1-picrylhydrazyl) were procured from Sigma Aldrich, methanol, chloroform, ethyl acetate, acetone, hexane, gallic acid, ascorbic acid, petroleum ether, Folin-ciocalteau reagent, Sodium carbonate, Silica-gel (60-120 mesh size) obtained from SISCO laboratories pvt Ltd. TLC Plates [Silica gel 60 F_{254} (Merck)]. All other chemicals used were of analytical grades.

Collection of sample

Leaves and seeds of *M. senegalensis* were collected from Dongargaon village, Tal. Ahmednagar, Dist. Ahmednagar (MS) India. The plant was authenticated by taxonomist of the Dept. of Botany, Ahmednagar College, Ahmednagar. Plant samples were deposited in Ahmednagar Herbarium (Index No. 202). Leaves and seeds were thoroughly washed by distilled water and dried in incubator at 37ºC. Completely dried leaves and seeds were pulverized into fine powders by using grinder mixer. Fine powder was preserved at room temperature in a moisture free environment for further study.

Preparation of extracts

Extraction of active components from leaves and seeds powder was performed sequentially in different polar and non-polar solvents such as methanol, acetone, ethyl acetate, chloroform and petroleum ether. Fine powder was soaked in each solvent 1:10 (w/v) and mixed well by continuously stirring at room temperature for 2-3 hrs and then centrifuged at 5000 rpm for 15 min. The supernatants were collected and solvent of each extract was removed by vacuum rotary evaporator at room temperature. The remaining residues were collected and preserved at -10^{o} C for further experiment.

Estimation of total phenolics

Total phenolic compounds present in different solvent extracts were estimated by using Folin-Ciocalteau assay (Jaiwal et al., 2012). Equal volume of each solvent extract was mixed with Folin-Ciocalteau reagent (0.5 ml) and incubated at room temperature for 3 min after which 1ml Na_2CO_3 (20% w/v) was added and incubated for 1 min in boiling water bath. Aliquots were cooled under tap water and optical density was recorded at 650nm. Simultaneously, increasing concentration of gallic acid was taken in other aliquots for standard. Amounts of phenolics were determined by using standard graph and the concentration of phenolics were expressed as gallic acid equivalents (GAE mg/ml).

Detection of radical scavenging activity

Antioxidant activity of methanol, ethyl acetate, acetone, chloroform and petroleum ether extracts was detected by using DPPH radical scavenging assay (Yamaguchi et al., 1998). In a typical reaction, 100µl plant extracts and 2 ml DPPH reagent (100 µg/ml in acetone/ethanol 1:1 v/v) were incubated at ambient temperature for 15 minutes. Simultaneously, two aliquots were kept separately one containing ascorbic acid in place of plant extract (standard antioxidant) and the other containing only DPPH reagent for control. The plant extracts containing antioxidant activity was visually detected by the change in color from purple to yellow of DPPH reagent.

DPPH radical scavenging assay

DPPH radical scavenging assay of extracts was quantitatively estimated by using a protocol of Yamaguchi et al (1998) as discussed above. A typical reaction contains 2 ml of DPPH in ethanol /acetone (1:1 v/v) was mixed with various concentrations of each extract such as 25, 50, 75, 100 and 125 µg. Ascorbic acid was used as standard reference antioxidant. Reaction mixtures were incubated in dark condition for 15min and thereafter the optical density was recorded at 517nm. For the control, DPPH in ethanol/acetone was taken another aliquot without plant extract. The decrease in optical density of DPPH in addition of the test samples (as compare to control) was used to calculate the antioxidant activity and the percentage of scavenging activity was calculated by using the following equation.

$$\text{Effect of scavenging (\%)} = \left[\frac{1 - \text{A sample (517nm)}}{\text{A control (517nm)}}\right] \times 100$$

Thin layer chromatography (TLC)

The separation of compounds from each crude extract was carried out on TLC (Aluminum sheet coated with silica). The composition of mobile phase was used according to polarity of solvent used for extraction. The 5-10 µl sample was applied on TLC by pipette. After separation of compounds on thin layer chromatography, the TLC plate was observed under UV-light for visualization of resolved compounds.

Column chromatography

Isolation of antioxidant from *M. senegalensis* leaves ethyl acetate extract was performed by using silica gel column chromatography (Chunpeng et al., 2011). Silica (60-120 mesh size) slurry was prepared in hexane: ethyl acetate (1:9 v/v) and was packed in a glass column (2×15 cm). Ethyl acetate extract (1 gm) was loaded on column and eluted sequentially in order to increase linear gradients of ethyl acetate in hexane. In all, 10 fractions were collected (each 50 ml) and the fraction no. 4, 5, 6, 7, 8 and 9 exhibited antioxidant activity. According to TLC pattern the fraction no. 4, 5 and 6 were pooled together and reloaded on the same type of column and eluted using the same gradients. Total 6 fractions were collected (each 50 ml), according to TLC pattern fraction no.1, 2 and 3 were pooled together and were applied on preparative TLC. Compounds 3rd (1.8 mg) and 4th (1.95 mg) were afforded. Finally, the remaining three fractions were pooled together and applied on preparative TLC and the compounds 1st (2.5mg) and 2nd (2.2 mg) were afforded.

HPLC (High performance liquid chromatography)

HPLC was used for the confirmation of single compound purification and the analysis was carried out according to a well-established method (Lillian et al., 2012). HPLC binary system (Chemito) was equipped with a Rheodyne injector, 20µl sample loop, UV detector (Model: LC 6600) and Iris34 software for data acquisition. Stationary phase was C18 analytical column (250*4.5 mm) with a particle Eurospher 100-5. Mobile phase for chromatographic analysis was methanol: acetonitrile (1:1 v/v) at constant flow rate of 1 ml/min.

Fourier Transform Infrared Spectroscopy (FTIR) and Elemental analysis

Functional groups of the compound 1st were detected by using FTIR (Make: Bruker, Germany; Model: 3000 Hyperion Microscope with Vertex 80 FTIR System). Further, elemental analysis was done by using CHNS (O) analyzer (Make: Thermo finnigan, Italy; Model: FLASH EA 1112 series).

Statistical analysis

All experiments were conducted and analyzed in triplicate. Means and standard deviations were calculated and compared. Analysis was performed using Microsoft Excel.

Results and discussion

Amount of total phenolic compounds from leaves and seeds

M. senegalensis have been investigated to exhibit different medicinal properties. In order to study the antioxidant property, the leaves and seeds were procured and allowed to completely dry. Bioactive constituents were removed from the powdered seeds and leaves using polar and non-polar solvents resulting in a total of 10. Dried residues were obtained by completely removing the solvent from extracts and calculated their % yield was calculated by weighing of residues. Amount of extracted % yields (w/w) were in order of leaves methanol extract (7.6%) > leaves petroleum extract (5.56%) > leaves chloroform extract (2.21) > leaves ethyl acetate extract (1.24%) > leaves acetone extract (1.05%) and seeds methanol extract (5.54%) > seeds petroleum extract (3.92%) > seeds acetone extract (2.90%) > seeds chloroform extract (1.16%) > seeds ethyl acetate extract (1.01%) as shown in Table 1. The amount of extracted residues in methanol was found the highest as compared to other residues from other solvents.

The amounts of total phenolics in each residue were estimated by Folin-Ciocalteau assay and amount was expressed as gallic acid equivalent (GAE mg/ml). The various concentrations of phenolics were observed among extracts and they were in order of leaves methanol extract (15.8 mg/ml) > leaves acetone extract (2.9 mg/ml) > leaves ethyl acetate extract (2.3mg/ml) > leaves chloroform extract (0.9mg/ml) > leaves petroleum extract (0.5 mg/ml) and seeds methanol extract (15mg/ml) > seeds ethyl acetate extract (1.9 mg/ml) > seeds acetone extract (1.5 mg/ml) > seeds chloroform extract (0.5 mg/ml) > seeds petroleum extract (0.25 mg/ml) as shown in Table 1. The highest amount of phenolics was observed in methanolic extracts while negligible amount of phenolics was observed in petroleum ether solvent. Therefore methanol was a better solvent for the removal of bioactive constituents including phenolics from *M. senegalensis*.

Table 1. Different polar and non-polar solvent extracts were prepared from leaves and seeds of *M. senegalensis*, their % yields, total phenolics, antioxidant activities and IC_{50} values.

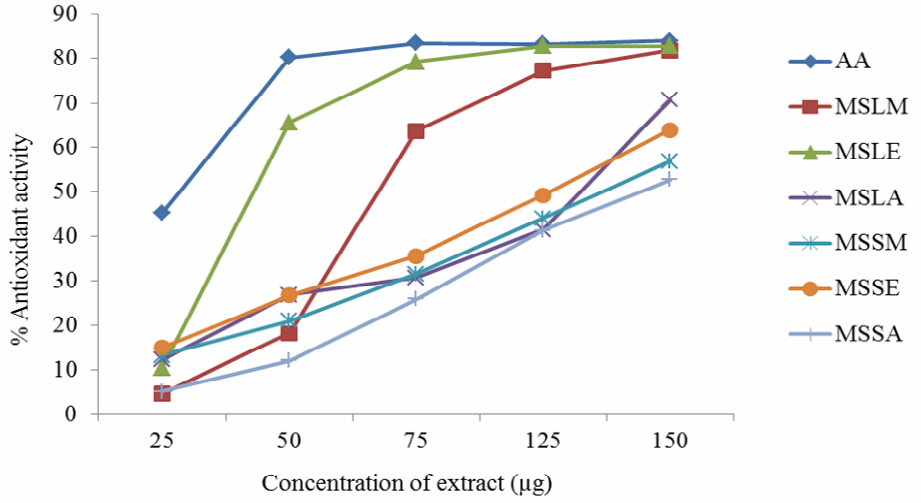
Test samples	% yield (w/w)	Total phenolics (mg/ml)	Antioxidant activity	IC_{50} (µg/ml)
Leaves methanol extract	7.6	15.8	+	21.6
Leaves ethyl acetate extract	1.24	2.3	+	14.58
Leaves acetone extract	1.05	2.9	+	35.41
Leaves chloroform extract	2.21	0.9	-	-
Leaves petroleum ether extract	5.56	0.5	-	-
Seeds methanol extract	5.54	15	+	37.50
Seeds ethyl acetate extract	1.01	1.9	+	33.33
Seeds acetone extract	2.90	1.5	+	40
Seeds chloroform extract	1.16	0.5	-	-
Seeds petroleum ether extract	3.92	0.25	-	-
Ascorbic acid	-	-	+	9.33
+= Presence of antioxidant activity, -= Absence of antioxidant activity.				

Polyphenols exhibit the different biological properties and receive much attention as natural antioxidant and they could be useful as free radical scavengers. Phenolic compounds are exhibit redox properties of hydrogen donors and singlet oxygen quenchers, these properties of phenolics are responsible for antioxidant activity (Rice-evans et al., 1995). According to World Health Organization phytomedicines have been considered as medicinal value from traditional health care system and greater than 80% of world population depends on traditional medicine (Duraipandiyan et al., 2006). Medicinal plants contain different polyphenols such as flavonols, isoflavones, flavones, tannins, phenolic acids and anthocyanins are responsible for multiple bioactivities including free radical scavenging activity and therefore they are commonly consumed (Hasanuzzaman et al., 2013). Glycosylation influenced by chemical and physical properties of phenolics in human body including bioavailability and absorption that lead broad spectrum of different activities beneficial for positive effects in many diseases (Scalbert and Williamson, 2000).

Table 2. Identification of specific frequency regions characteristic and functional groups by FTIR of purified compound 1st from ethyl acetate extract of leaves.

Sl. No.	Frequency area (cm^{-1})	Characteristic of frequencies (cm^{-1})	Assignment
1.	3422.57	O-H Stretch	Phenolics
2.	2925.05	C-H Stretch	Aromatics
3.	1630.70	C=O Stretch	Amides
4.	1028.33	C-O Stretch	Alcohols
5.	670.98	C-I Stretch	Alkyl halides

Fig. 1: Graphical representation of % DPPH radical scavenging activity of different solvent extracts of leaves and seeds of *M.senegalensis* (Lam) Excell. AA: Ascorbic acid, MSLM: Methanol extract of leaves, MSLE: Ethyl acetate extract of leaves, MSLA: Acetone extract of leaves, acid, MSSM: Methanol extract of seeds, MSSE: Ethyl acetate extract of seeds and MSSA: Acetone extract of seeds.



DPPH free radical scavenging activity

Free radical scavenging activities of extracts were determined by using DPPH reagent. Methanol, ethyl acetate and acetone extracts of leaves and seeds exhibited free radical scavenging activities while chloroform and petroleum ether extracts does not exhibited free radical scavenging activities. The various concentrations of each extract were used for in-vitro for evaluation of antioxidant efficacies. According to IC_{50} values the antioxidant potentials were observed in order of leaves ethyl acetate extract (IC_{50} 14.58 µg/ml) >leaves methanol (IC_{50} 21.6 µg/ml) > leaves acetone extract (IC_{50} 35.41 µg/ml) and seeds ethyl acetate (IC_{50} 33.33 µg/ml) > seeds methanol extract (IC_{50} 37.50 µg/ml) > seeds acetone extract (IC_{50} 40 µg/ml) shown in Table 2. The antioxidant potentials of different solvent extracts of leaves and seeds were slightly lower as compared to antioxidant potential of standard ascorbic acid (IC_{50} 9.33 µg/ml) (Fig. 1).

Fig. 2: Graphical representation of % antioxidant activities of compound 1st and 2nd purified from ethyl acetate extract of *M. senegalensis*.

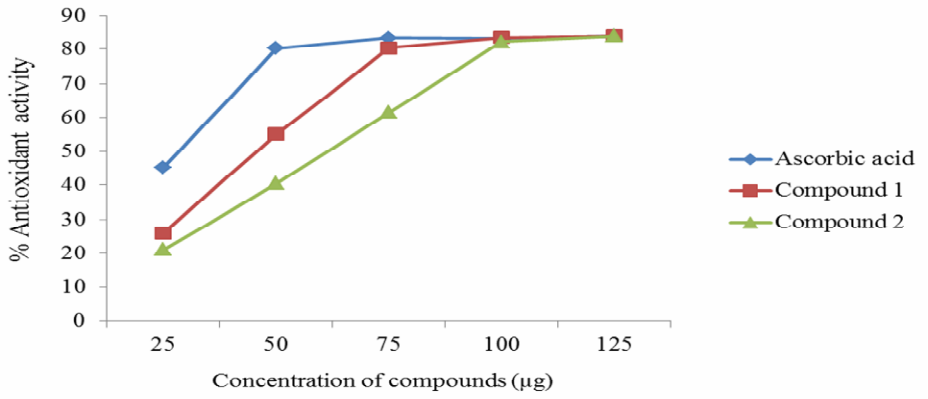


Fig. 3: HPLC chromatogram of purified compounds (A) 1st (B) 2nd (C) 3rd and (D) 4th.

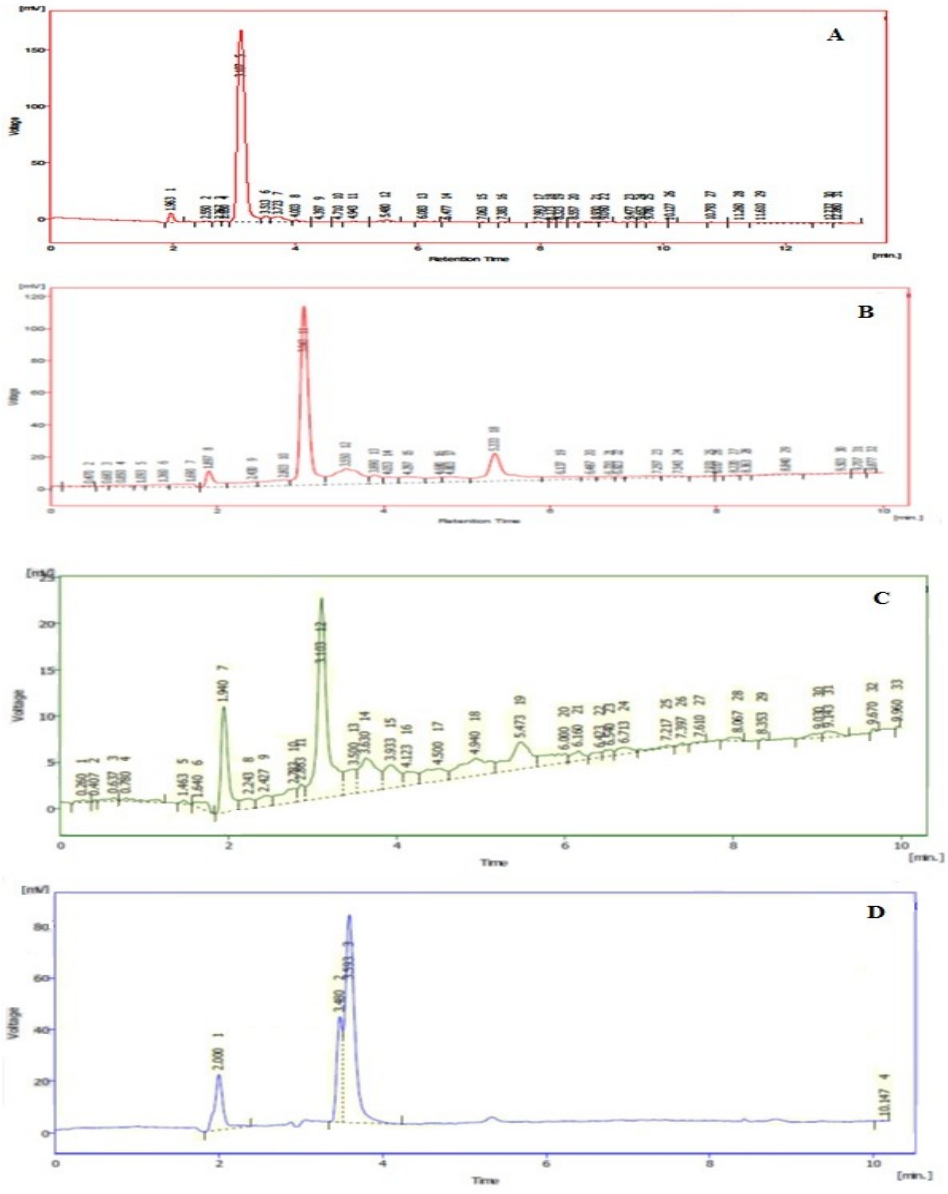
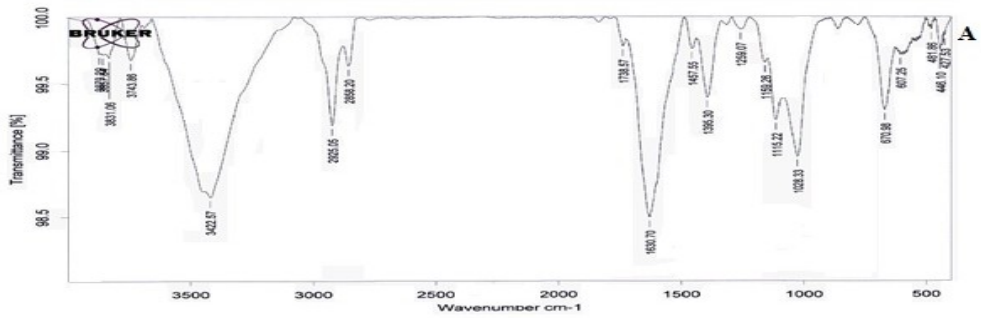
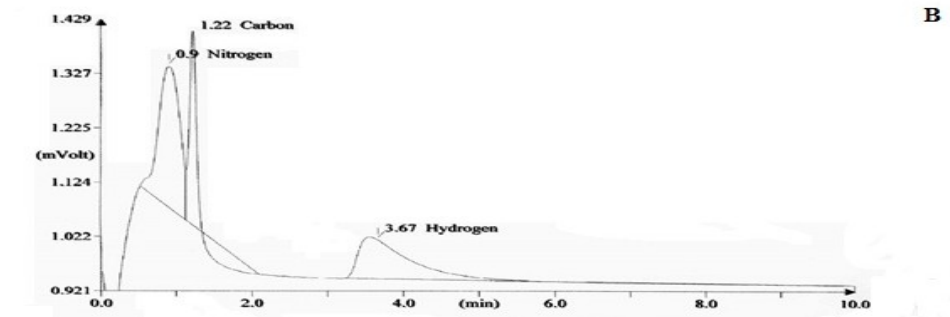


Fig. 4: FTIR spectrum and elemental analysis of compound 1st. (A) Peaks display frequencies of functional groups (B) Peaks showing % of carbon, hydrogen and nitrogen (C) Peak showing % of O.





Peak Number (#)	Retention Time (min)	Area (.1*uV*sec)	Element %	Component
1	0.900	51286	1.245	Nitrogen
2	1.217	12553	0.136	Carbon
3	3.667	34474	0.128	Hydrogen
		98313	1.509	

Peak Number (#)	Retention Time (min)	Area (.1*uV*sec)	Element %	Component
1	0.817	44086	0.286	Oxygen
		44086	0.286	

Plant antioxidant reacts with stable free radicals of DPPH and acts as an electron paired off and bleaching of the color stochiometrically. Our findings indicate that the plant antioxidant reduces free radial involvement in various disorders and developed scavenging system in living organism. It has been found that the antioxidant properties of compounds are due to only scavenging of free radicals (Aswatha et al., 2008). Ethyl acetate extract exhibited the highest antioxidant potential than other extract. Our results were similar to earlier reported study of antioxidant activity in some medicinal plants such as *P. cubeba* Linn, *Eugenia caryophyllus*, *Zingiber officinale*, *Trigonella foenum-graecum* and Black tea (*Camelia sinensis*) (Aneta et al., 2007). In present study the prominent correlation was observed between amount of total phenolics and antioxidant activity similar to earlier reported (Yu et al., 2003).

Purification of antioxidant

Purification of antioxidant compounds were carried out by silica gel column and thin layer chromatography by using different gradients of ethyl acetate in hexane. Four spots were isolated and their R_f values were observed as 0.20 (1^{st}), 0.42 (2^{nd}), 0.69 (3^{rd}) and 0.81 (4^{th}) by using mobile phase chloroform: methanol (9:1v/v). Compounds 1^{st} and 2^{nd} exhibited antioxidant activities and compounds 3^{rd} and 4^{th} not exhibited antioxidant activities.

Purification of single compound was confirmed by single peak of HPLC chromatogram. According to HPLC chromatogram the single compound purification of 1^{st} and 2^{nd} with the retention times 3.107 and 3.043 were observed as shown in Fig. 3. Slight impurities were observed with compound 3^{rd} and 4^{th} and their retention times were 1.940, 3.103, 2.000, 3.480 and 3.593. Compound 1^{st} exhibited more antioxidant potential (IC_{50}15.12 µg/ml) as compared to compound 2^{nd} (IC_{50} 22.56 µg/ml) (Fig. 2).

FTIR spectral analysis

The compound 1^{st} was analyzed by FTIR because it exhibited higher antioxidant potential than the other compounds. Functional groups associated with 1^{st} compound were identified by effective peaks. FT-IR spectrum gave more intense peaks assigned to aromatics (3422.57 cm^{-1}), phenolics (2925.05 cm^{-1}), amides (1630.70 cm^{-1}), alcohols (1028.33 cm^{-1}) and alkyl halides (670.98 cm^{-1}) as shown in Table 2.

The data suggests that the antioxidant properties of compound 1^{st} can be associated with hydrogen-donating hydroxyl groups on the aromatic ring of the phenolic group. Similar results have been already investigated in leaves extract of *Elaeis guineensis* (Vijayarathna and Sasidharan, 2012). This study supports in further research for the investigation of other antioxidant compounds from *M. senegalensis.*

Elemental analysis

Percentage elemental analysis of compound 1^{st} was also carried out. Fig. 4 clearly indicated that the 1^{st} contain Carbon, Hydrogen, Oxygen and Nitrogen and their percentage were 0.136, 0128, 0.286 and 1.245 respectively.

Conclusion

The results obtained in the present study indicate that the *M. senegalensis* leaves and seeds extract exhibit free radical scavenging activities. The overall antioxidant activity might be due to polyphenolic constituents. Methanol is a better solvent for extraction of phenolic compounds from leaves and seeds of *M. senegalensis*. According to FTIR data the purified compound (**1^{st}**) from leaves is a phenolic/aromatic type and its scavenging potential slightly lower than ascorbic acid. Our findings of the present study, suggest that *M. senegalensis* extract could be a potential source of natural antioxidant that could have great importance as therapeutic agent in preventing aging and age associated oxidative stress related degenerative diseases.

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