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Original Article

Evaluation of Antioxidant and Immunomodulatory Potential of Methanolic Extract of *Juniperus squamata* Buch.-Ham. ex D.Don

Rohaya Ali[1], Nasreena Sajjad[1], Sabia Qureshi[2], Sumaya Hassan[3], Muzaffar Ahmad[1], Showkat Ahmad Ganie[3], Rabia Hamid[1*]

ABSTRACT

Background: Antioxidant plays a vital role in scavenging of free radicals, thus, providing protection against oxidative stress. Now the modern research is directed towards the discovery of herbal antioxidants and immnomodulators due to their lesser side effects. **Objective:** The aim of the present study is to evaluate the antioxidant and immunomodulatory potential of methanolic extract of *Juniperus squamata* from the Apharwat region of Gulmarg. **Materials and method:** The antioxidant activity of methanol extract of *Juniperus squamata* was evaluated by using 1, 1-diphenyl, 2-picrylhydrazyl (DPPH) scavenging, reducing power, hydroxyl radical scavenging, hydrogen peroxide scavenging activity and lipid peroxidation. Furthermore, the immunomodulatory potential of the extracts was investigated through the Delayed type hypersensitivity and phagocytic carbon clearance assay. **Results:** The highest phenolic content of 780 GAE/g (Total phenolic content) was observed in the methanolic extract, while the lowest Total phenolic content of 459 mg GAE/g was achieved in the petroleum ether extract. At a concentration of 700 g/mL, DPPH radical scavenging activity was found to be highest in methanolic extract (82.12%). Methanol extract was found to be an efficient scavenger of hydrogen peroxide radical and lipid peroxidation with IC_{50} values of 161.44 ± 0.08 µg/mL and 78.65 µg/mL respectively. Administration of plant extract (100 and 200 mg/kg body weight) increased the DTH response and phagocytic carbon clearance significantly. **Conclusion:** The results indicate that the methanolic leaf extract of *Juniperus squamata* has good antioxidant and immunomodulatory potential.

Key words: Carbon clearance, Delayed hypersensitivity, Fenton reaction, *In vitro* antioxidant, Lipid peroxidation.

Rohaya Ali[1], Nasreena Sajjad[1], Sabia Qureshi[2], Sumaya Hassan[3], Muzaffar Ahmad[1], Showkat Ahmad Ganie[3], Rabia Hamid[1*]

[1]Department of Biochemistry, University of Kashmir, Srinagar, Jammu and Kashmir, INDIA.
[2]Department of Microbiology and Immunology, Skuast Kashmir, Srinagar, Jammu and Kashmir, INDIA.
[3]Clinical Biochemistry, University of Kashmir, Srinagar, Jammu and Kashmir, INDIA.

Correspondence

Dr. Rabia Hamid and Dr Showkat Ahmad Ganie

Department of Biochemistry, University of Kashmir, Srinagar, Jammu and Kashmir, INDIA. Clinical Biochemistry, University of Kashmir, Srinagar, Jammu and Kashmir, INDIA.

Phone no: 9419548985; 9419972678

E-mail: rabeyams@yahoo.co.in, showkat_ganie786@yahoo.com

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INTRODUCTION

Plants are vital sources of natural medicine. A number of modern drugs have been isolated from them. An increasing interest in the herbal remedies has been observed in several parts of the world. Many of the herbal remedies have been incorporated into orthodox medicinal plant practice.[1] Today in this modern world, even though synthetic drugs are readily available and highly effective in curing various diseases, there are people who still prefer using traditional folk medicines because of their less harmful effects.[2]

Plants contain a wide range of secondary metabolites with potent antioxidant properties. Such metabolites have an immense potential in pharmaceutical and food sectors. Natural antioxidants are interesting green alternatives to artificial antioxidants mostly because of the safety concerns.[3]

Plant based drugs have been used for the treatment and prevention of different types of illnesses, especially in developing countries where infectious diseases are endemic and health services and hygiene facilities are inadequate. They can act as antibacterial, antioxidant, antiulcer, anti-inflammatory, antiviral and anticancer agents.[4]

Phytochemical screening of various plants has been reported by many workers. These studies have revealed the presence of numerous chemicals, including alkaloids, flavonoids, steroids, phenols, glycosides, and saponins.[5] The number of phytochemicals varies considerably from species to species and even from plant to plant, depending on the age and various ecological and climatic factors. The phenolic compounds are one of the largest and most ubiquitous groups of plant metabolites. Many studies have focused on the biological activities of phenolic compounds, which are antioxidants and free radical scavengers. Therefore, it is also necessary to have knowledge of chemical constituents of plants before using it as medicine.[6]

Reactive oxygen species (ROS) and other free radicals are responsible for many diseases, such as arteriosclerosis, heart diseases, aging process and cancer. They may lead to cell damage through membrane lipid peroxidation and DNA mutations and as a consequence

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of that many diseases such as cancer may develop.[7] The antioxidant activity of plant phenolic compounds is found to be mainly due to their scavenging and redox properties, through neutralizing and quenching free radicals. The importance of reactive oxygen species (ROS) has attracted attention globally over the past decade.[8] The immunomodulatory agents are being used as adjuvant therapy in oxidative stress induced various diseases or vice versa.[9]

Immunomodulatory activity means the biological or pharmacological effects of compounds on humoral or cellular aspects of the immune response. For maintaining a disease-free state, modulation of immune response either through stimulation or suppression is required.[10]

Immunomodulation by utilizing natural medicinally important herb can provide an alternative to chemotherapy for various diseases. There is the great possibility for the discovery of more specific immunomodulators that mimic the biological impact of cytokines and interleukins. Refinement of assays for these mediators will create specific and sensitive screens. There is a need for integrated, systematic research on standardized products of many plants with the aim of developing commercially viable phytomedicines.[11] Most commonly, this activity is being studied by using different parameters like inhibition of histamine release from mast cells, mitogen induced lymphocyte proliferation assay, inhibition of T cell proliferation. The *in vivo* study, methods include hemagglutination antibody titer assay, arthus-type immediate hypersensitivity, delayed type hypersensitivity, adjuvant arthritis in rats, etc.[12] Different reports on the use of standard immunosuppressive drugs suggest the incorporation of cyclophosphamide as reference immunosuppressive drug.[13]

Juniperus L. (family Cupressaceae), a genus of evergreen aromatic shrubs is distributed in temperate and cold regions of the Northern Hemisphere. Depending on taxonomic viewpoint, between 50 and 67 species of Juniper are widely distributed throughout the Northern Hemisphere, from the Arctic, south to tropical Africa in the old world, and to the mountains of central America, Pakistan, India and China. Leaves remain green for several years and even after drying may remain on the shoots for an indefinite period. Flowers are unisexual. In some species flowers of both the sexes occur on the same plant. All the species can be propagated through seeds; seeds retain viability for several years when stored in cool dry place. Common Juniper species of Himalayan range include: *J. communis, J. indica, J. recurva* and *J. squamata*.[14]

Juniperus squamata Buch-Ham. ex D.Don commonly known as weeping blue juniper is a prostrate or decumbent shrub or a small tree of drooping habit attaining a height of 9-12 meters and is found throughout alpine Himalayas and Assam. In Kashmir it is found in the Apharwat region of Gulmarg and on the route from Sonamarg to Amarnath cave at an 82 altitude of 3000-4000 meters. The wood, leaves and twigs are all aromatic and are burnt as incense. Smoke from the green plant is considered to be emetic.[15]

MATERIALS AND METHODS

Chemicals

1, 1-Diphenyl-2-picrylhydrazyl (DPPH), gallic acid, Folin-Ciocalteu reagent and ascorbic acid were purchased from Sigma-Aldrich. All other chemicals were of analytical grade and obtained from Himedia Company.

Plant Material

J.squamata was collected from the higher altitudes of Gulmarg (Apharwat), Jammu and Kashmir State, India, in the months of September and October 2014, and identified by the Centre of Plant Taxonomy, Department of Botany, University of Kashmir. A reference specimen has been retained in the herbarium of the Department of Botany at the University of Kashmir under reference number 2211-KASH.

Extract Preparation

The entire plant material was dried in the shade. The dried material was ground into a powder using a grinder. The dried powder (200g) obtained was successively extracted with different solvents like petroleum ether, methanol, ethanol, ethyl acetate, and water for 48 h using a Soxhlet (60–80°C) (Figure 1). The extract was then concentrated with the help of a rotary evaporator under reduced pressure and the solid extract was stored in the refrigerator for further use.

Determination of Total Phenolic Content

The total phenolic content of the methanolic extract of *J.squamata* was determined by Folin-Ciocalteu method with some modification.[16] An aliquot of 0.5 mL of sample solution was mixed with 1.0 mL Folin-Ciocalteu reagent (10 times dilution before use) and allowed to react at 30°C for 5 min in the dark. Then 2.0 mL of saturated Na_2CO_3 solution was added and the mixture could stand for 1 h before the absorbance of the reaction mixture was read at 747 nm. A calibration curve, using Gallic acid with a concentration range of 0.01–0.10 mg/mL, was prepared. The TPC of the samples was standardized against Gallic acid and expressed as mg Gallic acid equivalent (GAE) per g of sample on a dry weight basis.

DPPH Radical Scavenging Activity

DPPH method was carried out according to the method modified by Kim *et al*.[17] To 1 mL of the sample (100, 200, 300, 400, and 500 μg/mL) was added 3 mL of a 0.1 mmol/L methanol solution of DPPH. The absorbance of all the samples was determined at 517 nm after an incubation period of 30 min. BHT was used as the standard (100 mg/10 ml). The percentage of radical inhibition was calculated by the following formula:

$$\% \text{ inhibition} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance without sample, and A_1 is absorbance with sample

Antioxidant Activity against Oxidative Damage to DNA

Hydroxyl radicals generated by Fenton reaction were used to induce oxidative damage to DNA. The reaction mixture (15 μL) contained 25 mg of calf thymus DNA in 20 mM phosphate buffer saline (pH 7.4) and different concentrations of plant extract (10, 30, 50 and 80 μg) were added and incubated with DNA for 15 min at room temperature. The oxidation was induced by treating DNA with 20 mM ferric nitrate and 100 mM ascorbic acid and incubated them for 1 h at 37°C. The reaction was terminated by the addition of loading buffer bromophenol blue (0.25%) and glycerol (30%) and the mixture was subjected to gel electrophoresis in 0.7% agarose/TAE buffer run at 100 V. DNA was visualized and photographed by gel doc.

Thin layer chromatography analysis of antioxidant activity

The antioxidant constituents were analyzed using thin layer chromatography (TLC) followed by DPPH (2, 2- Diphenyl-1-picrylhydrazyl) technique. About 2.5 μL of methanolic extracts (1 mg/mL) were loaded on TLC plates. The plates were developed in 10% chloroform in methanol and Methanol: Chloroform: Hexane (7:2:1) to separate the various constituents of the extracts. The developed plates were air dried and observed under visible and UV light (240 and 300 nm). Various separated spots were noted as their Rf values. After this examination, 0.05% of DPPH solution in methanol was sprayed on the surface of developed TLC plates and incubated for 10 min at room temperature. The active antioxidant constituents were detected as yellowish white spots produced by bleaching of DPPH.

Microsomal Lipid Peroxidation

Liver was washed in ice cold 1.15% KCl and homogenized in a homogenizing buffer (50 mM Tris-HCl, 1.15% KCl pH 7.4) using Teflon homogenizer. The homogenate was centrifuged at 9,000 ×g for 20 min to remove debris. The supernatant so obtained was further centrifuged at 15,000 rpm for 20 min at 4°C to get post mitochondrial supernatant (PMS). Microsomes were obtained by centrifuging the portion of prepared PMS by using Sorvall Ultracentrifuge at 105,000 ×g for 1 hr at 4°C to obtain the microsomal fraction. This fraction was resuspended in 0.25 M sucrose and stored frozen until use.

Rat liver microsomal lipid peroxidation was carried out according to the method of Urata *et al*, with little modifications.[18] The test sample (20–100 µg/mL) was added to 1 mL of liver microsomes. Lipid peroxidation was induced by adding 100 µL of ferric nitrate (20 mM) and 100 µL of ascorbic acid (100 mM). After incubation for 1 hr at 37°C, the reaction was stopped by the addition of 1 mL of TCA (10%) and 1 mL of (1.67%) TBA was added and the reaction mixture was boiled for 15 min, cooled, and centrifuged and the absorbance of the supernatant was measured at 532 nm.

Hydroxyl Radical Scavenging Assay

Hydroxyl radical scavenging activity was measured by the ability of the different concentrations of extract to scavenge the hydroxyl radicals generated by the Fe^{3+}-ascorbate-H_2O_2 system (Fenton reaction).[19] The reaction mixture contained; 500 µL of 2-deoxyribose (2.8 mM) in phosphate buffer (50 mM, pH 7.4), 200 µL of premixed ferric chloride (100 mM), 100 µL of H_2O_2 (200 mM) with or without the extract solution (100–500 µg/mL). The reaction was triggered by adding 100 µL of 300 mM ascorbate and incubated for 1 h at 37°C. 0.5 mL of the reaction mixture was added to 1 mL of TCA (10%), then 1 mL of 1% TBA was added to the reaction mixture. The mixture was heated for 15 min on a boiling water bath. After the mixture being cooled, the absorbance at 532 nm was noted against a blank (the same solution but without reagent).

Reducing Power Test

The reducing power test based on Fe (III) to Fe (II) transformation in the presence of the solvent fractions was carried out by using the method of Oyaizu.[20] The Fe (II) can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Various concentrations of the sample (2 mL) were mixed with 2 mL of phosphate buffer (0.2 M, pH 6.6) and 2 mL of potassium ferricyanide (10 mg/mL). The mixture was incubated at 50°C for 20 min followed by the addition of 2 mL of trichloroacetic acid (100 mg/L). The mixture was centrifuged at 1500 ×g for 10 min to collect the upper layer of the solution. A volume of 2 mL from each of the mixture earlier mentioned was mixed with 2 mL of distilled water and 0.4 mL of 0.1% (w/v) fresh ferric chloride. After 10 min reaction, the absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicates a higher reducing power.

Superoxide radical scavenging activity

The assay was based on the capacity of the extracts to inhibit formazan formation by scavenging the superoxide radicals generated in Riboflavin-light-NBT system. The reaction mixture contained 50 mM phosphate buffer (pH7.6), 20 µg riboflavin, 12 mM EDTA, and NBT 0.1 mg/3 mL, added in sequence. Reaction was started by illuminating the reaction mixture with different concentrations of sample extract/ standard for 90 sec. Immediately after illumination, the absorbance was measured at 590 nm. BHT was used as positive control.

Immunomodulatory activity

Animals

Wistar rats of either sex, weighing 90–100 g, housed in standard conditions of temperature, humidity and light were used. They were fed with standard rodent diet and water *ad libitum*. The study was approved by IAEC (Institutional Animal Ethics Committee) department of Pharmaceutical Sciences, University of Kashmir under registration no. 801/03/CA/CPCEA.

Drugs

Weighed quantity, 50 mg/10 ml of *J.squamata* methanolic was suspended in 1% sodium carboxymethylcellulose to prepare the suitable dosage form. The control animals were given an equivalent volume of sodium carboxymethylcellulose vehicle orally.

Antigen (SRBC)

Fresh SRBC collected aseptically from the jugular vein of sheep were stored in cold sterile Alsever's solution, washed three times with pyrogen free sterile normal saline (0.9% NaCl w/v), and adjusted to a concentration of 5×10^9 cells/mL for immunization and challenge at the required time schedule (DTH response).

In vivo phagocytosis using carbon clearance

The phagocytic activity was carried out following the procedure of Benacerraf *et al*.[21] Rats were divided into four groups (6 animals in each group). The control group received vehicle (water), while the standard group treated with levamisole at dose 50mg/kg bw for 5 days orally. The remaining two groups (treated groups) were treated with plant extract (100 and 200 mg/kg) orally. After 48 h of the last dose on 5th day, rats were injected with 0.1 ml of Indian ink intravenously through the tail vein. Blood samples were collected from retro-orbital plexus just before and at 0 and 15 min after injection. Blood samples were lyzed with 2 ml of 0.1% sodium carbonate and absorbance of samples recorded at 660 nm. The phagocytic index was calculated using the formula:

$$\text{Phagocytic index} = K=(\text{In } OD_1 - \ln OD_2)/t_2\text{-}t_2$$

Where, OD1= optical density at time ($t_{1)}$ and, OD_2= optical density at time ($t_{2).}$

DTH response

To determine the effect of the drugs on cell-mediated immunity, the delayed hypersensitivity to sheep RBC was assessed in rats. Animals were sensitized with 10% sheep RBC (1×10^8 cells) at day 0 and day 7 subcutaneously (s.c.). Rats were divided into four groups (6 animals in each group). The control group received vehicle (water), while the standard group treated with levamisole at dose 50mg/kg bw. The remaining two groups (treated groups) were treated with plant extract (100 and 200 mg/kg) orally for 28 days. All the four groups of SRBC immunized rats were then challenged by subcutaneous administration of SRBC 0.25ml/100g body weight in right hind foot pad on 28th day and 0.2 ml of 0.9% normal saline was similarly injected into the left hind foot pad as a control. The cell mediated immune response was measured at 24 h after SRBC challenged on the 28th day in terms of increase in paw volume (plethysmometrically). The DTH response was expressed as the mean percent increase in paw volume between the right foot pad injected with SRBC and left foot pad injected with normal saline

Statistical analysis

Results were expressed as mean ± SEM. Data were analyzed using One-way analysis of variance (ANOVA) followed by Dunnett test. Value of $p<0.05$ was considered to be statistically significant.

RESULTS

Total Phenolic Content

Phenolic compounds are considered to be the most important antioxidants and are widely distributed among various plant species. Phenolic compounds

in plants are powerful free radical scavengers that can inhibit lipid peroxidation by neutralizing peroxyl radicals generated during the oxidation of lipids These phenols play important roles in plants such as protection against herbivores and pathogens, cementing material joining phenolic polymers to cell wall polysaccharides, regulation of cell growth and cell division.[22]

The TPC of the different extracts of *J. squamata* was assayed by the Folin-Ciocalteu method using gallic acid as standard. The highest TPC of 780 mg GAE/g was obtained in the methanolic fraction, whereas the lowest TPC of 459 mg GAE/g was achieved in the petroleum ether extract.

Antioxidant Assay

Different *in-vitro* assays were used to measure the antioxidant activity of crude methanolic leaf extracts. The DPPH radical scavenging assay has been widely used as a model system to investigate the radical scavenging activity of natural compounds. DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule.[23] The antioxidants reduce the stable DPPH radical to a yellow colored diphenyl-picrylhydrazine with maximum absorption at 517 nm. The percent radical scavenging activity of methanolic extracts of *J.squamata* under study was found to be about 82.12% at conc. of 700ug/ml (Figure 2). Moreover, the methanolic fraction exhibited the highest scavenging with IC50 (50% inhibitory concentration) value (235.43±1.13µg /ml) as compared to the other fractions of *J. squamata* extract (Table 1). The results are represented relative to butylated hydroxytoluene (BHT), a reference standard with IC50 value of 119±3.67 µg/ml.

Thin layer chromatography analysis of antioxidant activity

DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay was used as a rapid thin layer chromatography screening method to evaluate the antioxidant activity of the methanolic extracts due to free radical scavenging. DPPH is a purple coloured stable free radical, which on reduction gives yellow coloured diphenyl picryl hydrazine compound. In the present study, separated compounds (bands) with antioxidant activity stained yellow and the remainder of the TLC plate stained purple. Three main dark yellow bands and few lighter yellow bands were observed indicating that the plant possesses a good antioxidant activity (Figure 3).

Antioxidant Activity against Oxidative Damage to DNA

The protective effect of *J. squamata* methanol extract on calf thymus DNA is shown in Figure 4. Hydroxyl radicals generated by Fenton reaction were found to induce DNA strand breaks in calf thymus DNA. H_2O_2 alone did not cause DNA strand cleavage. However, H_2O_2 in the presence of ferric nitrate and ascorbic acid induces DNA strand breaks. The Fentons reaction involves the reaction between hydrogen peroxide and Fe^{2+} to form hydroxyl radical. The results showed complete degradation of DNA treated with Fentons reagent which is indicated in lane 2. The effect of plant extracts on the DNA damage caused by Hydroxyl radical was indicated from lane 3-7. The intensity of the DNA damage was reduced in a concentration dependent manner. The DNA damage was protected in a dose dependent manner which may be due to the possible ability of polyphenolic compounds of plant *J. squamata* that could be responsible for the protection against oxidative damage to DNA (Figure 4).

Hydrogen peroxide scavenging capacity of Juniperus squamata

The methanolic extract of *J. squamata* was capable of scavenging hydrogen peroxide in a concentration dependent manner. 300 µg/ml of extract exhibited 81.23% scavenging activity on hydrogen peroxide (Figure 5). On the other hand, using the same amounts, BHT exhibited 88.63 % hydrogen peroxide scavenging activity. The IC50 value of methanolic extract was found to be 161.44 ± 0.08 µg/ml. (Table 1)

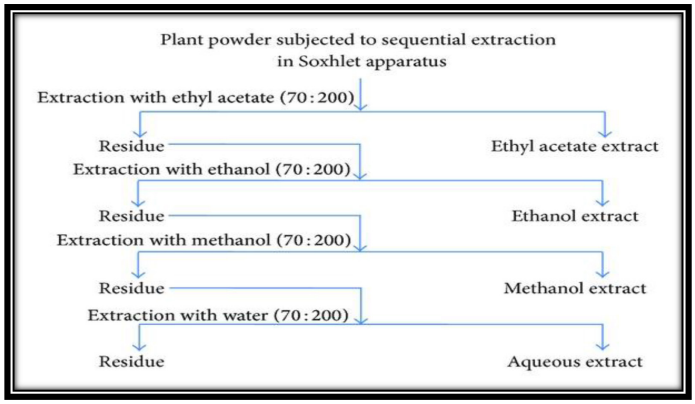


Figure 1: Systematic representation of preparation of different solvent extracts of *Juniperus squamata* by sequential extraction method.

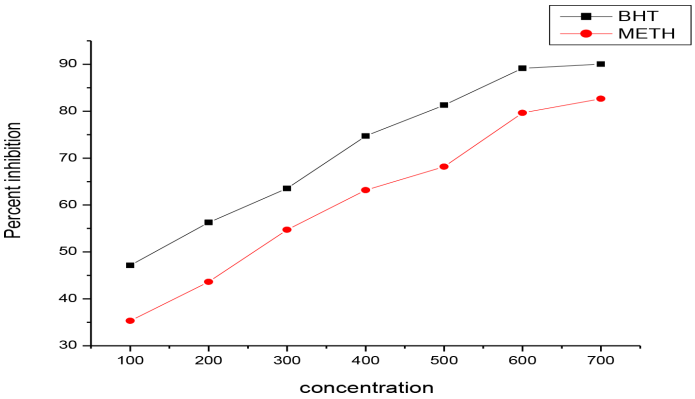
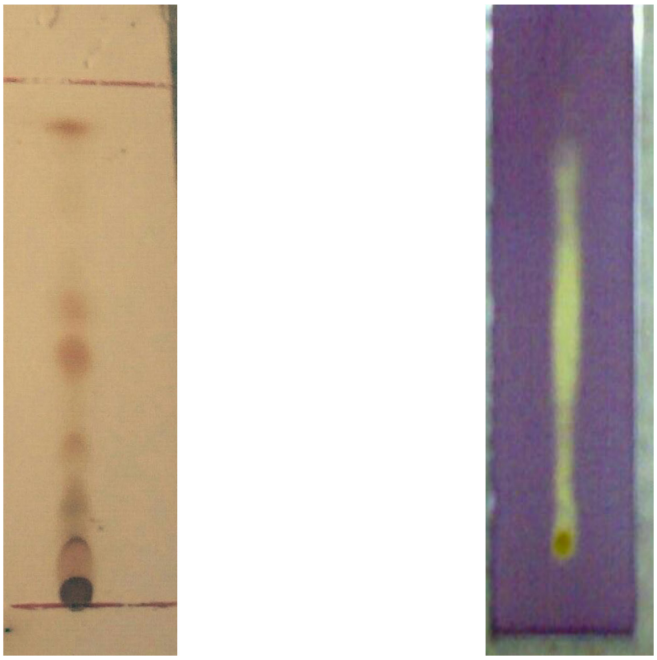


Figure 2: The effect of methanolic extract of *J. squamata* and known antioxidant(BHT) on DPPH radical scavenging activity. The results represent mean ± SD of 3 separate experiments.



a)TLC before DPPH spray b)TLC after DPPH spray

Figure 3: TLC determining antioxidant activity of methanolic extract of *J. squamata*.

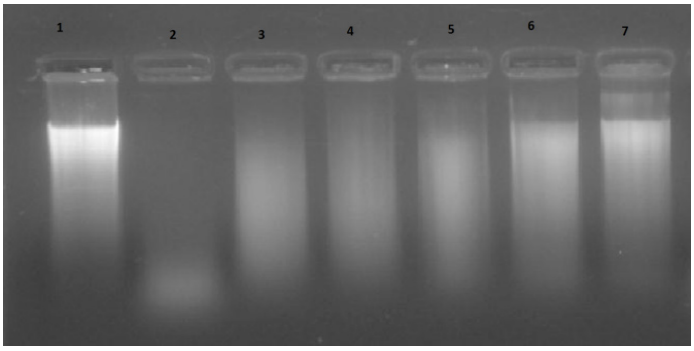


Figure 4: Protective effect of methanolic extract of *J. squamata* on oxidative damage to calf thymus DNA. Lane 1: control, Lane 2: +ve control, Lanes 3-6: Plant extract (10, 20, 30, and 40 mg /ml), lane 7: catechin (10 mg /ml)

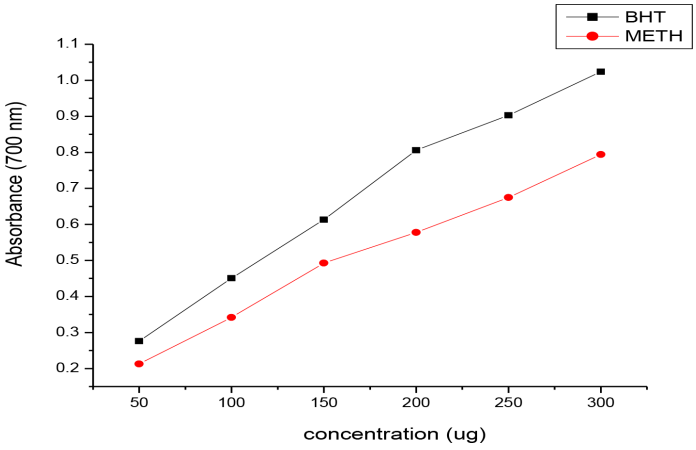


Figure 7: The effect of methanolic extract of *J. squamta* on reducing power activity. The results represent mean ± SD of 3 separate experiments.

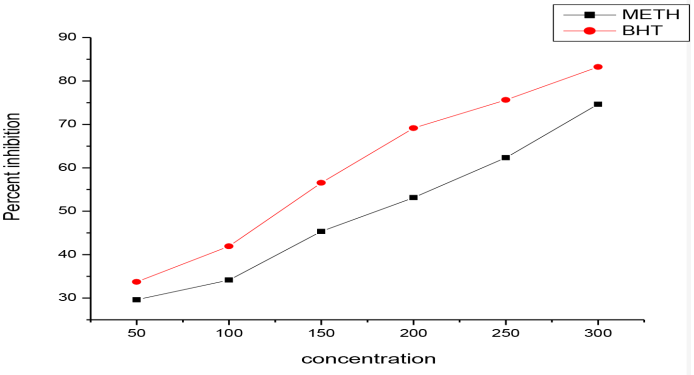


Figure 5: The effect of methanolic extract *J. squamata* and BHT on hydrogen peroxide scavenging activity. The results represent mean ± SD of 3 separate experiments.

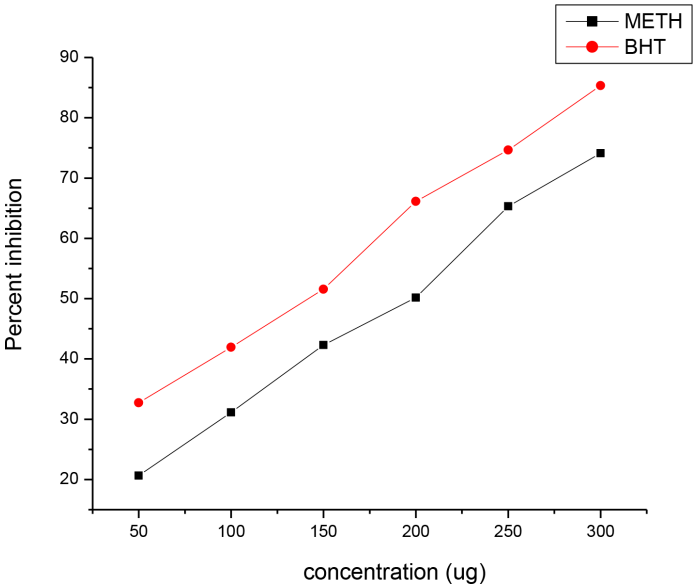


Figure 8: The effect of methanolic extract of *J. squamta* and BHT on hydroxyl radical scavenging activity. The results represent mean ± SD of 3 separate experiments.

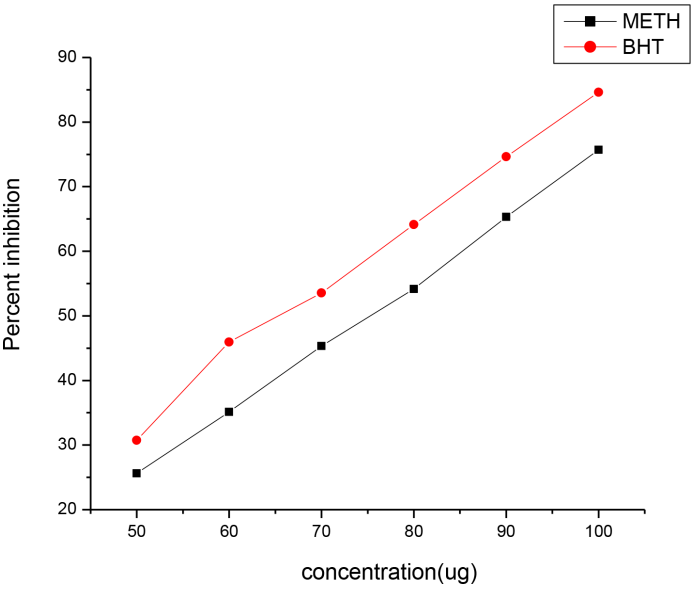


Figure 6: The effect of methanolic extract of *J. squamata* and BHT on lipid peroxidation activity. The results represent mean ± SD of 3 separate experiments.

Inhibition of Lipid Peroxidation

The protective effect of methanolic extracts against $FeSO^4$ induced lipid peroxidation was assessed in rat liver mitochondrial samples. During lipid peroxidation, polyunsaturated fatty acids present in lipid membrane undergo oxidation, resulting in formation of malonaldehyde (MDA) which reacts with two molecules of thiobarbituric acid (TBA) to form TBARS, a pinkish red chromagen which is read at 532nm. Lipid peroxidation is the oxidative degradation of polyunsaturated fatty acids and involves the formation of lipid radicals leading to membrane damage. Free radicals induce lipid peroxidation in polyunsaturated lipid rich areas like the brain and liver and can also damage DNA, proteins and other biological molecules. Initiation of lipid peroxidation by ferrous sulphate takes place either through ferryl-perferryl complex or through hydroxyl radical by Fenton reaction depending upon the reaction conditions.[24] The extracts showed the *in vitro* lipid peroxidation inhibition effects in a concentration dependent manner (Figure 6). Lipid peroxidation is the oxidative degradation of polyunsaturated fatty acids and

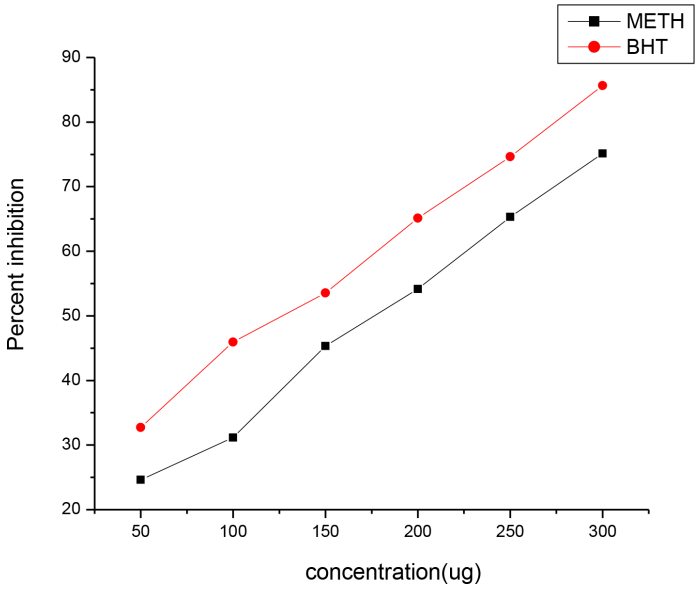


Figure 9: The effect of methanolic extract of *J. squamta* and BHT on superoxide anion scavenging activity. The results represent mean ± SD of 3 separate experiments.

involves the formation of lipid radicals leading to membrane damage. Free radicals induce lipid peroxidation in polyunsaturated lipid rich areas like the brain and liver and can also damage DNA, proteins and other biological molecules. Initiation of lipid peroxidation by ferrous sulphate takes place either through ferryl-perferryl complex or through hydroxyl radical by Fenton reaction depending upon the reaction conditions. The extracts showed the *in vitro* lipid peroxidation inhibition effects in a concentration dependent manner and the results are expressed in terms of percentage inhibition and IC_{50} values for the same were calculated from the regression equation respectively. Methanol extract had the greatest inhibiting activity, with the lowest IC50 value of 78.65 µg/Ml (Table 1)

Reducing Power

In reducing power assay, potential antioxidants reduce the Fe^{3+}/ ferricyanide complex to its ferrous form which can then be monitored spectrophotometrically at 700 nm.[25] Increased absorbance of the reaction mixture indicates increased reducing power. The antioxidant activities of natural components may have a reciprocal correlation with their reducing powers. The reducing power values were found to be correlated with the concentration of the extract. The reducing power of methanolic extract of *J. squamata is* summarized in Figure 7. The data shows that the reducing power of extract increased in a dose dependent manner.

Hydroxyl Radical Scavenging Activity Radical

The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells. The ability of the above-mentioned extracts to quench hydroxyl radicals seems to be directly related to the prevention of propagation of the process of lipid peroxidation and seems to be good scavenger of reactive oxygen species.[26]

A significant decrease in concentration of hydroxyl radical was observed due to *J. squamata extracts* with maximal inhibition of 74.13 ± 1.17% with methanol extract.BHT used as standard inhibits the hydroxyl radical up to 85.32% with the same concentration (Figure 8).

Superoxide anion radical scavenging activity

Superoxide radicals generated from riboflavin-light-NBT system and can be measured by their ability to reduce NBT. The decrease in absorbance at 590 nm with the plant extracts and the reference compound BHT indicates their abilities to quench superoxide radicals in the reaction mixture. We observed superoxide radical scavenging increased in a concentration dependent manner. At the higher concentration of the plant extract (300 µg/ml), we observed 75.15% inhibition for methanolic extract of *J. squamata*. BHT a known antioxidant showed 85.63% inhibition at the same concentration (Figure 9). The IC50 value of methanolic extract was found to be 187.56 ± 0.65 µg/ml (Table 1).

Carbon clearance assay

Administration of PE (100 and 200 mg/kg bw) increased the clearance of carbon particles from blood as indicated by a significant increase in the phagocytic index when compared with the control group as shown in Table 2. The levamisol (50 mg/kg bw) also showed a significant effect on the phagocytic index in the carbon clearance assay.

Delayed type hypersensitivity responses

Administration of PE (100 and 200 mg/kg bw) increased the DTH response significantly. This is indicated in terms of increase in the difference of paw thickness when compared with the control group. DTH requires the specific recognition of a given antigen by activated T lymphocytes, which subsequently proliferate and release cytokines. These in turn increase the vascular permeability, induce vasodilatation, macrophage accumulation, and activation, promoting increased phago-

Table 1: **IC_{50} values (µg/ml) of Methanolic extract of *Juniperus squamate*.**

Methanolic Extract	IC50 Value
DPPH	235.43± 0.12
Superoxide Radical	187.56±0.65
Hydroxyl radical	109.33±0.08
Hydrogen peroxide	161.44±0.90
Lipid Peroxidation	78.65±0.67

Table 2: **Phagocytic Carbon clearance.**

S.NO	TREATMENT	DOSE mg/kg	PHAGOCYTIC INDEX ±SEM
1	Vehicle	-	0.0410±0.0051
2	P.E	100	0.0597±0.0063
3	P.E	200	0.0787±0.0029
4	Standard(Levimasole)	50	0.0969±0.0062

Values are mean±SD, n=6 in each group. *P<0.05 when compared with respective control group (Dunnett's test).

Table 3: **Effect of methanolic extract on SRBC-induced delayed type hypersensitivity reaction in rats.**

Groups	Dose (mg/kg)	DTH response (% increase in paw volume)
Control	-	30.17
P.E	100	38.21
P.E	200	45.97
Levimasole	50	57.23

p<0.05 as compared to vehicle treated group. (Statistically analysed by One-way analysis of variance (ANOVA) followed by (Dunnett) multi comparis test

cytic activity and increased concentrations of lytic enzymes for more effective killing. When activated TH1 cells encounter certain antigens, viz. SRBC,they secrete cytokines that induce a localized inflammatory reaction called delayed type hypersensitivity.[27] DTH comprises of two phases, an initial sensitization phase after the primary contact with SRBC antigen. A subsequent exposure to the SRBCs antigen induces the effector phase of the DTH response, where TH1 cells secrete a variety of cytokines that recruits and activates macrophages and other non-specific inflammatory mediators. The delay in the onset of the response reflects the time required for the cytokines to induce the recruitment and activation of macrophages. Therefore, increase in DTH reaction in rats in response to T cell dependent antigen revealed the stimulatory effect of extract on T cells.[28] DTH response was checked by increased footpad thickness using digital vernier caliper. The animals treated with the *J.squamata* extracts showed a significant change in DTH response as compared to control animals. (Table 3)

DISCUSSION

Antioxidants fight against free radicals and protect us from various diseases. They exert their action either by scavenging the reactive oxygen species or protecting the antioxidant defense mechanisms. The increasing evidence suggests that oxidative damage has great impact on the immune system of humans. Free radical components have a relevant pathophysiological role in several types of autoimmune human diseases such as heart failure, shock, atherosclerosis, etc.[29] It is essential to counteract this oxidative stress and thereby enhance immunity of the body system. The immunomodulatory agents are being used as adjuvant therapy in oxidative stress induced diseases. A very few modern medicines are available to treat the oxidative stress and immune senescence which are costly and possess serious side effect. On the contrary, the natural antioxidants are safer choice in the treatment of oxidative stress and immune senescence.

Immunological agents of plant origin enhance the immune responsiveness of an organism against a pathogen by non-specifically activating the immune system. Natural antioxidants can act as potential therapeutic agents against many diseases resulting from oxidative stress. Nowadays there has been increasing interest in the investigation of medicinal plants for the discovery of new antimicrobial, immunomodulatory and antioxidant agents.[30] Abila *et al* have worked on the exploration of high value medicinal and aromatic plants of Kashmir Himalayas for various biological activities. The have found that the essential oil of *Juniperus recurva*, one of the species of juniper exhibits strong antimicrobial activity against pathogens of clinical significance.[31]

In the present study, we tested different extracts of *J. squamata* for their antioxidant activity using DPPH, H_2O_2, OH and lipid peroxidation assays. The immunomodulatory activity was determined by Carbon clearance and DTH assay.

DPPH test is a widely used method to evaluate the free radical scavenging effect of plant extracts. This method is based on the reduction of DPPH solution in the presence of antioxidant resulting in change of colour of solution from purple to yellow.The degree of discoloration indicates the scavenging potential of the extract.[32]

In the present study, among all the extracts tested, the highest capacity to neutralize DPPH radicals was found for the methanolic extract followed by ethyl acetate and aqueous extract. This activity could be due to the large number of phenolic compounds found in these fractions. The estimation of phenolic compounds revealed that methanolic fraction exhibited the highest phenolic content. The evaluation of the phenolic compounds uses the Folin-Ciocalteau reagent, which forms blue complexes in the presence of reducing agents. The content of phenolic compounds in the methanolic extract was calculated using the standard curve of Gallic acid. Our results revealed that there is a strong and significant correlation between TPC and DPPH• free radical scavenging activity of different extracts of *J. squamata*. Similar results were reported by Sharad and Gupta, while studying the bioactive compounds and antioxidant activity of *Moringo olefera* hydroethanolic extract.[33] Further it was concluded that *J. squamata* possesses a good reducing power ability. The ferric reducing power activity of different extracts of *J. squamata* seems to be due to the presence of polyphenols which is correlated to its total phenolic content. Our results are in tune with the results reported by Sharad Vats, while studying the antioxidant activity of *Azadirachta indica*.[34]

In our study we also tested the extracts for superoxide scavenging activities. Superoxide anion is one of the most important representatives of free radicals. It acts as a precursor of more reactive oxidative species such as single oxygen and hydroxyl radicals that have the potential of reacting with biological macromolecules and thereby inducing tissue damage and plays a vital role in peroxidation of lipids.[35] Methanol extract with least IC50 showed strong super oxide radical scavenging activity than ethyl acetate and aqueous extract. However, the values remain below the BHT used as known superoxide radical scavenger.

Hydrogen peroxide (H_2O_2) is a biologically relevant, non-radical reactive oxygen species and is inevitably generated as a by-product of normal aerobic metabolism. However, when concentration increases under stress conditions, (H_2O_2) could be detrimental for cells. Thus, H_2O_2 scavenging activity becomes a crucial characteristic of total antioxidant activity.[36] In this study, methanol extract was found to be efficient scavenger of hydrogen peroxide radical while ethyl acetate extract and aqueous extract were least efficient. The results strongly suggest that these extracts contain the necessary compounds for radical elimination.

In this study, we also measured the potential of *J. squamata* extracts to inhibit lipid peroxidation in rat liver microsomes induced by the Fe^{2+} /ascorbate system. Lipid peroxidation in biological systems has long been thought to be a toxicological phenomenon that can lead to various pathological consequences. Fe^{2+}-ascorbic acid mixture is well known to stimulate lipid peroxidation in rat liver *in vivo* and in microsomes and mitochondria of rat liver *in vitro*. Since it is believed that lipid peroxidation is one of the causes of the occurrence of cardiovascular disease and cancer, its high inhibition by extracts of plants may represent an indicator of their high therapeutic potential.[37] Different extracts protected against lipid peroxidation induced by Fe^{2+} considerably reduced MDA content in a concentration-dependent manner. Similar results were reported by Gul *et al*., were studying the antioxidant and antiproliferative activities of *Abrus precatorius* leaf extract.[38]

J.squamata extracts were also found to exhibit a dose dependent hydroxyl radical scavenging activity and prevent the Calf thymus DNA damage. Methanolic extract was to possess highest hydroxyl radical scavenging activity. Hydroxyl radical is generated by a mixture of Fe^{3+}- H_2O_2 and ascorbic acid and is assessed by monitoring the degraded fragments of deoxyribose, through malonaldehyde (MDA) formation and DNA strand breaks in Calf thymus DNA. If any plant extract or drug scavenges the hydroxyl radical, they may either scavenge the radical or may chelate the Fe^{2+} ion, making it unavailable for the Fenton's reaction.[39]

As it is noted from the above results that the extracts of *J.squamata* exhibit good antioxidant activities, the results are highly correlated with immunomodulatory properties .Administration of *J.squamata* methanolic extract (100 and 200 mg/kg bw) increased the clearance of carbon particles from blood in wistar rats as indicated by a significant increase in the phagocytic index when compared with the control group. Similarly, the animals treated with the *J.squamata* methanolic extract showed a significant change in DTH response as compared to control animals. DTH response was checked by increased footpad thickness using digital vernier caliper. Similar results were reported Patel *et al*, while studying immunomodulatory activity of aqueous extract of *Trapa bispinosa*.[40]

CONCLUSION

This work is an attempt to identify the biological potential of *J. squamata* growing in the Himalayan region for the first time. The methanolic fraction of *J.squamata* was observed to harbour considerable antioxidant potential and, in case of immune modulating studies, the methanolic fraction was observed to show remarkable immune suppressive as well as immune stimulating potential, which could prove to be of immense value in auto-immune diseases as well as in immune compromised patients. However, the phytoconstituents responsible for their significant activity are still unknown. The results obtained in this work provide the basis for designing future experimentation on this species for better understanding of its antioxidative and enhance the immune system and could be used as a potential immunomodulatory agent for tumor immunotherapy.

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CONFLICT OF INTEREST

The authors have no personal or financial conflicts of interest associated with this work.

ABBREVIATIONS

LPO: Lipid peroxidation; **DPPH:** 1, 1-Diphenyl-2-picryl-hydrazyl; **ROS:** Reactive oxygen species **GAE:** Gallic acid; **BHT:** Butylated hydroxytoluene; **EDTA:** Ethylene diamine tetra acetic acid; **TAE:** Tris acetate; **NBT:** Nitro blue tetra zolium test; **TBARS:** Thiobarbituric acid reactive substances; **TPC:** Total phenolic content; **MDA:** Malondialdehyde; **H_2O_2:** Hydrogen peroxide.

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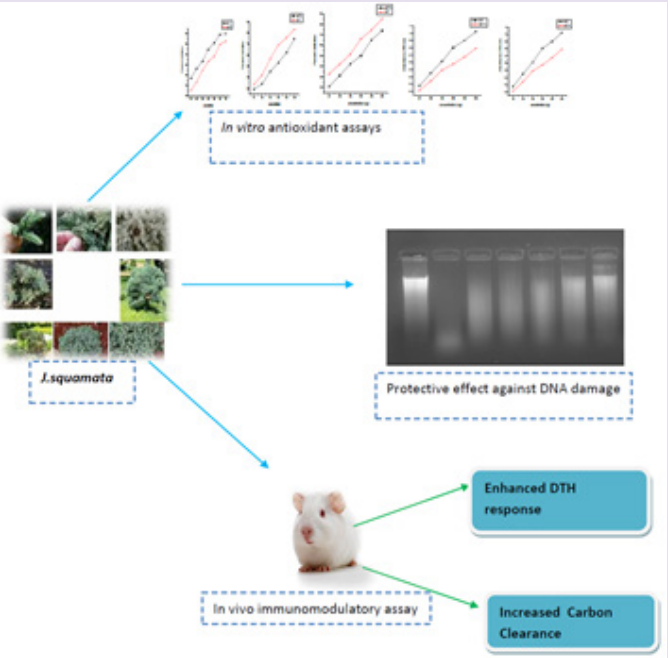
GRAPHICAL ABSTRACT

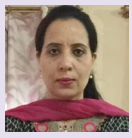


SUMMARY

- The methanolic extract of *J. squamata* showed high content of total phenolic compounds as compared to ethyl acetate and other extract. It also exhibited strong *in vitro* antioxidant potential on DPPH radical scavenging activity, reducing power, LPO and other assays. The extract was tested for hypersensitivity and carbon clearance using rats as the animal model. It was noted to exhibit a dose related increase in the hypersensitivity reaction, to the SRBC antigen. Administration of *J. squamata* methanolic extract also increased the clearance of carbon particles from blood in wistar rats as indicated by a significant increase in the phagocytic index when compared with the control group. Methanolic extract thus exhibits potent antioxidant and immunomodulatory activity which might be useful for therapeutic purposes to prevent ROS disorders and enhance the immune system. It could be used as as an agent for tumor immunotherapy.

ABOUT AUTHORS

Rohaya Ali: Is a research scholar at the University of Kashmir India, where she obtained her masters in Biochemistry. Her research is focused on the evaluation of antioxidant and immunomodulatory activity of *Juniperus squamata*. Besides, she is also working on exploration of new therapeutic bioactive compounds from *Juniperus squamata*.



Dr. Rabia Hamid: Has obtained her Ph.D degree from the Department of Biochemistry, University of Kashmir Srinagar, India. She works as Associate Professor in Department of Biochemistry, University of Kashmir. The author has a vast experience in the area of medicinal plants research, free radical biology and protein chemistry. Currently the focus of the lab is on searching new therapeutically active drugs from medicinal plants of Kashmir valley.

Dr. Showkat A Ganie : Has obtained his Ph.D degree in 2010 from Department of Biochemistry University of Kashmir, Srinagar India. He is also Assistant Professor in Department of Clinical Biochemistry Kashmir University. Dr. Showkat has experience in the area of Medicinal plant research, working mainly for searching new therapeutic active drugs from various medicinal of Kashmir valley.

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