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# Antioxidant, In-Vitro Anti-Inflammatory and Anti-Alzheimer Activities of Methanolic Fraction of *Salvia Miltiorrhiza* Flower

**Lokraj Subedee[1], Binod Kumar Yadav[2], Rohit Sarawat[3]**

[1]PhD Scholar, Department of Pharmacology, OPJS University Churu- Rajasthan, 331303 India

[2]Lecturer, Peoples Dental College and Hospital, Kathmandu, Nepal

[3]Professor, Department of Pharmacology, OPJS University Churu- Rajasthan, 331303 India

**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

## 1. 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 seekpotent, 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, anti-inflammatory and anti-Alzheimer potential of Salvia miltiorrhiza by in vitro method. Therefore, the present study aimed to investigate the anti-oxidant, anti-inflammatory and anti-Alzheimer potential of Salvia miltiorrhiza by various reliable and effective in vitro protocols.

## 2. 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, Hexadecyltrimethylammonium bromide (HTAB), O-dianisidine and 1, 1, 3, 3-Tetramethoxypropane were


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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 phytochemicals screening for the detection of various phytoconstituents 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 µg/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 µg 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


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absorption at 230 nm with molar absorbtivity 81 $molL^{-1}cm^{-1}$.The plant extract (50-250 µg/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 µg/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 (50-250µg/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 (Ethylenediaminetetraacetic acid) was used as standard chelating compound.

**In-Vitro Anti-inflammatory activity**

***Membrane stabilization***

***Preparation of Red Blood cells (RBCs) suspension***

The Blood was collected from healthy human volunteer who has not taken any NSAIDs (Non Steroidal Anti-Inflammatory Drugs) for 2 weeks prior to the experiment and transferred to the centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10min and were washed three times with equal volume of normal saline. The volume of blood was measured and re constituted as 10% v/v suspension with normal saline.

***Heat induced haemolysis***

The reaction mixture (2ml) consisted of 1 ml test sample of different concentrations (100 - 500 µg/ml) and 1 ml of 10% RBCs suspension, instead of test sample only saline was added to the control test tube. Aspirin was used as a standard drug. All the centrifuge tubes containing reaction mixture were incubated in water bath at 56 ºC for30min. At the end of the incubation the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants was taken at 560 nm (19). The experiment was performed in triplicates for all the test samples. The Percentage inhibition of Haemolysis was calculated as follows:

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

***Hypotonicity-induced haemolysis***

Different concentration of extract (100-500µg/ml), reference sample, and control wereseparately mixed with 1ml of phosphate buffer, 2ml of hyposaline and 0.5ml of HRBC suspension. Diclofenac sodium (100µg/ml) was used as a standard drug. All the assay mixtures were incubated at 370c for 30minutes and centrifuged at 3000rpm. The supernatant liquid was decanted and the haemoglobin content was estimated by a spectrophotometer at 560nm (19). The percentage hemolysis was estimated by assuming the haemolysis produced in the control as 100%.

% protection = 100- (OD sample/OD control) x 100

***Anti-lipoxygenase activity***

Anti-Lipoxygenase activity was studied using linoleic acid as substrate and lipoxidase as enzyme. Test samples were dissolved in 0.25ml of 2M borate buffer pH 9.0 and added 0.25ml of lipoxidase enzyme solution (20,000U/ml) and incubated for 5 min at $25^0$C. After which, 1.0ml of linoleic acid solution (0.6mM) was added, mixed well and absorbance was measured at 234nm. Indomethacin was used as reference standard (19). The percent inhibition was calculated from the following equation,

% inhibition= [{Abs control- Abs sample}/Abs control] x 100

A dose response curve was plotted to determine the IC50 values. IC50 is defined as the concentration sufficient to obtain 50% of a maximum scavenging capacity. All tests and analyses were run in triplicate and averaged.

**In-vitro Anti-Alzheimer activity**

***Determination of AChE Inhibitory activities***

The AChE inhibitory activity was preformed according to the colorimetric method of Ellman's method using acetylcholine iodide as a substrate. For enzyme source, bovine brain were homonized with 10 volumes of extraction buffer [50mM Tris-HCl (pH 7.4) which contain 50M NaCl, 50mM MgCl2 and 1 % Triton X-100] and centrifuged at 10000 rpm for 30 minute. The resulting supernatant collected and treated with super saturated ammonium sulfate solution, left for few hours at $4^o$C to complete precipitation of proteins and again centrifuged at 12000 rpm for 20 minutes. The resulting precipitation was solubilized with extraction buffer. This solution used as an enzyme source. AChE inhibitory assay was carried out by modified Ellman's method. For positive control 200µl enzyme solution, fruit extract and extraction buffer incubated for 2 hours at room temperature 200µl (20).

***Cell culture maintenance and harvesting***

SH-SY5Y neuroblastoma cells (ATCC no. CRL-2266) were purchased from the American Type Culture Collection and


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cultured in 75 cm3 culture flasks at 37°C under an atmosphere of 5% CO2 and humidified air. Cells were grown in Ham's F12 medium, supplemented with 10% heat-inactivated foetal calf serum (FCS) and 1% penicillinstreptomycin. The medium was replaced every 2-3 days, as required. Once cells reached a confluency of ≈ 80%, medium was discarded and cells were washed with phosphate buffered saline (PBS). Cells were detached using a 0.125% Trypsin/Versenesolution and harvested by centrifugation at 200 × g for 5 min. Cells were resuspended in 1 ml of medium and viable cells were counted using trypan blue (0.4% w/v in PBS).

***Cytotoxicity and effects on rotenone-induced cytotoxicity***

Cells (100 µL, 1 × 105 cells/ml) were pre-seeded into 96-well plates followed by the addition of 80 µL of 2% FCS supplemented medium and incubated for 24 h (21). To determine the cytotoxicity profiles of the extract or rotenone alone, a volume of 20 µL of either plant extract (final exposure concentrations of 0.78-100 µg/ml) or rotenone (final exposure concentrations of 0.128 nM-50 µM) was added to the plates and incubated for 72 h, after which the SRB assay was performed. Vehicle controls were exposed to 0.05% (v/v) DMSO in culture medium and wells containing culture medium only served as blanks. To assess the effects of the plant extracts on rotenone-induced cytotoxicity, cells were pre-treated for one hour with four non-toxic concentrations of each of the plant extracts prior to being exposed to rotenone for 72 h at concentrations of 10 nM, 50 nM and 100 nM. Minocycline (10 µM), which is known to counteract rotenone toxicity (Faust et al., 2009), was used as treatment positive control throughout the study. All subsequent experiments evaluating mechanistic parameters utilized a rotenone concentration of 50 Nm.

After the 72 h exposure period, 100 µL of the supernatant was aspirated from the wells and replaced with 100 µL of cold trichloroacetic acid (TCA) solution (30% w/v). Each plate was then incubated at 4°C for 1 h to fix cells to the plate. After incubation, the plate was gently washed with water (four times) to remove excess TCA. The plate was dried in a low-temperature oven, after which 100 µL of 0.057% (w/v) SRB solution was added to wells to stain thecellular protein contents. The plate was incubated for 30 min at 4°C and washed twice with 200 µL of a 1% acetic acid solution (v/v) to remove excess unbound dye. The platewas allowed to dry, after which the bound dye was dissociated using 200 µL of a 10mM Tris base solution (pH 10.5). Absorbance was measured at 540 nm with a reference wavelength of 630 nm, using a Biotek ELx 800UV Universal plate reader. Preliminary experiments indicated that rotenone induced approximately 50% cell death at a concentration of 50 nM. For this reason rotenone was used at a concentration of 50 nM for all subsequent mechanistic studies (21).

***Intracellular reactive oxygen species***

Briefly, following 24 h exposure to the extract, 20 µL of 2', 7'-dichlorodihydrofluorescein diacetate (H2DCF-DA; 20 µM) in PBS solution was added to the relevant wells and the plates incubated for 30 min at 37ºC. Plates were then washed once with 100 µL of PBS to remove excess H2DCF-DA solution. An additional 100 µL of PBS was added to each well and the fluorescence intensity measured using a BMG Fluostar Optima fluorescent plate reader set at excitation and emission wavelengths of 492 nm and 525 nm, respectively. The values are expressed as the mean absorbance normalized to a percentage of the untreated control value (22). An established ROS-inducing agent, 2, 2'-azobis-2-methyl-propanimidamide dihydrochloride (AAPH) (150 µM), was included as an additional positive control to ensure that the assay produced expected results.

***Intracellular glutathione levels***

Following 24 h exposure to the relevant treatment, 20 µL of monochlorobimane (40 µM) in PBS solution was added to all the wells. The plates wereincubated for 2 h at 37°C, followed by a 100 µL PBS washing step. After the additionof 100 µl of PBS, fluorescence intensity was recorded using a BMG Fluostar Optima setat excitation and emission wavelengths of 360 nm and 460 nm, respectively. The glutathione depleting agent, N-ethylmaleimide (NEM) (10 µM), was used as an additional positive control to ensure that the assay produced expected results (23).

***Mitochondrial membrane potential***

Briefly, after treatment of cells for 24 h, 100 µL of the supernatant was discarded and 20 µL of 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1; 10 µM) in PBS was added before plates were incubated for 30 min at 37°C and 5% CO2 in the dark. Excess dye was washed off using 100 µL PBS and an additional 100 µL PBS was added for fluorescence measurements. Fluorescence intensity was measured using a BMG Fluostar Optima fluorescence microplate reader set at excitation wavelengths of 492 nm and 520 nm, and emission wavelengths of 544 nm and 590 nm for the monomeric and aggregate forms of JC-1, respectively. The ratio of the fluorescence intensities at 590 nm (J-aggregates) / 520 nm (J-monomers) was used as an indication of MMP. The mitochondrial uncoupler, valinomycin (20 µM), was used as an additional positive control to ensure that the assay produced expected results (24).

***Apoptosis***

Briefly, following exposure, cells were lysed with 25 µL of a cold lysis buffer [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mM ethylenediaminetetraacetic acid (EDTA), 5 mM3-[(3-cholamidopropyl) dimethylammonio]-1propanesulfonate (CHAPS), 5 mM betamercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] and kept on ice for30 min. Thereafter, 100 µL of reaction buffer (20 mM HEPES, 2 mM EDTA, 5 mM β-mercaptoethanol, 0.5 mM PMSF, 10 µM of a 7-amino-4-methylcoumarin-coupled caspase-3 substrate) was added to wells followed by an overnight incubation at 37ºC. Fluorescence intensity was measured using a BMG Fluostar Optima set at excitation and emission wavelengths of 360 nm and 460 nm, respectively. Staurosporine (11 µM), a general apoptosis inducer, was used as positive control to ensure that the assay produced expected results (24).

**Statistical analyses**

For cell viability, the concentration that produces 50% cell death (LC50) was calculated by fitting a four-parameter Hill


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equation to the observed results. Two constraints (top = 100; bottom = 0) and a variable slope were used for fitting the non-linear model. Calculated LC50 values are expressed as the mean ± the standard error of the mean (SD). All experiments were carried out in triplicate on three separate occasions. Background signals (blanks) were deducted in all experiments. Depending on the normality of the data, either Mann–Whitney or Student's t-tests were performed to test for significant differences between the means of the various groups. Results for endpoints assays were normalized to a percentage of the mean of vehicle controls and are presented as mean ± SD. Significant differences from vehicle controls are indicated by p value < 0.05. Significant differences between treatment groups and rotenone treatment alone are indicated by p value < 0.05. GraphPad Prism 6.0 was used for all statistical manipulations.

## 3. 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 dependent 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 miltiorrhizademonstrated 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


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of hydro-peroxides (ROHO). As a very reactive species, hydroxyl radical attacks almost every molecule in the physiological system and initializes the process of peroxidation of cell membrane lipids yielding malondialdehyde, which is a hallmark sign of cell damage.

In vitro, MESM demonstrated hydroxyl radical scavenging activity in a concentration dependent 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)**.

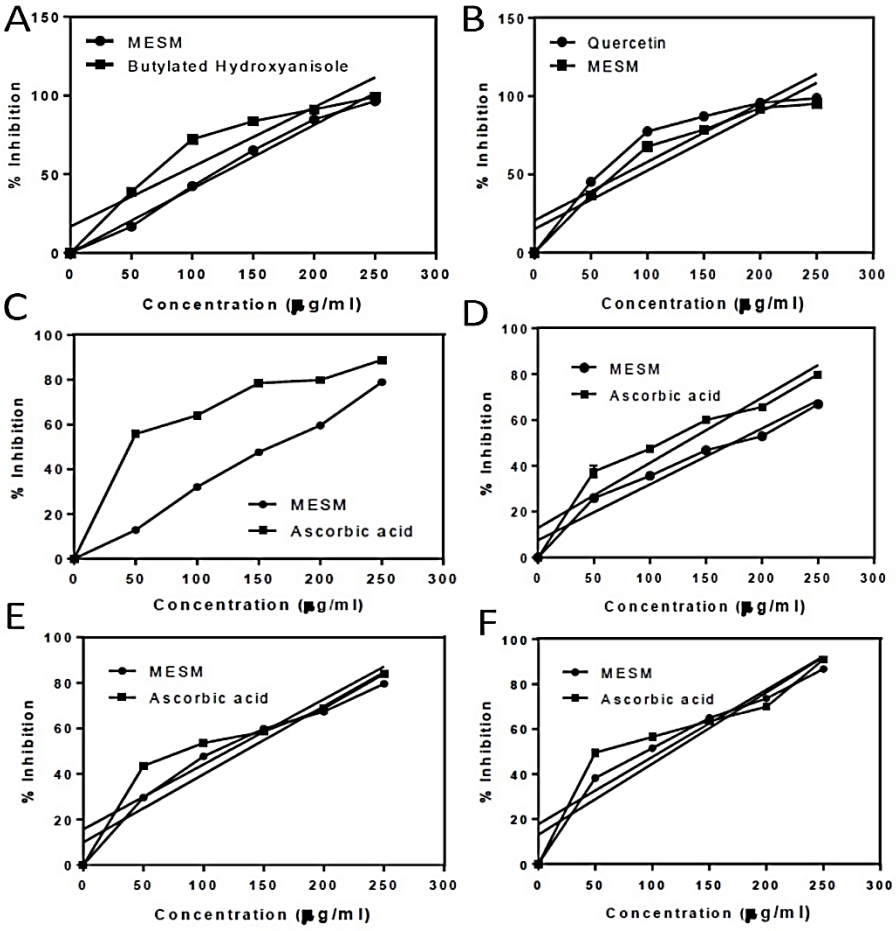


**Figure-2:** In-vitro Antioxidant activity. (A)Effect on DPPH radical scavenging activity, (B) ABTS radical decolorization assay, (C) Effect on Superoxide anion scavenging activity, (D) Effect on Nitric oxide scavenging activity, (E) Effect on Hydrogen peroxide scavenging activity and (F) Hydroxyl radical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

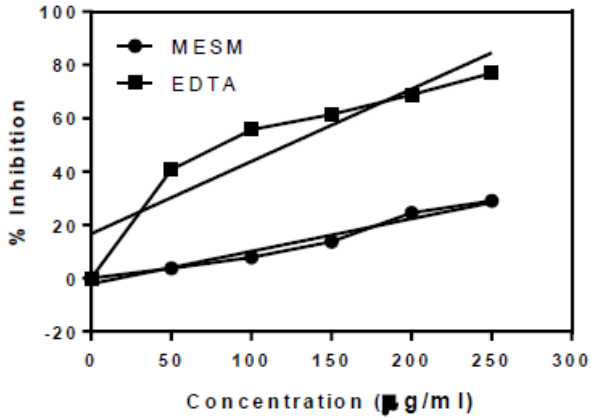


**Figure 3:** Metal chelating activity

**In vitro anti-inflammatory activity**

***Membrane stabilization***

The HRBC membrane stabilization has been used as a method to study the in vitro anti-inflammatory activity because the erythrocyte membrane is analogous to the


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lysosomal membrane and its stabilization implies that the extract may well stabilize lysosomal membranes. Stabilization of lysosomal is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil, such as bacterial enzymes and proteases, which causes further tissue inflammation anddamage upon extra cellular release. The lysosomal enzymes released during inflammation produce a various disorders. The extra cellular activity of these enzymesare said to be related to acute or chronic inflammation. The non-steroidal drugs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane.

***Heat induced haemolysis***

The extract MESM was effective in inhibiting the heat induced haemolysis at different concentrations. The results showed that MESM at concentration 400 and 500 µg/ml protect significantly ($p<0.05$) the erythrocyte membrane against lysis induced by heat (**Table-1**). Aspirin 100µg/ml offered a significant ($p<0.01$) protection against damaging effect of heatsolution.

**Table 1:** Effect of MESM on heat induced haemolysis of erythrocyte

| Treatment | Concentration (µg/ml) | Absorbance at 660 nm | % Inhibition of haemolysis |
|---|---|---|---|
| Control | - | 0.35 ± 0.02 | - |
| MESM | 100 | 0.29 ± 0.03 | 17.14 |
| MESM | 200 | 0.26 ± 0.04 | 25.71 |
| MESM | 300 | 0.24 ± 0.04 | 31.43 |
| MESM | 400 | 0.22 ± 0.03 | 37.14 |
| MESM | 500 | 0.20 ± 0.05 | 42.86 |
| Aspirin | 100 | 0.14 ± 0.05 | 60.00 |

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

***Hypotonicity-induced haemolysis***

The results showed that MESM at concentration range of 200-500µg/ml protect significantly ($p<0.01$) the erythrocyte membrane against lysis induced by hypotonic solution (**Table-2**). Diclofenac sodium (100µg/ml) offered a significant ($p<0.01$) protection against the damaging effect of hypotonic solution. At the concentration of 500µg/ml, MESM showed maximum of 66.67 % protection, whereas, Diclofenac sodium (100µg/ml) showed 44.44% inhibition of RBC haemolysis when compared with control.

**Table 2:** Effect of MESM on hypotonicity induced haemolysis of erythrocyte

| Treatment | Concentration (µg/ml) | Absorbance at 660 nm | % Inhibition of haemolysis |
|---|---|---|---|
| Control | - | 0.36 ± 0.03 | - |
| MESM | 100 | 0.27 ± 0.01 | 25 |
| MESM | 200 | 0.21 ± 0.03 | 41.67 |
| MESM | 300 | 0.19 ± 0.04 | 47.22 |
| MESM | 400 | 0.17 ± 0.05 | 52.78 |
| MESM | 500 | 0.12 ± 0.04 | 66.67 |
| Aspirin | 100 | 0.20 ± 0.02 | 44.44 |

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

***Anti-lipoxygenase activity***

The plant lipoxygenase pathway is in many respects equivalent to the 'arachidonic acid cascades' in animals. For thisreason, the in vitro inhibition of lipoxygenase constitutes a good model for thescreening of plants with anti-inflammatory potential. LOXs are sensitive to antioxidants and the most of their action may consist in inhibition of lipid hydroperoxide formation due to scavenging of lipidoxy or lipid peroxy- radical formed in course of enzyme peroxidation. This can limit the availability of lipid hydroperoxide substrate necessary for the catalytic cycle of LOX. MESM has been checked at 100,200,300,400, 500µg/ml, it showed 8.89, 15.56, 22.22, 42.22, 55.56% antilipoxygenase inhibition respectively. From these result, the strongest inhibition was obtained at concentration 500µg/ml. The standard Indomethacin showed a 75.56% inhibition at a concentration of 100µg/ml. At the concentration of 100 and 200µg/ml, MESM not showed significant difference ($p>0.05$) when compared with control. The results obtained from our studies on MESM have shown a potential anti-inflammatory activity. The MESM extracts inhibited the lipoxygenase enzyme activity. This indicates that plant MESM is more useful in studies of inflammation and in various related physiological studies, aging and diseases such as cancer, neurological disorder etc.

**In-vitro Anti-Alzheimer activity**

***Determination of AChE Inhibitory activities***

Extract (MESM) of Salvia miltiorrhiza shows AChE inhibitory activity. Reduction of ACh in the CNS is a major characteristic of AD. Therefore, inhibition of AChE level is an important strategy to treat AD. The inhibitory activity of MESM was determined by Ellman's method. This method based on the reaction between acetylthiocholine iodide, DINB, MESM and the enzyme solution. The enzymatic activity measured spectrophotomatrically, when yellow color complex produced by the decline byreaction of DINB ion. MESM was found to inhibit 65.21% of AChE at a concentration of 100 ug/mL, which indicated it was a potential source of cholinergic inhibitor.

***Cytotoxicity and effects on rotenone-induced cytotoxicity***

The effect of MESM on viability, when toxicity was induced with 50 nM rotenone, was less pronounced than that observed at 10 nM, but greater than that at 100 nM (**Figure-11**). MESM did not produce protective effect against toxicity induced with 100 Nm rotenone.MESM was found to contain polyphenolic compounds such as flavonoids, tannins, and sterols as suggested by preliminary phytochemical screening. The protective effect of MESM might be due to the presence of these polyphenols. Phytochemical screening of MESM revealed the presence of alkaloids and glycosides such as saponins. These compounds are known for their cytotoxic properties, which may have been enhanced at high (100 nM) rotenone concentrations. MESM was observed to exert protective effects against rotenone-induced toxicity in a dose-dependent manner


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(**Figures-11**). At 3.125 and 6.25 µg/mL MESM was protective against toxicity induced with 10 nM of rntenone (**Figures 11**). A decrease in extract concentrations resulted in increased cell viability at 10 nM of rotenone (Figures 11B). The protective effect of this extract was observed at 3.125 and 6.25 µg/mL when toxicity was induced with 10 nM rotenone (**Figures-11**).Low concentrations of MESM offered protective effects in SH-SY5Y cells exposed to low concentrations of rotenone. Furthermore, the cytotoxic effect produced by MESM was comparable to that of rotenone control (**Figures-11**).

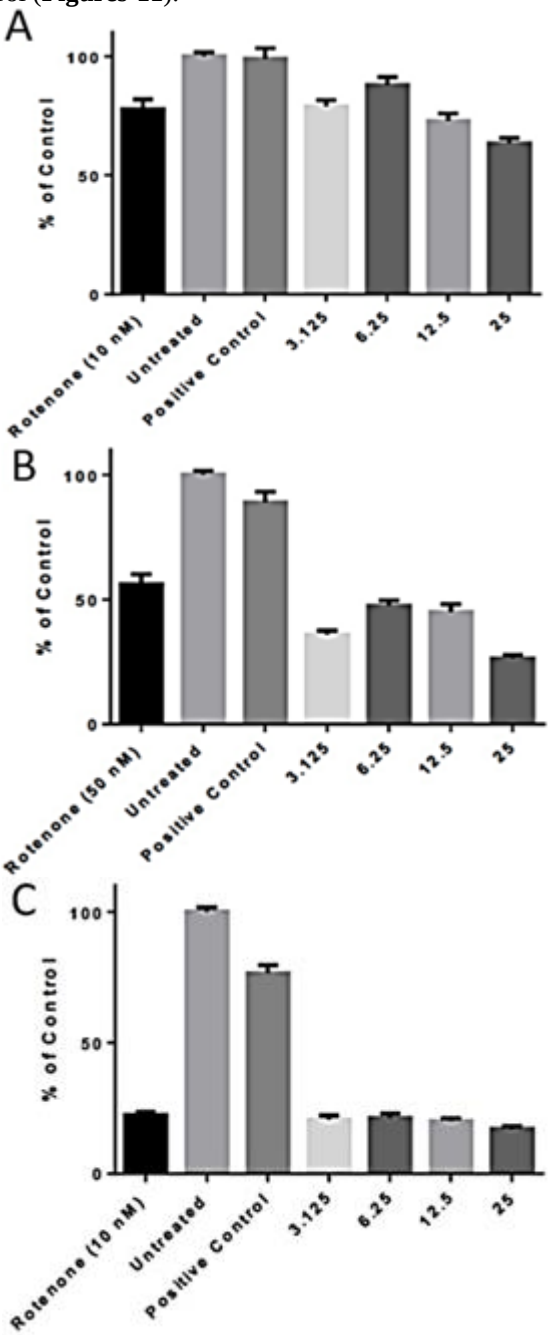


**Figure 4:** Effect of MESM on the survival of SH-SY5Y cells after 72 h exposure to rotenone at A) 10 nM, B) 50 nM and C) 100 nM, using the sulforhodamine B assay. Significant differences from the rotenone control are indicated by * representing p value < 0.05, while significant differences from untreated cells are indicated by •, also representing p value < 0.05. PC = positive control (minocycline at 10 µM).

***Intracellular reactive oxygen species***

Mitochondrial ETC complex I is the main site of ROS production from mitochondria and ROS production can be enhanced by a defective mitochondrial ETC complex. Rotenone, a specific mitochondrial ETC complex I inhibitor, caused no intracellular ROS production in the present study. In contrast, ROS generation has been reported after treatment of both undifferentiated and differentiated human stem cells with 8 µM rotenone after a 24 h exposure period. The rotenone concentration (50 nM) used in the present study, might have not been high enough to achieve similar results, regardless of the exposure time.

A significant ($p < 0.05$) intracellular ROS production was observed in cells exposed to AAPH, when compared to the untreated cells, indicating that the assay performed as expected (Figure 12). Compared to the untreated cells, there was no intracellular ROS production in cells exposed to rotenone alone for 24 h. Rather, rotenone exposure resulted in significant ($p < 0.05$) decreased intracellular levels of ROS. Minocycline, at a concentration of 10 µM, was able to counteract the effects of ROS after 24 h of exposure. However, minocycline caused a significant ($p < 0.05$) increase in intracellular ROS levels, when compared to the untreated cells, at 24 h exposure time (**Figure-5**). MESM countered the decrease in intracellular ROS caused by rotenone after 24 h exposure. Methanol extracts were reported to be more potent at decreasing intracellular ROS levels than other extracts, presently MESM showed potent decrease in intracellular ROS levels (**Figures-5**).The authors demonstrated that the generation of ROS from activated microglia induced by rotenone, was mediated by NADPH oxidase. The latter is a membrane bound enzyme complex that generates superoxide radicals by transferring electrons from NADPH inside the cells to oxygen, across the membrane. Molina-Jimenez et al. (2003) showed rotenone-induced ROS production in SH-SY5Y cells after 16 h treatment with 5 µM rotenone. It would appear as if rotenone-induced ROS generation in vitro may be influenced by the type of cell line used, concentration of rotenone used and time of exposure to rotenone.

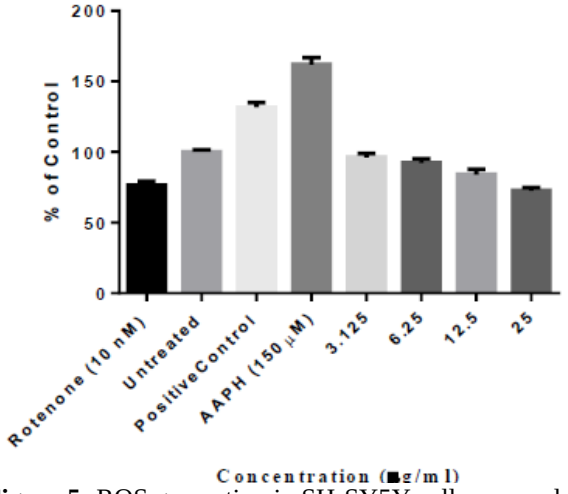


**Figure 5:** ROS generation in SH-SY5Y cells exposed to rotenone at 50 nM after pretreatmentwith MESM following 24 h exposure period


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***Intracellular glutathione levels***

Antioxidants are known to counteract the deleterious actions of ROS by scavenging, detoxifying and suppressing ROS formation, thus preventing cell damage. An increase in ROS production can result in depletion of the antioxidant status of the cell. Reduced glutathione acts as an antioxidant in dopaminergic cells and aids in combating ROS induced cellular damage, thus protecting the cell against the deleterious effects of ROS. In the present study no ROS generation was observed in cells treated with rotenone alone, when compared to the untreated cells (**Figure-7**). However, reduced intracellularglutathione content was observed in cells treated with rotenone alone and this effect was counteracted by minocycline (10 µM), which increased intracellular glutathione content (**Figure-7**).

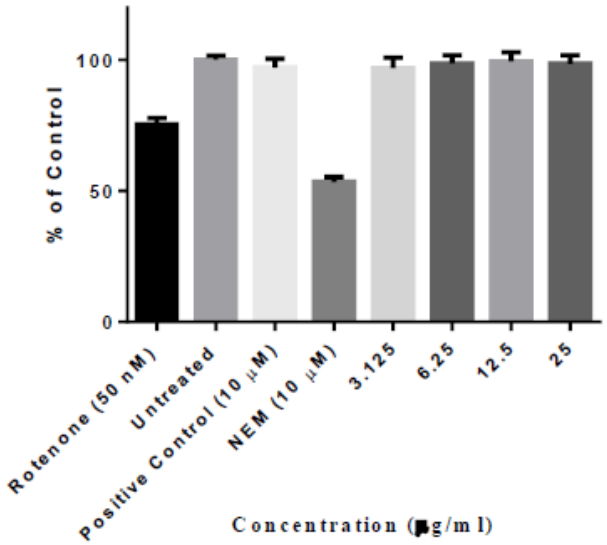


**Figure 7:** Intracellular glutathione content in SH-SY5Y cells exposed to rotenone at 50 nM after pretreatment with MESM following 24 h exposure period

The positive control, N-ethylmaleimide (NEM) (10 µM), which decreases intracellular glutathione by conjugation, was observed to decrease intracellular glutathione content, indicating that the assay performed as expected (**Figure-7**). MESM increased intracellular glutathione content more effectively (**Figure-7**). MESM resulted in intracellular glutathione content comparable to that observed in the untreated cells (**Figure-7**). MESM produced a response comparable to that of minocycline at 6.25 and 12.5 µg/ml concentrations (Figure 13). It is also possible that the studied plant extract (MESM) may contain precursors of glutathione like cysteine or could stimulate the synthesis of glutathione.

**Mitochondrial membrane potential**

In vitro models used to study mitochondrial dysfunction have been developed and provide insights into the biochemical pathways involved in disorders of the like. In the present study, rotenone was used to induce mitochondrial dysfunction by uncoupling the MMP in the SH-SY5Y cells. Compared to the untreated controls, cells exposed to rotenone alone showed a significant ($p < 0.05$) reduction in the MMP (**Figure-8**). Minocycline (10 µM) was not able to counteract the effect observed in cells exposed to rotenone alone (**Figure-8**). Valinomycin (20 µM) caused a significant ($p < 0.05$) reduction in the MMP, compared to the untreated cells, indicating that the assay performed as expected (**Figure-8**). MMP levels were observed to be more effectively reduced by MESM (**Figure-8**). Therefore, methanol did extract compounds that enhanced MMP reduction in SH-SY5Y cells. Compared to rotenone, MESM significantly ($p < 0.05$) reduced MMP levels at all extract concentrations tested. MMP is critical for cell survival and homeostasis. Rotenone is known to inhibit the function of mitochondrial complex I, reducing ATP production from the ETC. Reduced ATP production depolarizes the inner mitochondrial membrane, causing the MMP to dissipate. Moreover, formation of an irreversible MPT pores on the inner mitochondrial membrane allows influx and efflux of ions and other solutes resulting in further MMP depolarization. Furthermore, loss of glutathione and increased intracellular cytosolic calcium can occur. All of these events ultimately contribute to apoptotic cell death. Therapeutic agents that can inhibit MPTpore formation would be beneficial in preventing MMP reduction and reduction in ATP production, thus suppressing cell death and promoting cell survival.

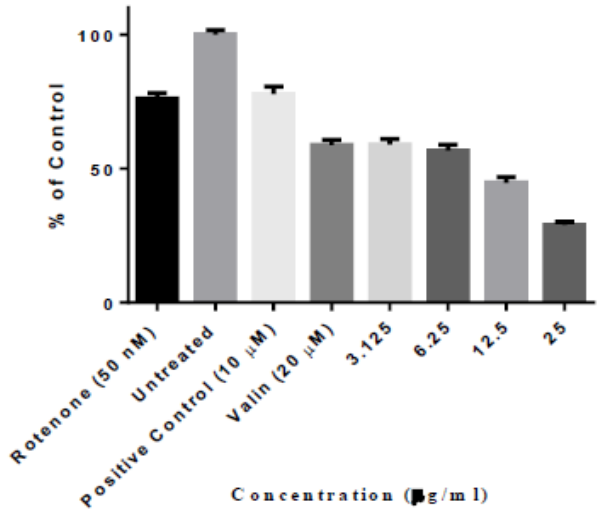


**Figure 8:** Mitochondrial membrane potential (MMP) in SH-SY5Y cells exposed torotenone at 50 nM after pre-treatment with MESM following 24 h exposure period

The plant extracts did not prevent/reduce the MMP uncoupling effect of rotenone **(Figure-8).** This may be due to the irreversible formation of MPT pore that is reported to occur within 20 min of the rotenone apoptogenic effect, once mitochondrial dysfunction results. All of these events commit a cell to apoptotic cell death. Therefore, the time of rotenone exposure in cells is very critical in studying mitochondrial dysfunction. Moreover, MMP is not a static parameter. It is dynamic and fluctuates with the respiratory needs of the cell. If a cell requires more energy (high ATP utilization) the MMP will decrease as ATP production increases. If less energy is required, the opposite will happen. The fact that the plant extracts further decreased MMP may indicate that the extracts increased ATP utilization.

**Apoptosis**

Staurosporine, a general apoptosis inducer, caused a significant ($p < 0.05$) increase in caspase-3 activity,


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indicating that the assay performed as expected (**Figure-9**). Rotenone also up-regulated caspase-3 activity, when compared to the untreated controls. This action was counteracted by minocycline, which reduced caspase-3 activity (**Figure-9**). MESM was observed to significantly ($p< 0.05$) reduce rotenone induced caspase-3 activity at all extract concentrations tested (**Figure-9**). MESM was observed to reduce caspase-3 activity more efficiently. MESM decreased caspase-3 activity in a dose-dependent manner (**Figure-9**).

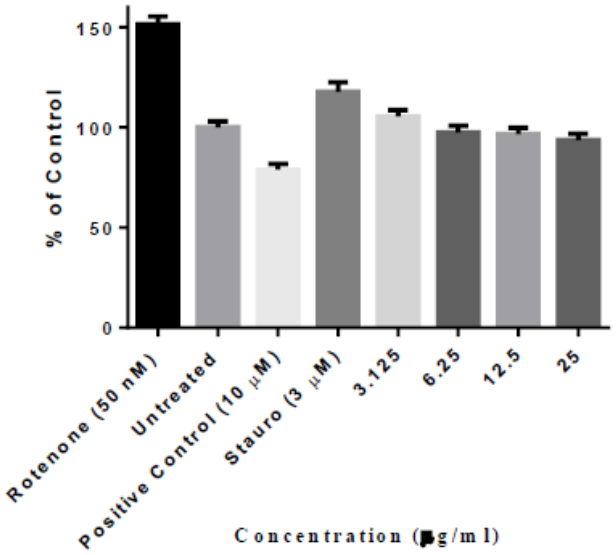


**Figure 15:** Caspase-3 activity in SH-SY5Y cells exposed to rotenone at 50 nM after pre-treatment with MESM following 24 h exposure period.

## 4. 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. The extract clearly demonstrated anti-inflammatory activities in terms of protein denaturation, Antiproteinase action and Membrane stabilization. MESM also revealed potential therapeutic activity in alzheimers's disease in vitro models in terms of AChE Inhibitory activities. MESM also has been indicated as significantly protective in cell line studies involving SH-SY5Y neuroblastoma cells.

Rotenone produced cytotoxic effects on the SH-SY5Y cells. This effect was counteracted by MESM. The neuroprotective effect of MESM may be attributed to their anti-apoptotic effect. The latter resulted due to inhibition/suppression of caspase-3 activity thus promoting cell survival. Moreover the pesticide reduced intracellular glutathione content, depolarization and up-regulated caspase-3 activity in the SH-SY5Y cells. MESM depolarized MMP further. MMP depolarization is known to correlate with increased cellular energy demands. During cellular respiration, some electrons leak out of the ETC and are converted into free radicals that can enhance oxidative stress. MESM in the present study, preserved the intracellular glutathione content and inhibited caspase-3 activity potently. Suppression of caspase-3 activity by the extracts may be attributed to the inhibition of the MTP pore formation which in turn resulted in the prevention of intracellular glutathione leakage out of cells, thus preserving the cells.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.

## 5. Declaration of Interest

None Declared

## 6. Acknowledgement

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