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Original Research Paper

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INVITRO ANTIOXIDANT ACTIVITIES OF METHANOLIC FRACTION OF RED SAGE FLOWER.

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ABSTRACT The present study aimed to investigate the antioxidant, in-vitro anti-inflammatory and Anti-Alzheimer activities of methanolic extract of Salvia miltiorrhiza (MESM) in various in vitro models. Preliminary phytochemical screening was also performed. Antioxidant activity and free radical scavenging activity of MESM has been evaluated by employing various in vitro models including reducing power, Total antioxidant activity, DPPH radical, ABTS radical, superoxide radical, nitricoxide, hydrogen peroxide, hydroxyl radical scavenging activity and metal chelating activity.Anti-inflammatory activity has been evaluated by various in vitro methods including protein denaturation, anti-proteinase action and membrane stabilization. The plant extract (MESM) revealed significant antioxidant activity with lower IC50 values. Metal chelating activity was found poor. In inflammation modelS, MESM demonstrated significant anti-inflammatory activity. Acetylcholinesterase (AChE) inhibitory activity was also estimated. Anti-Alzheimer profile was also evaluated along with the necessary observations using neuroblastoma cell lines. Results demonstrated a neuroprotective profile of the methanol extract of Salvia miltiorrhiza as well as revealed a protective efficacy in Alzheimer's disease (AD). The results indicated that the plant could be a potential source of antioxidant and could find a use in the herbal therapy of inflammation also, which further supports the protective efficacy in AD.

KEYWORDS : Salvia Miltiorrhiza, Salvia, Antioxidant, Inflammation,anti Alzhimer's, Red Sage, Danshen.

INTRODUCTION

The demand for natural product-based medicines and herbal products, such as nutraceuticals, herbal supplements and cosmetics is continuously increasing globally. The reasons behind this increase in demand are their properties, such as non-toxic nature, less or no side effects, biocompatibility and ease of availability.According to an estimation, about 80% of healthcare needs in developing countries are met through traditional medicines or natural products [1].With the increasing dependence on the herbal prescription, it ends up relevant to seek potent, effective and relatively safe plant medicines. In addition, validated scientific methods should also be establish in order to enhance their safety and efficacy.
Alzheimer's disease is a neuropathological process consists of neuronal loss and atrophy in the temporoparietal and frontal cortex with an inflammatory response [2, 3]. There is an increased presence of monocytes/macrophages in the cerebral vessel wall and activated microglial cells in the nearby parenchyma [4, 5].To date, very little scientific data on traditional medicinal plants used for CNS disorders is available. Few studies including pre-screening approach for anti-inflammatory and anti-Alzheimer activity screening of potential medicinal plants have been reported.

In the present investigation, flower of Salvia miltiorrhiza (also known as Danshen) has been used.To date, Salvia miltiorrhiza have been investigated for its potential application in various therapeutic conditions [6-9]. As per the literature review, there is no significant studies that has been done to evaluate the anti-oxidant,. Therefore, the present study aimed to investigate the anti-oxidant, of Salvia miltiorrhiza by various reliable and effective in vitro protocols.

MATERIALS AND METHODS

PLANT

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

CHEMICAL

Acetic acid, N-butanol, Thio barbituric acid, Tris buffer, Chloroform and Diethyl ether were purchased from LOBA chemicals, Mumbai, India.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. Acetylthiocholine, Sodium hydroxide, Sodium hydrogen carbonate and Di-potassium hydrogen phosphate were purchased from Central Drug House (CDH) Pvt Ltd, New Delhi, India. Napthylethylene diamine dihydrochloride, Griess reagent, Hexadecylt rimethylammonium bromide (HTAB), O-dianisidine and 1, 1, 3, 3-Tetramethoxypropane were purchased from Suvchem, Mumbai, India. Di thionitrobenzoic acid, Pyridine, Sodium nitrate, Sodium nitrite and Sulphanilamide were purchased from RANKEM Pvt Ltd, Haryana, India. All other chemicals and reagents were of reagent grade and available commercially (SRL Mumbai, E. Merck India).

PREPARATION OF THE EXTRACT

The flowers of Salvia miltiorrhiza were collected from Mandi district of Himanchal Pradesh, India 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 (MSRSY/2018/13). The flowers were dried and pulverization. Fordefatting purpose, the powdered flowers (2 kg) were macerated twith petroleum ether three times at room temperature for 48 h. Following maceration, the final product was extracted usingmethanol as an extracting solvent. The methanol extract was collected and concentrated under reduced pressure at 45 – 50°C. Concentrated methanol extract of dark green brownish color was obtained (yield 0.85%, w/w with respect to the dried starting material). Finally, the final product wasthen stored at 4°C for further use.

PHYTOCHEMICAL SCREENING

The methanolic extract was subjected to preliminary phytoch

emicals screening for the detection of various phytocon stituents such as alkaloids (Mayer's, Dragendorffs, Wagner's and Hager's test), steroids(Froth test), flavonoids (Shinoda, Ammonia and Lead acetate solution test), sterols (Salkowaski reaction, Liebermann's test and Liebermann-Burchard's reaction), Carbohydrates (Molisch's, Fehling's solution and Benedict's solution test), tannins (Ferric chloride reagent and Lead acetate test), proteins and amino acid (Ninhydrin and Millon's test) and Triterpenoids (Libermann-Burchard and Salkowski's test) (10,11).

ANTIOXIDANT ACTIVITY

DETERMINATION OF DPPH RADICAL SCAVENGING ACTIVITY

A 0.1mM solution of DPPH in ethanol was prepared and 1ml of this solution was added to 3ml of extract solution in water atdifferent concentrations (50-250 g/mL).The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm in a spectrophotometer (UV -1601 Shimadzu, Japan). Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The percent DPPH scavenging effect was calculated using the following equation:

DPPH scavenging effect (%) = 100 − [(A0 – At / A0) × 100]

Where A0 was the absorbance of the control reaction and At was the absorbance in the presence of the standard sample or extract. All the tests were performed in triplicate andgraph was plotted with the mean ± SD values. BHA was used as standard antioxidant compound (12).

ABTS RADICAL DECOLORIZATION ASSAY

ABTS was dissolved in water to make a concentration of 7 mM. ABTS· was produced by reacting the 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. For the test of samples, the ABTS· stock solution was diluted with phosphate-buffered saline 5 mM (pH 7.4) to an absorbance of 0.70 at 734 nm. After the addition of 1.0 ml of diluted ABTS· to 20 µl of sample, the absorbance reading was taken 5 min after the initial mixing (13). This activity is given as percent ABTS· -scavenging that is calculated as follows:

% ABTS·-scavenging activity = [Control absorbance – Sample absorbance] / [Controlabsorbance] × 100

ASSAY OF SUPEROXIDE RADICAL SCAVENGING ACTIVITY

The assay was based on the capacity of the methanolic extract to inhibit blue formazon formation. Superoxide radical were generated in riboflavin-light-NBT (Nitroblue tetrazolium) system(14). The total volume of the reactant mixture was 3 ml. Each 3 ml of this reaction mixture contained 50 mM sodium phosphate buffer (pH 7.6), 20 g riboflavin, and 12 mM EDTA, and 0.1 mg NBT and 1 ml sample solution. Reaction was started by illuminating the reaction mixture with different concentrations of the methanolic plant extract (50-250 µg/ml) for 90 seconds. Immediately after illumination, the absorbance was measured at 590 nm. The reactionassembly was enclosed in an aluminium foil lined box. Unilluminated identical tubescontaining reaction mixture served as blank. The percentage inhibition of superoxide anion generation was calculated using the following formula:

% Inhibition = (A0 – At / A0) × 100

Where, A0 was the absorbance of the control (without extract) and At was the absorbance in the presence of the extract or standard. All the tests were performed in triplicate and graph was plotted with the mean ± SD values. Ascorbic acid was used as standard compound.

ASSAY OF NITRIC OXIDE SCAVENGING ACTIVITY

Sodium nitroprusside (10 mM) in phosphate buffered saline was mixed with different concentrations of methanolic extract of the plant dissolved in methanol and then incubated at room temperature for 150 minutes. In the same way, a reaction mixture was prepared without the methanolic extract but with equivalent amount of methanol was added. Thisserved as control. After the incubation period, 0.5 ml of Greiss reagent (1 % Sulphanilamide, 2 % H3PO4 and 0.1 % N-(1-naphthyl) ethylenediamine dihydrochloride) was added to the mixture. The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthyl ethylenediamine was measured at 546 nm. Standard solutions of ascorbic acid treated in the same way as tests with Greiss reagent served as positive control (15). The percentage of inhibition was calculated by using the following formula:

% Inhibition = (A0 – At / A0) × 100

Where, A0 was the absorbance of the control (without extract) and At was the absorbance in the presence of the extract. All the tests were performed in triplicate and graph was plotted with the mean ± SD values.

HYDROGEN PEROXIDE SCAVENGING ACTIVITY

Hydrogen peroxide (H2O2) scavenging ability of the extract was measured using a method described previously (16).A solution of hydrogen peroxide (2mmol/L) was prepared in phosphate buffer (pH 7.4). Concentration of hydrogen peroxide was determined spectrophotometrically from absorption at 230 nm with molar absorbtivity 81 $molL^{-1}cm^{-1}$.The plant extract (50-250 g/ml) were added to H2O2 solutions (0.6 ml). Absorbance of H2O2 at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without H2O2. The percentage inhibition was calculated using the following formula:

% Inhibition = (A0 – At / A0) × 100

Where, A0 was the absorbance of the control (without extract) and At was the absorbance in the presence of the extract or standard. All the tests were performed intriplicate and graph was plotted with the mean ± SD values. Ascorbic acid was used asstandard compound.

HYDROXYL RADICAL SCAVENGING

The reaction mixture containing 2-deoxy-d-ribose (1 mM), phenyl hydrazine (0.2 mM), (in phosphate buffer, pH 7.4) and different concentration of the test samples (50-250 g/ml) were incubated for 4h at 37 C. The reaction was stopped by the addition of 2.8 % (w/v) trichloroacetic acid solution, followed by centrifugation at 5000 rpm (for 10 min). The supernatant was mixed with aqueous 1% (w/v) thiobarbituric acid (TBA). The TBA reactive product thus formed was directly measured at 532 nm (17).

METAL CHELATING ACTIVITY

The chelating of ferrous ions by the methanolic plant extract was measured by the method described previously (18).Different concentrations of the extract (50-250 g/ml) were added to a solution of FeCl2 (0.05 ml, 2 mM). Then the reaction was initiated by addition of 5 mM ferrozine (0.2 ml). The reaction mixture was then shaken vigorously and allowed to stand at room temperature for 10 minutes. The absorbance of the solution was then measured at 562 nm. The percentage inhibition of ferrozine –ferrous complex formation was calculated by using the following formula:

% Inhibition = (A0 – At / A0) × 100

Where, A0 was the absorbance of the control (without extract) and At was the absorbance in the presence of the extract or standard. All the tests were performed in triplicate and graph was plotted with the mean ± SD values. EDTA (Ethylenedia minetetraacetic acid) was used as standard chelating compound.

RESULTS AND DISCUSSION

PREPARATION OF THE EXTRACT

The flowers of Salvia miltiorrhizawere collected from Mandi district, Himanchal Pradesh, India (Figure-1).A dark green brownish concentrated methanol extract of flowers of Salvia miltiorrhiza (MESM) was obtained (yield 9.56 %, w/w with respect to the dried starting material). The final product was then stored at 4°C prior to use.

Figure-1. Salvia miltiorrhiza plant

PHYTOCHEMICAL SCREENING

Phytochemical test revealed the presence of alkaloid, saponins, steroids, carbohydrates, tannins, triterpene, flavonoids, fatty acid and glycoside.

IN-VITRO ANTIOXIDANT ACTIVITY

In this present study, Butylated HydroxyAnisole (BHA) was used as a standard radical scavenger. **Figure 2A** shows the decrease in concentration of DPPH radical due to scavenging capability of the extract and standard compound (BHA) at different studied concentrations (50-250 µg/ml). The present study utilized BHA as standard radical scavengers. DPPH radical scavenging ability of MESM was found to be comparable to that of BHA which demonstrated stronger scavenging ability than Salvia miltiorrhiza. The percent DPPH scavenging ability were found to be 96.46 % (MESM) and 98.89 % (BHA) at the concentration of250 µg/mL. The results revealed the strong DPPH radical scavenging ability of the plant comparable to standards. The IC50 values of Salvia miltiorrhiza and BHA were found to be 122.68 µg/ml and 87.72 µg/mL, respectively.The scavenging ability of ABTS·radical for MESM and standard antioxidant compounds were found to be profound and concentration dependent. The calculated IC50 values of the MESM and quercetin were found to be 64.48 µg/mL and 27.03 µg/mL, respectively (**Figure-2B**).

Phenolic compounds particularly flavonoids and catechins are found to be important antioxidants and superoxide scavengers. The scavenging efficiency of these compounds mainly depends on the concentration of phenol and the numbers and locations of the hydroxyl groups.(Ashokkumar, et al., 2008a, Erasto, et al., 2007b). Superoxide anion is a highly toxic species and generated by different biological reactions in the physiological system. The present study revealed the decrease in absorbance at 590 nm with antioxidants samples which confirmed the consumption of superoxide anion in the reaction mixture. With the increasing concentration of MESM and the standard compound, the percentage inhibitions of superoxide radical generation were found to be increased. Salvia miltiorrhiza demonstrated significant superoxide radical scavenging activity with lower activity than the ascorbic acid. The IC50 values of MESM and ascorbic acid were found to be 161.75 µg/mL and 88.07 µg/mL, respectively (**Figure-2C**).

Nitric oxide or reactive nitrogen species (ROS) are very reactive compounds which can change pathologically the structural and functional behavior of many cellular components.(Ashokkumar, Thamilselvan, GP, Mazumder and Gupta, 2008a) The reduction of linear time-dependent nitrite production in the sodium nitroprusside–PBS system was investigated for evaluating the scavenging of nitric oxide by MESM. The nitric oxide scavenging activity of MESM and standard compound were found to be concentration depen dent and also demonstrated lower scavenging activity of MESM than the ascorbic acid. The percentage inhibitions of MESM and standard compound were found to be 63.81 ± 0.19 and 73.96 ±0.40% at the concentration of 250 µg/mL, respectively. Upon linear regression analysis of the data, the IC50 values, calculated were found to be 174.07 µg/mL and 130.53 µg/mL for Salvia miltiorrhiza and ascorbic acid, respectively (**Figure-2D**).

Hydrogen peroxide gives rise to hydroxyl radicals. Removing hydroxyl radicals (˙OH) is very essential for the protection of living system as they react with mostbiomolecules and other cellular components to cause tissue damage leading to cell death. (Dhuley, et al., 1993, Reddy, et al., 2010) Different concentrations of MESM (50 – 250 µg/mL) were evaluated for their H2O2 scavenging ability. Salvia miltiorrhizade mons trated potential H2O2 scavenging activity as compared to standard ascorbic acid. The percentage H2O2 scavenging activity at a concentration of 250 µg/mL of MESM and ascorbic acid were obtained as 79.63 ± 0.3308 % and 83.95 ± 0.2464 %, respectively. The IC50 values were calculated as 133.83 µg/mL and 120.15 µg/mL for Salvia miltiorrhiza and ascorbic acid, respectively (**Figure-2E**). Hydroxyl radicals are considered as highly reactive short-lived entity. However, they are an integral part of reactive oxygen species as well as radical biology. Most notably hydroxyl radicals are generated upon decomposition of hydro-peroxides (ROHO). As a very reactive species, hydroxyl radical attacks almost every molecule in the physiological system and initializes the process of peroxi dation of cell membrane lipids yielding malondialdehyde, which is a hallmark sign of cell damage. In vitro, MESM demonstrated hydroxyl radical scavenging activity in a concentrationdependent manner (50 – 250 µg/mL). The IC50 values were found to be 116.21 µg/Ml and 107.96 µg/mL for MESM and ascorbic acid respectively **(Figure-2F)**.

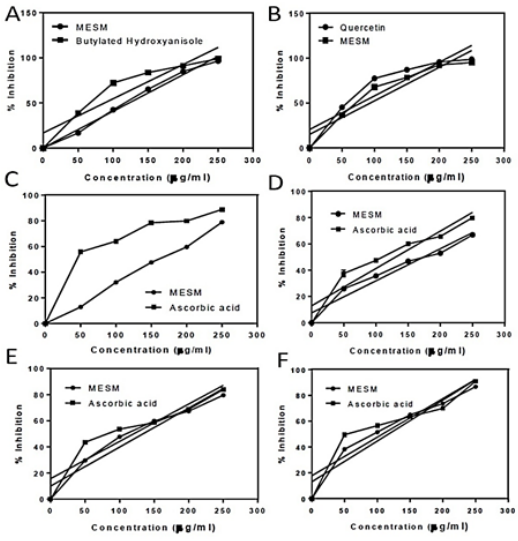

Figure-2: In-vitro Antioxidant activity. (A)Effect on DPPH radical scavenging activity, (B) ABTS radical decolorization assay, (C) Effect on Superoxide anion scavenging activity, (D) Effect on Nitric oxide scavenging activity, (E) Effect on Hydrogen peroxide scavenging activity and (F) Hydroxyl radical scavenging activity.

The method of determination of metal chelating ability is based on chelation of Fe2+ ions by the ferrozine reagent.(Dinis, Madeira and Almeida, 1994, Kumaran and Joel Karunakaran, 2006) A complex with Fe2+ ions is formed in the reaction which gives absorbance. This formation of the complex is disturbed in the presence of other agents with metal chelating property and absorbance decreases with the

reduction of formation of red coloured complex. Measurement of the rate of reduction of the colour, therefore allows estimation of the chelating activity of the co-existing chelator. The reaction of ferrous complex formation with ferrozine reagent is key to the assay of metal chelating activity.In this present setup, the reaction of ferrous complex formation with ferrozine reagent was interfered by both MESM and EDTA, the standard compound. The metal chelating activity of MESM was found to be concentration dependent. The percentage metal chelating activity was found to be increased with increasing concentration of MESM and EDTA. The IC50 values werecalculated from linear regression analysis and found as 408.50 μg/mL and 122.65 μg/mL for MESM and EDTA, respectively (**Figure-3**).

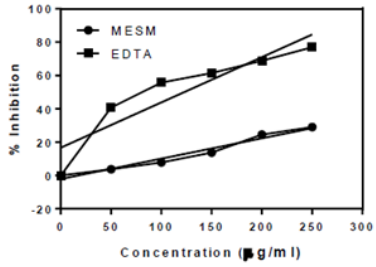

Figure-3. Metal chelating activity

CONCLUSION

Based upon the results from different in vitro antioxidants models it is evident that MESM has an effective and considerable antioxidant profile. The possible mechanism of action for these different antioxidant activities includes hydrogen-donating ability, reducing ability, scavenging ability of superoxide anion radical, nitric oxide, hydrogen peroxide, DPPH radical and chelating ability of metals. The presence of different phytochemicals or phytoconstituents in MESM such as flavonoids, tannins and phenolic compounds may be responsible for the antioxidant mechanisms. The preliminary phytochemical investigation also suggested the above mentioned phytochemicals. We have already undertaken a further investigation on the isolation and identification of antioxidant compounds in this plant, which may lead to new chemical leads with potential for clinical use and further investigation.

DECLARATION OF INTEREST

None Declared

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