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Research Article

EVALUATION OF ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITY OF *STACHYS LAVANDULIFOLIA*

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ABSTRACT

The present study deals with the antioxidant and anti-inflammatory activities of *Stachys lavandulifolia* (*S. lavandulifolia*). Total phenolic content was assessed using Folin-Ciocalteu's method. The antioxidant activity was determined by measuring the scavenging activity of DPPH, ABTS, FRAP and lipid peroxidation assay. *In vitro* anti-inflammatory activity was evaluated using 15-lipoxygenase (15-LO) inhibitory assay. Quercetin was used as a standard drug for the study of anti-inflammatory activity. The methanolic extract of *S. lavandulifolia* showed highest total Phenolic content. The methanol and ethyl acetate extracts possessed strong scavenging activity in DPPH, ABTS and FRAP methods. Both ethyl acetate and methanol extracts showed Dose-Dependent protective effect against lipid peroxidation and free radical generation in liver homogenates. All extracts of *S. lavandulifolia* screened for anti-inflammatory LOX assay and hexane extract showed significant LOX inhibition compare to quercetin as positive control . For further investigation hexane extract was subjected to thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). One of TLC fraction showed significant LOX inhibition. The results obtained in this study confirm the traditional use of *S. lavandulifolia* for the treatment of painful and inflammatory conditions.

Keywords: *Stachys lavandulifolia*, Anti-inflammatory, Antioxidant activity, HPLC, Lipid peroxidation, LOX.

INTRODUCTION

The use of plants as source of remedies for the treatment of diseases date back to prehistoric times and people on all continents are used to this old tradition. Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effect. The research into plants with traditional use as anti-inflammatory agents should be viewed as a fruitful and logical research strategy in the search for new anti-inflammatory drugs.

The *Labiatae* family (*Lamiaceae*) is one of the largest and most distinctive families of flowering plants, with about 220 genera and almost 4000 species worldwide. This family has an almost cosmopolitan distribution. The *Labiatae* family is best known for the essential oils common to many members of the family. Many biologically active essential oils have been isolated from various members of this family. The plants of this family also famous for the diterpenoids. Evidence from archeological excavations shows that some species of this family, which are now known only as wild plants, had been cultivated at local scales in the past (1). *Stachys* species have been used as a medicine for centuries throughout the world. *Stachys lavandulifolia* is of interest to researchers because the constituents such as betulinic acid, oleanolic acid, rosmarinic acid, and ursolic acid are similar to other *Stachys* species commonly used as an alternative in medicinal preparations. *Stachys lavandulifolia* is grown in many parts of Iran, Iraq and Anatolia (2). The plant is known as Chaye-kuhi in Iran and its' English name is Betony. It is used as the herbal tea in gastrointestinal disorders (3).

Inflammation is the complex pathophysiological process. Inflammatory diseases including different types of rheumatic disorders are very common throughout the world (4). Inflammatory diseases are accompanied by the chronic release of cytokines and nitrogen species. The reactive oxygen and nitrogen species may further increase the tissue injury (5). There is much evidence has shown that the production of reactive species occurs at the site of inflammation and contributes to the tissue damage (6). Although several modern drugs are used to treat these types of disorders but their prolonged use may cause several adverse effects like gastric intestinal irritation (7), consequently there is a need to develop new anti- inflammatory agents (8). Herbal medicines are being accepted and used increasingly by general populations in both Eastern and Western countries because of their medicinal properties (9). Several plants are being used in the traditional medicine for treating the inflammatory disorders. Cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) are both key enzymes involved in the arachidonic acid (AA) cascade (10). *In vitro* bioassay systems have been extensively used to monitor biological activities of medicinal plant extracts used in traditional medicines. In this study, we have demonstrated a correlation between antioxidant efficiency and phenolic composition of the different extracts. The anti-inflammatory action was determined by a bioassay system that tested the inhibitory effect on soybean 15-lipoxygenase (15-LO) inhibitory assay.

MATERIAL AND METHODS

Materials

15-lipoxygenase (15-LO), Quercetin, Linoleic acid, (1, 1-diphenyl, 2-picrylhydrazyl radical (DPPH), 2, 2-Azinobis (3-ethyl benzothiazoline-6-sulfonic acid) (ABTS), Ascorbic acid, Galic acid, 2,4,6-tripyridiyl-striazine (TPTZ), Folin-Ciocalteu *phenol* reagent (FC reagent), Thiobarbituric acid (TBA), potassium persulfate, ferrous sulphate, Trichloroacetic acid (TCA), were purchased from Himedia, Mumbai, India; Methanol, ethyl acetate, DMSO and hexane were purchased from Fisher Scientific, Bangalore, India.

Plant material

The areal parts of *S. lavandulifolia* were collected in May, 2010 from Hamadan in Iran and authenticated by a plant taxonomist.

Extraction method

The fresh areal part of *S. lavandulifolia* washed to remove adhering dust and shade-dried at room temperature and reduced to coarse powder. One hundred grams of the powder were sequentially extracted in a soxhlet apparatus with hexane, ethyl acetate, methanol and water to obtain the respective extracts. Extracts, with different polarities, were concentrated to dryness and the residues were kept at +4°C. The water extract lyophilized and residue was stored at +4°C until required.

Total Phenolic Content Estimation

The total phenolic content of the different extract of S. *lavandulifolia* was determined using the Folin-Ciocalteau reagent method (11). To 50 µl of each extract, 2.5 ml of Folin-Ciocalteau reagent (1/10 dilution) and 2 ml of 7.5% Na_2CO_3 (w/v) were added and mixed well. The blend was incubated at 45°C for 15 min. The absorbances of all samples were measured at 765 nm with Na_2CO_3 solution (2 ml of 7.5% Na_2CO_3 in 2.55 ml of distilled water) as blank. The results were expressed as mg of gallic acid equivalence (GAE)/ mg of extract.

DPPH free radical scavenging activity

DPPH free radical scavenging activity was determined according to the modified method of Sultanova *et al.* (12). The reaction mixture contained 5 µL of test samples (5 mg/ml) and 95 µl of DPPH in methanol. Different concentrations of test samples were prepared while the concentration of DPPH was 300 µM in the reaction mixture. These reaction mixtures were taken in 96-well microtitre plates and incubated at 37°C for 30 min. The absorbance was measured at 517 nm. Percent radical scavenging activity upon sample treatment was determined by comparison with a methanol treated control. All determinations were performed in triplicates. IC_{50} denotes the concentration of extract required to scavenge 50% DPPH free radicals. Ascorbic acid was used as positive control.

$$\text{Radical scavenging (\%)} = [(A_c - A_s)/ A_c] \times 100$$

Where, Ac is the absorbance of the control (methanol-treated) and A_s is the absorbance of the antioxidants in the crude extract and standard.

Ferric-Reducing Antioxidant Power Assay (FRAP)

This assay has been described by Benzie and Strain (13) and Katalinic et al. (14). In fact, Ferric Reducing Antioxidant Power (FRAP) assay measures the change in absorbance at 593 nm due to the formation of a blue colored complex of ferrous ion (Fe^2+) and 2,4,6-tripyridiyl-striazine (TPTZ). Prior to this, colorless ferric ion (Fe^3+) was oxidized to ferrous ion (Fe^2+) by the action of electron donating antioxidants. Freshly prepared FRAP reagent was warmed at 37°C in a water bath. This reagent was prepared by mixing 10 mM of 2,4,6- tripyridyl-s-triazine (TPTZ) in 40 mM HCl, 20 mM $FeCl_3$ and 0.3 M acetate buffer (pH 3.6) in the ratio of 1:1:10. An aliquot of 25 µl of extract was added to 475 µl of FRAP reagent. The mixture was incubated at 37°C for 30 min. Absorbance was read at 593 nm using a UV-Vis Spectrophotometer. A reducing ability in FRAP assay was calculated with reference to the reaction given by the $FeSO_4.7H_2O$. The values were expressed as mM of galic acid equivalents (GAE)/ mg of extract.

ABTS (2, 2'-azino-bis 3-ethylbenzthiazoline -6-sulfonic) assay

The scavenging activity of $ABTS^{\bullet+}$ was measured ($ABTS^{\bullet+}$assay) according to the method described by Re *et al.* (15) with some modification. ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. Because ABTS and potassium persulfate reacts stoichiometrically at a ratio of 1:0.5, this will result in incomplete oxidation of the ABTS. Oxidation of the ABTS commenced immediately, but the absorbance was not maximal and stable until more than 6 hours had elapsed. The radical was stable in this form for more than two days when stored in the dark at room temperature. The $ABTS^{\bullet+}$ solution was diluted with ethanol/methanol, to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30°C. Methanol was used as a negative control. After addition of 1.0 ml of diluted $ABTS^{\bullet+}$ solution (A734nm = 0.700 ± 0.020) to 10µl of antioxidant compounds or Ascorbic acid standards in methanol the absorbance was taken at 734 nm exactly one min after initial mixing and up to six min using the spectrophotometer. Appropriate solvent blanks were run in each assay. All determinations were carried out at least three times, and in triplicate, on each occasion and at each separate concentration of the standard and samples. The $ABTS^{\bullet+}$ scavenging capacity of extract compared with Ascorbic acid and percentage inhibition was calculated as ABTS radical scavenging activity

$$\text{Radical scavenging (\%)} = [(Abs_{control} - Abs_{sample})]/(Abs_{control})] \times 100$$

Where, $Abs_{control}$ is the absorbance of ABTS radical + methanol; Abs_{sample} is the absorbance of ABTS radical + sample extract/standard.

Microsomal membrane lipid peroxidation

Rat liver (1g) was homogenized in 5 ml (50 mM) tris buffer (pH 7.4) and microsomes were isolated by the calcium aggregation method (16). The pellet was resuspended in 0.1M phosphate buffer. Microsomal lipid peroxidation was assayed by the thiobarbituric acid method (17). To 100 µl of microsomes were added ferrous sulphate (100 µM) and ascorbic acid (100 µM) with or without *S. lavandulifolia* extract in 0.1 M phosphate buffer (pH 7.4) and incubated at 37 °C for 1 h. This was followed by the addition of 20% trichloroacetic acid (2 ml) and 1% thiobarbituric acid (2 ml). The mixture was heated in a boiling water bath for 10 min, cooled, centrifuged and the colour in the supernatant was read at 535 nm in a spectrophotometer. Percent inhibition was calculated against a control without the extract.

15-lipoxygenase (15-LO) inhibitory

15-LO from soybeans was used for peroxidation of linoleic acid, and inhibition was carried out as described by Lyckander and Malterud (18). To a solution of linoleic acid (final concentration 134 µM) in borate buffer (0.2 M, pH 9.00, 2.90 ml) was added 50 µl of test substance dissolved in DMSO or (for blanks) DMSO alone. 50 µl of 15-LO solution in borate buffer (10,000 U/ml) was added, and the increase in absorbance at 234 nm for 30–90 s was measured. The value for % inhibition of enzyme activity was calculated as 100 $[(\Delta A_1/\Delta t) - (\Delta A_2/\Delta t) / (\Delta A_1/\Delta t)]$, where $\Delta A_1/\Delta t$ and $\Delta A_2/\Delta t$ are values for increase in A_{234} for sample without test substance and with test substance, respectively.

Preliminary separation by TLC

Thin layer chromatography (TLC) was carried out with the crude extract on silica gel (TLC silica gel. 60, 20 x 20, 0.5 mm, Merck and Co, Inc) with hexane: ethyl acetate (8:2) solvent system. The hexane crude extract was spotted, and the solvent front was allowed to run for approximately 16 cm. The running lane was then dried thoroughly; elution of compound was detected at 365 nm and sprayed with anisaldehyde-sulphuric acid reagent. The bands corresponding to active compound by initial screening were scraped off the plates not being sprayed and the compounds were washed out of silica gel with hexane. The obtained hexane solutions were filtered and subjected to HPLC analysis.

Analytical high performance liquid chromatography

Analytical HPLC (Shimadzu, 10ATVP) was performed with a reversed phase C18 column using LC-10-ATVP double units pumps. The analytical chromatography was carried out under isocratic conditions by varying the percentage of acetonitrile and 0.1% H_3PO_4 in water using a flow rate of 0.8mL/min at Rom temperature. Chromatograms were recorded at 254nm using a SPD-10Atvp variable wavelength detector. Assay samples were dried and dissolved in methanol. A 20 µL sample was injected each time.

RESULTS

Total phenolic content

The total phenolic content of different fractions of *S. lavandulifolia* are presented in Table (1). The phenolic was calculated as quite high in methanol fraction other than ethyl acetate, water extract and hexane fraction.

In vitro antioxidant activity

The measures of antioxidant activity were obtained using three described methods. The reduction capacity of DPPH radicals was determined by the decrease of the absorbance induced by the antioxidant at 517 nm, which is induced by the antioxidant (Figure 1). From these result, the strongest DPPH activity was obtained by methanolic extract followed by ethyl acetate, water and hexane extracts. The lowest activity was obtained in hexane extract. Ascorbic acid was used as positive control.

The FRAP assay evaluates the ability of a substance to reduce Fe^{3+} to Fe^{2+}; since the antioxidant activity of a substance is usually correlated to its reducing capacity, this assay provides a reliable method to evaluate the antioxidant activity. The FRAP assay is very simple, fast and precise, and was recently developed to measure the total antioxidant power of biological fluids. Total antioxidant power was assessed by the reduction of Fe^{3+} to Fe^{2+}, which occurred rapidly with all reduction. Therefore, the values express the corresponding concentration of electron-donating antioxidants. In the present experiment, the FRAP value expressed in Table (1).

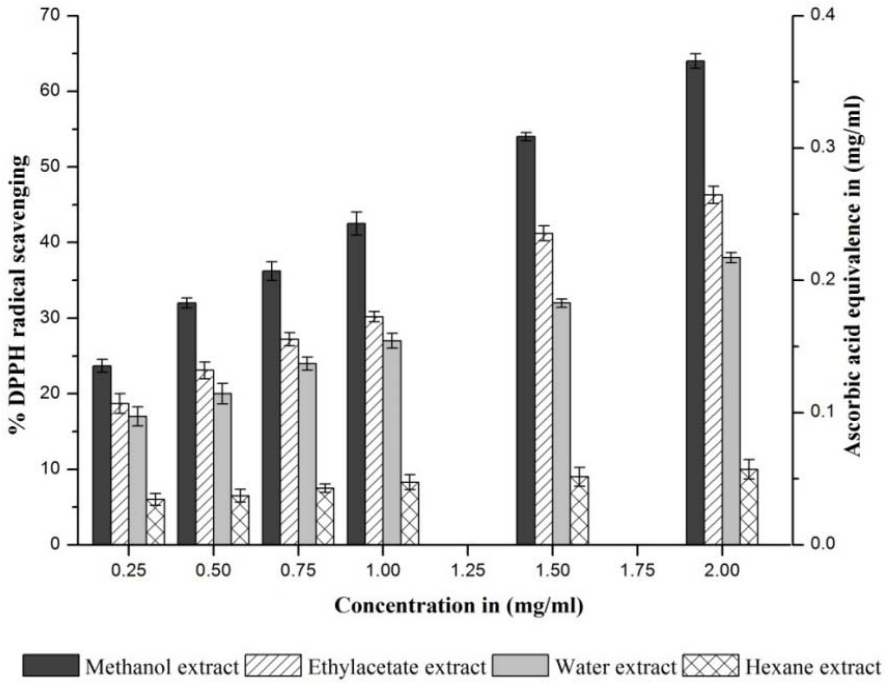


Fig. 1: DPPH- radical scavenging activity of S*. lavandulifolia* extracts in different concentrations with Ascorbic acid equivalence. The methanolic extract exhibited higher antioxidant activity than other extracts. Data expressed as mean ± SD (n=3).

The total antioxidant ability (ABTS) of *S. lavandulifolia* was measured by means of scavenging a protonated radical ABTS and compared with ascorbic acid standard. Methanol, ethyl acetate, hexane and water extracts of *S. lavandulifolia* were evaluated and compared for their ABTS scavenging capacities with ascorbic acid. All the tested extracts showed ABTS scavenging capacity (Figure 2). Higher concentrations of the extracts were observed to be more effective in quenching free radicals in the system. The moderate correlation between ABTS radical scavenging activity of the extract and total phenolic content implies that it is not only phenolic compounds that contributed to the radical scavenging action.

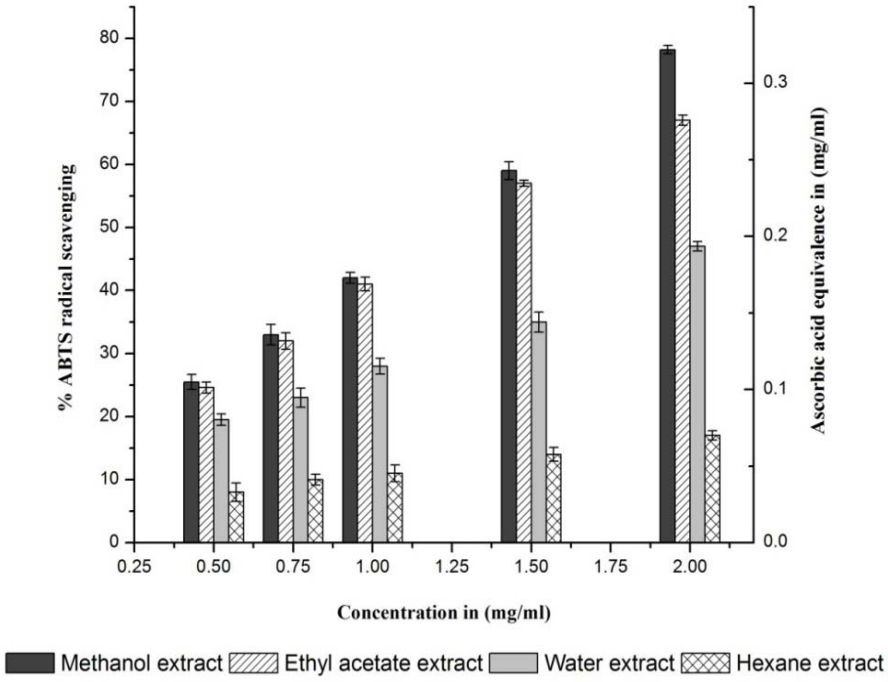


Fig. 2: ABTS$^{\cdot+}$ radical scavenging activity of S*. lavandulifolia* extracts in different concentrations with Ascorbic acid equivalence. The methanolic extract exhibited higher antioxidant activity than other extracts. Data expressed as mean ± SD (n=3).

Microsomal lipid Peroxidation (LPO) inhibition

Iron induced microsomal lipid peroxidation (LPO) inhibition was used as one of the methods to evaluate antioxidant capacity. Results are listed in Table 1 as the amount of each extract necessary to inhibit microsomal lipid peroxidation by 50 percent, (IC_{50}). As a result IC_{50} values for inhibition of lipid peroxidation were found to be 32.03± 0.21, 64.71± 0.13, 289.4± 0.032 and 312.66± 0.054 µg, for ethyl acetate, methanol, water and hexane extracts of *S. lavandulifolia*, respectively.

Table 1: Total phenolic content, FRAP and inhibition of lipid peroxidation (LPO) for different *S. lavandulifolia* extracts.

	Total phenolic content (mg of GAE/mg of extract)	FRAP mM of GAE/ mg of extract	Inhibition of LPO IC_{50} (µg)
Methanol extract	0.166± 0.0043	11.5± 0.005	64.71± 0.13
Ethyl acetate extract	0.151± 0.0032	8.6 ± 0.004	32.03± 0.21
H_2O_2 extract	0.068± 0.0046	5.33± 0.006	289.4± 0.032
Hexane extract	0.025± 0.0053	3.69± 0.005	312.66± 0.054

GAE: Galic Acid Equivalence

Values are mean ± SE for n = 3, (P< 0.05)

Inhibition of 15-LO

The lipoxygenase (LOX) activity was monitored as an increase in the absorbance at 234 nm, which reflects the formation of hydroperoxylinoleic acid. The highest inhibitory effect was obtained for hexane extract of *S. lavandulifolia*. By fractionation of hexane extract with TLC we got five different fractions with different Rf values. The LOX activity was monitored and the highest inhibitory activity toward 15-LO was observed in the first fraction of hexane extract from *S. lavandulifolia* with Rf value of 0.27. Quercetin was used as positive control (Table 2).

Semi-purification of hexane extract by TLC and HPLC

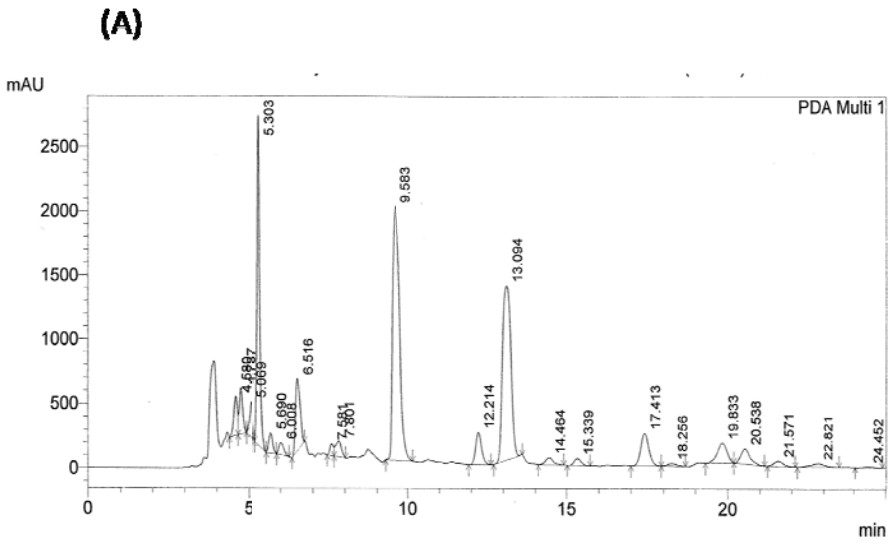
The hexane extract of *S. lavandulifolia* was preliminarily separated by TLC. The developed and dried TLC plate visualized under UV and sprayed with anisaldehyde-sulphuric acid reagent. All bands which detected by UV and anisaldehyde reagent were scraped off the plates not being sprayed and washed with hexane. The residue of hexane subjected to LOX assay along with hexane crude extract. After initial screening one band with Rf value of 0.27 demonstrated significant anti-inflammatory activity. For further study the active band residue subjected to HPLC.

HPLC chromatogram for crude hexane extract has shown four major peaks with retention time of 5.303, 6.516, 9.583 and 13.094. The active fraction of TLC also contains all those four peaks but the quantities of two peaks are higher than crude extract. We can estimate that the active compound is in one or both peaks (Figure 3A and 3B).

Table 2: Results of lipoxygenase inhibitory effects of *S. lavandulifolia* extracts and five TLC fractions of hexane extract tested.

Extract/ TLC fraction	Inhibition of LOX IC_{50} (µg/ml)
H_2O extract	185.184± 10.6
Methanol extract	161.29± 6.25
Ethyl acetate extract	151.515± 8.2
Hexane extract	64.102 ± 5.8
Quercetin	12.8± 2.3
TLC fraction of Hexane extract	15.7± 1.86
	17.8 ± 2.42
	18.69± 1.98
	27.05± 3.45
	22.46± 2.87

Lipoxygenase activity was determined as absorbance increase at λmax = 234 nm at 3 minutes of incubation with or without inhibitor tested. Results are presented as percent of control ± SD, n = 3.



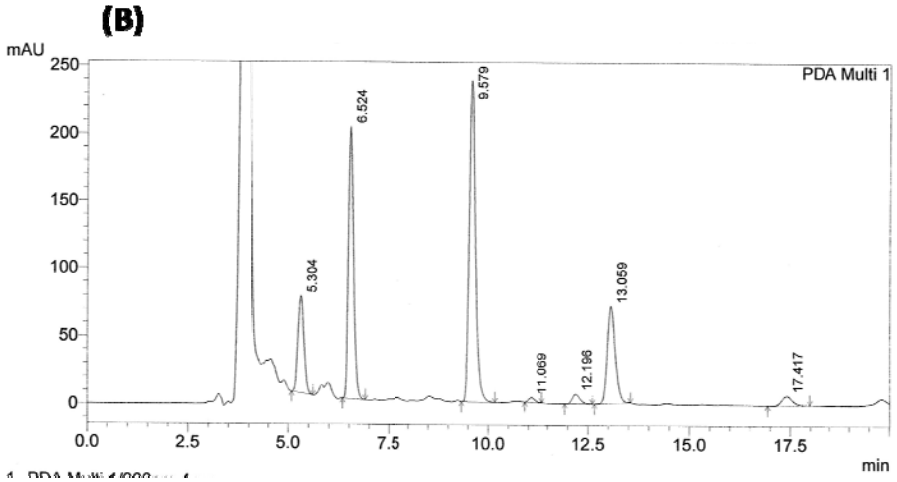


Fig. 3: HPLC chromatograms of (A) hexane soxhlet fraction and (B) hexane extract TLC fraction of *S. lavandulifolia* recorded at 254 nm.

DISCUSSION

Food producers are increasingly identifying specific genotypes and varieties of fruits and vegetables rich in functional ingredients comprising of nutritive and non-nutritive antioxidants. Many studies have demonstrated that cruciferous vegetables contain a wide array of phytochemicals.

This study showed that among the aqueous, methanol, ethyl acetate and hexane extracts of *S. lavandulifolia*, the methanolic extract possesses potent antioxidant activity and the potency of the extracts is in the order of methanol> ethyl acetate> aqueous> hexane. Hexane extract did not show any antioxidant activity in the DPPH and ABTS radical scavenging assay. Overall, the methanol extract of *S. lavandulifolia* is the most potent in scavenging the DPPH and ABTS. The presence of high levels of phenolic compounds in the methanol extract may have partly contributed to the observed antioxidant activities. It has been demonstrated in various in vitro assays that the phenolic compounds, extracted from plant materials, have good antioxidative properties due to which these compounds are finding increasing use as additional dietary supplements [19,20]. The phenolic compounds may contribute directly to the antioxidant action due to the presence of hydroxyl functional groups around the nuclear structure that are potent hydrogen donators. These phenolic compounds of plant origin show their antioxidative effect by various mechanisms including their ability to scavenge free radicals, chelate metal ions that serve as the catalysts for the production of free radicals or activate various antioxidant enzymes and inhibit oxidases [21-22]. The hydrogen donating potential is known to be one of the various mechanisms for measuring antioxidant activity.

LOXs are the family of the key enzyme in the biosynthesis of leukotrienes that are postulated to play an important role in the pathophysiology of several inflammatory disease. According to the currently used nomenclature, the LOXs are classified with respect to their positional specificity of arachidonic acid oxygenation (5-LOX, 9-LOX, 12- LOX, 15-LOX) [22]. LOX are sensitive to antioxidants, and the most common way of their action may consist of inhibition of lipid hydroperoxide formation due to scavenging of lipidoxyor lipidperoxy-radicals formed in course of enzymic peroxidation. This can limit the availability of lipid hydroperoxide substrate necessary for the catalytic cycle of LOX (23). This study provided evidence on the potential health benefits of *S. lavandulifolia.*

CONCLUSIONS

In this study the data obtained reveal that the activity of methanolic extract in DPPH, FRAP assay and ethyl acetate extract in lipid peroxidation assay is more potent than aqueous and hexane extracts of *S. lavandulifolia.* In LOX (lipoxygenase) assay hexane extract of *S. lavandulifolia* is more potent than other extracts of *S. lavandulifolia.*

The results obtained confirm the therapeutic potency of *S. lavandulifolia* used in traditional medicine. In addition, these results form a good basis for selection of the plant for further phytochemical and pharmacological investigation. The present studies support the folkloric usage of the studied plant and suggest that the plant extract possesses certain constituents with antioxidant, anti-inflammatory properties that can be used for the diseases such as aging, cancer and atherosclerosis. Therefore further investigations are necessary for purifying and characterization of active compounds.

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