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IN VITRO APPRAISAL OF ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF METHANOLIC FRACTION OF SALVIA MILTIORRHIZA

Pharmacology

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ABSTRACT

The present study aimed to investigate the antioxidant and anti-Inflammatory activities of methanolic extract of *Salvia miltiorrhiza* in various in vitro models. The work evaluated the antioxidant activities of the extract employing reducing power assay and total antioxidant activity assay in linoleic acid emulsion methods. Anti-inflammatory activities were evaluated utilizing two methods viz. inhibition of albumin denaturation and antiproteinase action. Preliminary phytochemical screening was also performed alongside total Phenolic content estimation using Gallic acid standard. The results demonstrated significant reducing power of the extract compared to the standard antioxidant compounds. Total antioxidant activity was also found to be significant. Anti-inflammatory activities were also found to be significant in terms of significant inhibition of albumin denaturation as well as antiproteinase action. The study demonstrated potent antioxidant as well as anti-inflammatory efficacy of the methanolic extract of *Salvia miltiorrhiza.*

KEYWORDS

Salvia miltiorrhiza, Salvia, antioxidant, inflammation, Red sage, Danshen.

INTRODUCTION

Inflammation is a complicated physiological process, which is commonly connected with pain and the increase of vascular permeability, increase of protein denaturation and membrane alteration. Upon damage or attack by microbes, physical agents or chemical agents, cell in the body get damaged due the stress caused by the event. As a defensive response to the stress, inflammation occurs in the tissue as a protective mechanism, which is further characterized by redness, pain, heat, and swelling and loss of function in the injured area (Kataki et al., 2014). Upon damage or assault, as a non specific defensive mechanism body tissues cause inflammation by releasing various mechanistic chemical mediators including kinins, prostroglandins and histamine. These work collectively to cause increased vasodilation (widening of blood capillaries) and permeability of the capillaries. This leads to increased blood flow to the injured site for the delivery of mediators of inflammation. These substances also act as chemical messengers that attract some of the body's natural defence cells a mechanism known as chemotaxis. Inflammation can be classified as either acute or chronic. Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes (especially granulocytes) from the blood into the injured tissues. A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue. Prolonged inflammation, known as chronic inflammation, leads to a progressive shift in the type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process. Oxidative stress or free radicals generated within the bodily functions or acquired from environment, have been considered significant modalities leading to inflammation or related disorders. There are several in vitro and in vivo experimental protocols of inflammation which are used for evaluating the potency of drugs. The management of inflammation related diseases is a real issue in the rural community; the population in these areas uses many alternative drugs such as substances produced from medicinal plants. Therefore quick, reliable and cost effective protocols are needed to evaluate the potency or efficacy of medicinal or folklore medicinal plants or other folklore modalities.

Salvia miltiorrhiza is medicinal and folklore plant used in various disorders throughout the world. It is also known as Danshen in Chinese traditional medicine which is also traditionally used for treatment of insomnia in Chinese herbal medicine. Grown body of research showed that the ether extract of *Salvia miltiorrhiza* can decrease the latency of sleep significantly and enhance sleeping time in mice model. Evidence also demonstrated that the water extract of *Salvia miltiorrhiza* is able to act on the BDZ (Benzodiazepine) binding sites of GABA-A receptors (Singh and Zhao, 2017). A survey of published literature revealed that there is no significant studies that has been done to evaluate the anti-inflammatory and antioxidant potential of *Salvia miltiorrhiza* by *in vitro* method. Therefore, the present study aimed to investigate the antioxidant and anti-inflammatory activity of *Salvia miltiorrhiza* by various reliable and effective *in vitro* protocols.

MATERIALS AND METHODS

Plant material

Plant Name : *Salvia miltiorrhiza*
Family : Labiatae; Lamiaceae.
Habitat : Native to the Mediterranean region; grown as an ornamental.
English : Red Sage.
Folk : *Salvia Sefakuss*.

Preparation of the extract

The flowers of *Salvia miltiorrhiza* were collected from Mandi district during the month of March and April. The plant was identified and authenticated by Dr. S. Sharma, Botanist, Research Institute in Indian System of Medicine (ISM), Joginder Nagar, Mandi, Himachal Pradesh. A voucher specimen (MSRSY/2018/13) has been preserved for further utilization. The plants were dried in shade and coarsely powdered. The coarsely powdered drug (5 kg) was successively extracted in the Soxhlet apparatus using methanol as the sole solvent for the complete extraction of the phytochemicals. The methanol extract was collected and concentrated under reduced pressure at 45 – 50°C. A dark green brownish concentrated methanol extract of *Salvia miltiorrhiza* (MESM) was obtained (yield 0.85%, w/w with respect to the dried starting material). The final product was then stored at 4°C prior to use.

Drugs and chemicals

1, 1 – diphenyl – 2 – picryl hydrazyl hydrate (DPPH) and 2, 2′ – azinobis –3 – ethylbenzothiazoline – 6 – sulfonic acid (ABTS) were purchased from Sigma – Aldrich (St. Louis, MO, USA). Quercetin was arranged as gift samples from authentic reliable sources. All other chemicals and reagents were of reagent grade and available commercially (SRL Mumbai, E. Merck India).

Ascertaining margin safety by acute oral toxicity studies in rats and mice (OECD guideline 423)

Whenever an investigator administers a chemical substance to a biological system, different types of interactions can occur and a series of dose-related responses result. In most cases these responses are desired and useful, but there are a number of other effects which are not advantageous. These may or may not be harmful to the patients. The

types of toxicity tests which are routinely performed by pharmaceutical manufacturers in the investigation of a new drug involve acute, sub-acute and chronic toxicity. Acute toxicity is involved in estimation of LD50 (the dose which has proved to be lethal (causing death) to 50% of the tested group of animals). Determination of acute oral toxicity is usually an initial screening step in the assessment and evaluation of the toxic characteristics of all compounds. The present study adapted OECD guideline 423 to perform the acute toxicity study for the extract of *Salvia miltiorrhiza.*

Determination of total phenolic compounds

Total soluble phenolic in the methanol extract were determined by using Folin – Ciocalteau method using quercetin as a standard phenolic compound described elsewhere (Slinkard and Singleton, 1977a). The concentration of total phenolic compounds in the extract was showcased as gram of quercetin equivalent (QE) using an equation obtained from linear regression analysis of the standard quercetin graph.

$Y = m x + c$

Where, y was the absorbance and x was the concentration.

Preliminary Phytochemical Screening

The methanolic extract from successive solvent extraction was subjected to preliminary phytochemicals screening for the detection of various phytoconstituents such as alkaloids, steroids, flavonoids, glycosides, tannins, phenolic compounds, carbohydrates, proteins, amino acids and fats. The following tests were carried out to identify the various phytoconstituents present in the extracts (Baxter et al., 1998).

Antioxidant activity Evaluation *In Vitro*

Reducing power

The reducing power of extract was determined according to the method described previously (Oyaizu, 1986b). The different concentrations of extract (50- 250µg/mL) in 1ml of distilled water was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [K3Fe(CN)6] (2.5 ml, 1%). The mixture was incubated at 50 ◦C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl3 (0.5 ml, 0.1%), and the absorbance was measured at 700 nm in a spectrophotometer (UV -1601 Shimadzu, Japan). Higher absorbance of the reaction mixture indicated greater reducing power. α-Tocopherol was used as standard antioxidant compound(Kataki et al., 2012).

Total antioxidant activity in linoleic acid emulsion system

The total antioxidant activity of the extract was determined according to the thiocyanate method (Kataki et al., 2012). Ten milligrams of extract was dissolved in 10 ml water. Different concentration of extract (50-250 µg/mL) or standard samples in 2.5 ml of potassium phosphate buffer (0.04 M, pH 7.0) was added to linoleic acid emulsion (2.5 ml) in potassium phosphate buffer (0.04 M, pH 7.0). Five millilitres linoleic acid emulsion consists of 17.5g Tween-20, 15.5µl linoleic acid, and 0.04M potassium phosphate buffer (pH 7.0). On the other hand, 5.0 ml control consists of 2.5 ml linoleic acid emulsion and 2.5 ml potassium phosphate buffer (0.04 M, pH 7.0). The mixed solution was incubated at 37◦C in a glass flask and in the dark. After the mixture was stirred for 3 min, the peroxide value was determined by reading the absorbance at 500 nm in a spectrophotometer (UV -1601 Shimadzu, Japan), after reaction with FeCl2 and thiocyanate at intervals during incubation. During the linoleic acid oxidation, peroxides formed. These compounds oxidize Fe2+ to Fe3+. The latter Fe3+ ions form complex with SCN−, which had maximum absorbance at 500 nm. Therefore, high absorbance indicates high linoleic acid oxidation. The solutions without extract or standards were used as blank samples. All data about total antioxidant activity are the average of triplicate analyses. The inhibition of lipid peroxidation in percentage was calculated by following equation:

$$\text{Inhibition}(\%) = (A0 - At/A0) \times 100$$

Where A0 was the absorbance of the control reaction and At was the absorbance in the presence of the sample. All the tests were performed in triplicate and graph was plotted with the mean ± SD values. α-Tocopherol were used as standard antioxidant compound.

Anti-inflammatory activity Evaluation *In Vitro*

Inhibition of albumin denaturation

The anti-inflammatory activity of MESM was studied by using inhibition of albumin denaturation technique which was studied according to Mizushima et al (Mizushima and Kobayashi, 1968) and Sakat et al (Sakat et al., 2010) followed with minor modifications. The reaction mixture was consists of test extracts and 1% aqueous solution of bovine albumin fraction, pH of the reaction mixture was adjusted using small amount of 1N HCl. The sample extracts were incubated at 37ºC for 20 min and then heated to 51ºC for 20 min, after cooling the samples the turbidity was measured at 660nm.(UV Visible Spectrophotometer Model 371, Elico India Ltd) The experiment was performed in triplicate. The Percentage inhibition of protein denaturation was calculated as follows: Percentage inhibition = (Abs Control –Abs Sample) X 100/ Abs control

Antiproteinase action

The test was performed according to the modified method of Oyedepo et al. and Sakat et al. (Sakat et al., 2010) The reaction mixture (2 ml) was containing 0.06 mg trypsin, 1 ml 20 mM Tris HCl buffer (pH 7.4) and 1 ml test sample of different concentrations (100 - 500 µg/ml). The mixture was incubated at 37oC for 5 min and then 1 ml of 0.8% (w/v) casein was added. The mixture was incubated for an additional 20 min. 2 ml of 70% perchloric acid was added to arrest the reaction. Cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer as blank. The experiment was performed in triplicate. The percentage inhibition of proteinase inhibitory activity was calculated.

Percentage inhibition = (Abs control –Abs sample) X 100/ Abs control

Statistical Analysis

Data were expressed as mean ± SD for 3 replicate analyses where applicable. GraphPad Prism V 6.0 has been used to analyse the data for statistical significance which has been set at $p < 0.05$.

RESULTS AND DISCUSSION

Preparation of Extracts

The plants were dried in shade and coarsely powdered. Flower 5 kg was successively extracted in the Soxhlet apparatus using water as the sole solvent for the complete extraction of the phytochemicals. The three extracts were dried in rota evaporator at 45 °C and the dried extracts were stored in vacuum desiccators containing anhydrous silica gel. The yield (%) of the extract was presented in table 1.

Table 1. The methanol extraction with percentage yield value.

S. No	Plant	Weight of Crude drug (Dry) in gm	Weight of the extract in gm	% Yield
1	*Salvia miltiorrhiza*	5000	478	9.56

Acute oral toxicity studies in rats (OECD guideline 423)

The extract w subjected to acute toxicity study for estimating the dosage schedule to be used further. The studied extract (MESM) did not demonstrate any mortality up to 2000 mg/kg dose level. Therefore, 1/20 th and 1/10th of the maximum dose (i.e., 100 and 200 mg/kg, p.o.) were chosen for the present study. At 2000 mg/kg dose level the studied extracts were found to be non toxic and it did not produce any toxic symptoms and lethality (Ld_{100}).

Table 2. Acute toxicity studies of MESM (Methanol Extract of *Salvia miltiorrhiza)*

Parameters	Day 1						Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
	30 min	1hr	2 hr	3hr	4 hr	8 hr													
Alertness	H-S	H-S	H-S	H-S	H-S	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√
	B-S	B-S	B-S	B-S	B-S	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√
	T-S	T-S	T-S	T-S	T-S	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√
Spontaneous motor activity	H-S	H-S	H-S	H-S	H-S	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√
	B-S	B-S	B-S	B-S	B-S	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√
	T-S	T-S	T-S	T-S	T-S	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√
Respiration	H-F	H-F	H-F	H-F	H-F	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√
	B-F	B-F	B-F	B-F	B-F	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√
	T-F	T-F	T-F	T-F	T-F	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√
Ataxia	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×
	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×
	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×

Body tremors	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×
	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×
	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×
Convulsions	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×	H-×
	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×	B-×
	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×	T-×
Reactivity to sound/touch	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√
	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√
	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√
Body tone	H-S	H-S	H-S	H-S	H-S	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√	H-√
	B-S	B-S	B-S	B-S	B-S	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√	B-√
	T-S	T-S	T-S	T-S	T-S	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√	T-√

Determination of total phenolic compounds

Phenolic and flavanoid type compounds present in plant extract was reported for the mostly observed and anticipated antioxidant activities of plant sources. In phenolic type of compounds the presence of hydroxyl groups imparts free radical scavenging ability to the plant extracts (Slinkard and Singleton, 1977b). In the present set up the extract (MESM) was subjected to a test for the evaluation of total phenolic content in the studied plant extract. The results revealed the presence of quantifiable phenolic contents (MESM = 594.67 ± 1.526 mg GAE/g of extract).

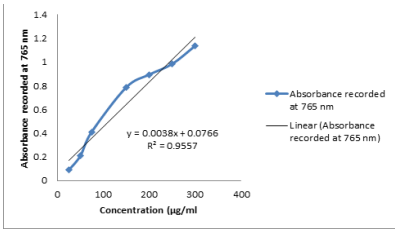

Figure 1. Total Phenolic Content Estimation using Gallic acid

Preliminary Phytochemical Screening

The results of the preliminary phytochemical screening revealed the presence and absence of various phytochemicals in the methanol extract. The tests were carried out using the methanol extract. Phytochemical tests showed the presence of alkaloids, flavonoids, triterpene polysaccharides, steroids, tannins, reducing sugar, and saponins. Data are presented in table 3.

Table 3. Results of preliminary phytochemical screening

S.No.	Constituents	MESM
1	Alkaloids	√
2	Saponins	√
3	Sterols	√
4	Carbohydrates	√
5	Tannins	√
6	Flavanoids	√
7	Fatty acid	√
8	Terpenoid	√
9	Glycosides	√

Antioxidant activity Evaluation In Vitro

Reducing power

The results of reducing power assay of MESM, sodium metabisulphite, quercetin and ascorbic acid were depicted in figures below. In this method of reducing ability measurement, the transformation of Fe^{3+} to Fe^{2+} was evaluated by using well known method described earlier (Oyaizu, 1986a). The reducing power of the studied plant extracts was found to be increased with the increasing concentration of MESM. The results demonstrated a comparable reducing power with the standard compounds used in the study.

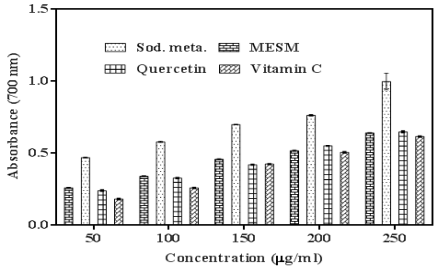

Figure 2. Reducing power assay of MESM

Total antioxidant activity in linoleic acid emulsion system

Total antioxidant activity of MESM along with the standard antioxidant compounds was evaluated by using a well established standard in vitro test model known as thiocyanate method. At the concentration level of 250 µg/ml, MESM were found to be potent antioxidant in terms of total antioxidant activity. In this method, the ability of the extract to inhibit the peroxidation of linoleic acid in the used emulsion was investigated and the results were depicted in figure 3. Initially the antioxidant efficacy was found to be increased, but at later time points the activity was found to be decreased slightly. The extract demonstrated higher antioxidant efficacy than α-Tocopherol but the activity was found to be lower than BHA (Butylated hydroxyanisole). MESM showed 63.17 % of inhibition at 12 hour time point and 56.79 % inhibition at 72 hour time point.

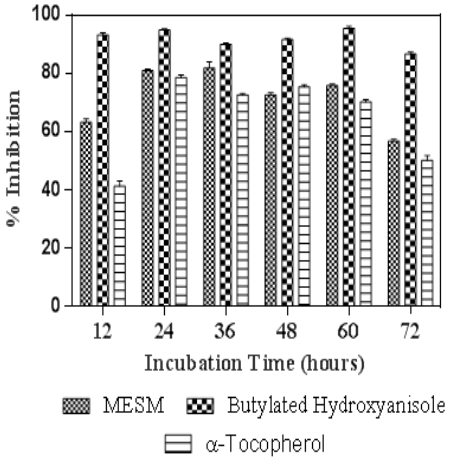

Figure 3. Total antioxidant activity MESM

Anti-inflammatory activity Evaluation *In Vitro*

Inhibition of albumin denaturation

Protein Denaturation is a process in which proteins lose their tertiary structure and secondary structure by application of external stress or compound, such as strong acid or base, a concentrated inorganic salt, an organic solvent or heat. Most biological proteins lose their biological function when denatured. Denaturation of proteins is a well documented cause of inflammation. As part of the investigation on the mechanism of the anti-inflammation activity, ability of plant extract to inhibit protein denaturation was studied. It was effective in inhibiting heat induced albumin denaturation. Maximum inhibition of 54.17% was observed at 500 µg/ml. Aspirin, a standard antiinflammation drug showed the maximum inhibition 52.08% at the concentration of 100 µg/ml compared with control (Table 4).

Table 4: Effect of MESM on heat induced protein denaturation

Treatment	Concentration (µg/ml)	Absorbance at 660 nm	% Inhibition of protein denaturation
Control	-	0.48 ± 0.06	-
MESM	100	0.36 ± 0.04	25
MESM	200	0.31 ± 0.02	35.42
MESM	300	0.27 ± 0.05	43.75
MESM	400	0.23 ± 0.06	52.08
MESM	500	0.22 ± 0.02	54.17
Aspirin	100	0.23 ± 0.03	52.08

Each value represents the mean ± SD. N=3, Experimental group were compared with control **p<0.01, considered extremely significant. MESM: Methanol Extract of *Salvia miltiorrhiza*

Antiproteinase action

Neutrophils are known to be a rich source of serine proteinase and are localized at lysosomes. It was previously reported that leukocytes proteinase play an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitors (Das and Chatterjee, 1995). MESM exhibited significant antiproteinase activity at different concentrations as shown in Table 5. It showed maximum inhibition of 65.63% at 500µg/ml. Aspirin showed the maximum inhibition 78.57% at 100µg/ml.

Table 5: Effect of MESM on proteinase inhibitory action

Treatment	Concentration (µg/ml)	Absorbance at 660 nm	% Inhibition of protein denaturation
Control	-	0.49 ± 0.05	-
MESM	100	0.41 ± 0.04	16.33
MESM	200	0.38 ± 0.03	26.83
MESM	300	0.34 ± 0.05	39.47
MESM	400	0.32 ± 0.05	50.00
MESM	500	0.28 ± 0.02	65.63
Aspirin	100	0.27 ± 0.06	78.57

Each value represents the mean ± SD. N=3, Experimental group were compared with control **p<0.01, considered extremely significant; *p<0.05, considered significant; ns p>0.05, non significant. MESM: Methanol Extract of *Salvia miltiorrhiza.*

CONCLUSION

In the present study, results indicate that the methanol extract of *Salvia miltiorrhiza* possess antioxidant and anti-inflammatory properties. These activities may be due to the strong occurrence of polyphenolic compounds such as alkaloids, flavonoids, tannins, steroids, and phenols. The extract serves as free radical inhibitors or scavenger or acting possibly as primary oxidants and inhibited the heat induced albumin denaturation and proteinase activity. The extract demonstrated potent antioxidant activities alongside anti-inflammatory activity. This study revealed that phytoconstituents in *Salvia miltiorrhiza* can be used as lead compound for designing a potent anti-inflammatory and antioxidant drug which can be used for treatment of various diseases such as neurological disorder, aging and inflammation.

DECLARATION OF INTEREST

None Declared

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