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Chemical Characterization and Protective Effect of Juniperus Squamata Buch.-ham.exd.don Against Cyclophosphamide-induced Immunosuppression in Albino Rats

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Abstract

Background: The current study was aimed to investigate the protective effect ofmethanolic extract ofJuniperussquamata leaf (JSME) against cyclophosphamide-induced immunosuppression in rats and the characterization of its bioactive compounds. JSME was first screened for its preliminary immunomodulatory activity throughin vitro lymphocyte proliferation assay and phagocytic activity of polymorphonuclear cells.

Methods: Immunoprotective of JSME was assessed through analysis of hematologicalparameters, phagocytic carbon clearance, and HA titer values in cyclophosphamide treated immunosuppressed Wistar albino rats. Silica gel column chromatography was used to isolate the bioactive fractions from JSME.

Results: Our in vitro studies revealed that JSME significantly enhanced lymphocyte proliferation as depicted from increased optical density values. It was also found to augment the phagocytic effect of the polymorphonuclear leucocytes by causing increased engulfment of Candida albicans cells. In the case of in vivo studies, cyclophosphamide was found to cause a noteworthy decline in the hemoglobin, RBCs, WBCs, and platelets count.Administration of JSME resulted in combating this myelosuppression by restoring hematological parameters to normal. Pretreatment with JSME also augmented the rate of clearance of carbon particles from the blood as depicted by a considerable reduction in the phagocytic index compared to cyclophosphamide treated group. JSME showed phagocytic index of 0.0497 ± 0.0063 and 0.0687±0.0029 at concentration of 100mg/kg and 200mg/kg body weight, respectively.

Conclusions: Three compounds (stigmasterol, apigenin, and luteolin) were isolated via chromatography techniques using different organic solvents.Further, the structures of the isolated compounds were elucidated by NMR. These compounds are being reported for the first time in Juniperus squamata. This work has to an extent, provided the pivot upon which future isolations and characterizations of the constituents of Juniperus squamata will anchor. Therefore, the constituents can serve as lead molecules for the development of agents with therapeutically useful immunogenic properties.

Keywords: Cyclophosphamide; immunomodulatory; isolatio n Juniperussquamata; macrophage ; myelosuppression; phagocytic index

Introduction

Juniperus squamata, commonly known as “Blue Juniper” and locally called as “Yathur” is a decumbent shrub with a tallness of 10-13 meters [1]. It is found all through Alpine Himalayas. In Kashmir, it is found mainly in regions like Gulmarg (Apharwat) and sonamarg at an altitude of 30000-4000 meters. Stem and leaves plant are highly aromatic. It is famous for its traditional use. In folklore medicine of Kashmir, it has been used for wound healing, cough, swelling, and sore throat. In Ayurvedic medicine, the berries of juniper are considered as one of the best diuretics for Vata constitution, dispelling excess Vata and improving digestion (Clutton). Its leaves are applied externally for swelling and arthritic pain. It is also recommended in diabetes, cough and infantile tuberculosis.

Besides, ash of the bark is used in treatment of skin diseases. It is also used in treating gastro-intestinal problems, urinary infection and respiratory problems. It has also been used as antispasmodic, aphrodisiac, antitoxic, antiseptic, astringent, depurative, sedative, carminative, and nevine. Its oil has been tested for antifungal activity .

Cedranediol isolated from its essential oil is used vasorelaxing and as an antiplatelet agent. Leaves of the plant have been screened for antibacterial activity and has been found to possess a broad spectrum activity against a panel of bacterial strains responsible for commom animal diseases. Phytochemical screening of the plant has revealed presence of

alkaloids, phenols, terpenoids, flavonoids, steroids etc. The plant has also been found to possess free radical scavenging activity and has also been assessesed for delayed-type hypersensitivity response in rats.

The engagement of natural compounds as medicine has been illustrated all through history as remedies, potions, oils, and traditional medicine, etc. However, several compounds among these with potential with bioactivity are still being unidentified.

The leading source of information of these natural compounds is the outcome of experimenting by trial and error for thousands of years through palatability trials or untimely death, probing for available foods for the treatment of diseases.

Plants have been documented for several years as a source of medicine, and are known to produce unique secondary metabolites to combat stress comorbidities and environmental triggers.

As per the World Health Organization (WHO), almost 80% of people are dependent on plant-based drugs for their health care. Around 80 % of these herbal drugs were related to their original ethnopharmacological rationale. A worldwide dependence on alternative medicine has resulted in the development of medicinal plant-based research [3]. Of these, finequantity of herbs has been employed for immune modulation from Ayurvedic formulation either alone or in combination.

It is a well-known fact that the immune system is involved in the etiology and pathological mechanisms of many diseases to endow protection against pathogens and various infectious agents. Modulation of the immune system could, therefore, offer strategies for therapy and management of diseases. The use of cyclophosphamide and other synthetic immunomodulatory drugs is associated with certain toxicities. Besides, they might increase the risk of immunosuppression related complications. The drug cyclophosphamide acts on both cyclic and inter-mitotic cells, resulting in the depletion of immune-competent cells. However, cyclophosphamide is reported to prompt several types of infections (Merwid). Several plant extracts are being researched for potential benefits in lessening or overcoming these adverse effects. The employment of herbal immunomodulators in the indigenous system of medicines, indeed, could modulate the body's defense mechanism [4]. Therefore, the use of medicinal plants to modulate immune function modulation has become an accepted therapeutic approach.

Plant-derived immunomodulators are reported to modulate immunity by affecting macrophage, NK cells, or complement functions. This is done via controlling homeostasis and through various adaptogenic activities. Various active constituents of plants like peptides, lectins, flavonoids, and tannins have been reported to modulate the immune system in different experimental models.

Kashmir valley is located in the lap of Western Himalayas. The flora of Kashmir is extremely rich in the lap of Western Himalayas and harbors a diverse variety of medicinal plants that could be exploited for the welfare of humankind.

However, the medicinal flora of Kashmir has not yet received the attention that it requires. It is in this backdrop, the current study wascarried out to screen Juniperussquamata, unexplored medicinal plants of the valley, for its protective role against cyclophosphamide-induced immunosuppression .Earlier our lab has carried preliminary phytochemical screening of this plant. Besides, we have also screened the plant for its antioxidant and delayed-type hypersensitivity response in rats . However, this study focuses on the isolation of bioactive constituents of JSME and its role as in immunoprotection [5].To the best of our knowledge, until now, no such scientific evaluations have been conducted for JSME [6].

Materials and methods

Experimental animals

Wistar rats, weighing 90-100g were used for the estimation of in vivoimmunomodulatory potential of JSME [7]. The rats were housed at standard laboratory conditions with a relative humidity of 55±10, a temperature of 25±2°C and fed with pellet diet (Lipton India Limited) and water was given ad libitum [8,9].

Experimental design

The experimental work on animals was carried out as per the guidelines set by the committee of the purpose of control and supervision of experiments on animals (CPCSEA). The quantity and number of animals employed for the investigation were duly approved by IAEC (Institutional Animal Ethics Committee), Department of Pharmaceutical Sciences, University of Kashmir under the registration no. 801/03/CA/CPCEA dated 21-08-2015.Tissues were collected only after euthanizing the rats or anesthetizing them till they turned unresponsive to all stimuli. Euthanasia was done employing cervical dislocation.

Preliminary toxicity study

The effectof plant extract on the safety and behavior of animals was done as per OECD (Organization of Economic Co-operation and Development) guideline number 423 [10]. Rats (3 females and 3 males) of either sexwere given a methanolic extract of Juniperussquamata at a dose of 1500mg/kg body weight orally by gavage . Then the animals were observed for 4 hours for any toxic symptoms. After 24 hours, the total number of survivors was calculated and kept under observation for 14 days. Any change in behavior, mortality weight, or physiological activity was noted down [11,12,13,14].

Collection of plant material

The whole plant of Juniperussquamatawas collected from Gulmarg (Apharwat)Jammu and Kashmir, in August. All the samples were acknowledged and validated byAkhtarHussain Malik, Curator, Centrefor Biodiversity and Taxonomy, University of Kashmir. A reference specimen has been preserved in the herbarium of the Department of Botany, University of Kashmir under voucher number 2211-KASH [15,16,17].

Preparation of extract

The plant material was cleaned and separated into root, stem, and leaf. Leaves of a plant were then dried under shade at room temperature. The dried leaf material was ground into a fine powder. The powder was extracted with different solvents using

a soxhlet extractor, filtered and concentrated usinga rotary evaporator. The boiling point of the respective solvent governed the temperature regulation of the soxhlet. The methanolic extract was stored at 4◦ C until further use [18,19,20].

Lymphocyte proliferation test

Spleen was collected under aseptic conditions in RPMI medium. Later, it was minced and allowed to pass via steel mesh to acquire a homogenous cell suspension.After that, the erythrocytes were lysed with ammonium chloride (0.8% w/v), and centrifuged (380× g) at 4°C for 10 minutes. The pelleted cells were washed three times with PBS and re-suspended in RPMI medium supplemented with 12 mM HEPES (pH7.1), 10% FBS, 0.05 mM 2-mercaptoethanol, 100µg/mL streptomycin, 100IU/mL penicillin). Finally, the cell number was counted by a hemocytometer usingthe trypan blue dye exclusion method.

To estimate the effect of JSME on the lymphocyte proliferation, the lymphocyte suspension was made (2×106 cells/ml) in RPMI-1640 supplemented with 50U/ml penicillin, 50U/ml nystatin, 50U/ml streptomycin, and 10%FBS. The cells were seeded into separate wells of 96 well culture plates in the presence of 5µg/ml CON A. To this, 50µl of different concentrations (20-100µg/ml in RPMI-1640) of JSME was added. The plate was incubated for 24 hours in a 5% CO2 humidified atmosphere at 37◦C temperature. After the incubation 10µl, MTT (5mg/ml) solution was added to each well. Then the plate was enclosed in an aluminum foil to avoid any exposure to light and was further incubated for 4 hours. Then 100µl solubilizing reagent was added to each well. Finally, the absorbance was measured at 570nm [21,22,23].

Phagocytosis test

Polymorphonuclear cell (PMN) function test was used to assess the immunomodulatory activity of JSME. Peripheral venous blood (7ml) was obtained in sterile heparinized tubes from the volunteers [24,25]. FicollHypaque density gradient sedimentation was used to isolate the neutrophils. RBC-PMN pellet was subjected to dextran sedimentation. Then the supernatant was collected. Finally,the dell density was adjusted to 1x105cells/ml. Candida albicans was used as a test organism and its cell density was adjusted to 1x106cells/ml using MEM medium, followed by mixing with PMN cells and incubated at 37˚C in 5% CO2 for 1 hour, in the presence of various concentrations of JSME. The control was the same solution but without JSME. After incubation, cytosmears were prepared. Fixing of smears was done with methanol and was then stained with Giemsa, followed by observation under 100x oil immersion to find out the phagocytic activity of PMN cells. Neutrophils were scanned and the cells with ingested Candida albicans were counted [26].

Cyclophosphamide induced myelosuppression

The method that described was employed for cyclophosphamide-induced myelosuppression assay. The animals were divided into four groups with 6 animals in each group. Group I and II were fed with normal saline. Group III and group IV were given test extracts (100-200 mg/kg) orally for 7 days. Cyclophosphamide (25mg/kg, IP) was injected in animals of Group II-IV, 1 hour after the administration of respective treatment on 4th, 5thand 6thday. Blood samples were then collected onthe 7th day of the experiment and analyzed for hematological parameters [27].

Macrophage carbon clearance assay

The experimental animals were divided into four groups with six animals in each group. Group I and group IIanimals were given normal saline. Then, animals in group III and group IV were given JSME for 7 days. Later, group II-IV animals were injected with cyclophosphamide, 1 hour after the administration of the respective treatment on 4th, 5th, and 6th day. After 24 hours of the final dose, the animals were injected with Indian ink (0.1ml) intravenously via the tail vein. Then, the blood samples were obtained from the animals through retro-orbital plexus at 5 and 15 minutes and mixed with a 3ml sodium carbonate solution (0.1%) for lysis of erythrocytes. Finally, absorbance was measured at 660nm [28].

Using the following formula, the phagocytic index was calculated:

Phagocytic index(K)= (lnOD1−ln OD2)/t2-t1

Where OD1 is the optical density at tme (t1) and, OD2 is the optical density at time (t2).

Haemagglutination assay

Fresh sheep red blood cells (SRBCs) were aseptically collected in Alsevier's solution from the jugular vein of animals housed at the farm, Faculty of Veterinary Sciences, SKUAST, Kashmir.The cells were washed 2-3 times by centrifugation (3000g for 10 minutes) in pyrogen-free saline. Finally, the washed SRBCs were taken and adjusted to a concentration of 109cells/ml with normal saline before used for immunization.

The experimental animals were divided into four groups with six animals in each group. Group I and II were given normal saline.The animals in groups III and IV were administered the test extracts orally (100-200mg/kg) for about 7 days.Group II-IV were injected with cyclophosphamide (25mg/kg,IP), 1 hour after the respective treatment on 4th, 5th, and 6th day. The animals were immunized by injecting 0.1ml of 20% fresh SRBC suspension through the intraperitoneal route on day 0. Then, the blood samples were collected via retro-orbital plexus on the 7th day and the serum was isolated.The levels of antibodies were calculated usinghaemagglutination assay. Equal volumes of serum samples of each group were pooled and two-fold dilutions were made in 25µl saline in the microtitration plate. Then, 25µl of 1% SRBC suspension wasadded to the plate. Finally, the plates were incubated for 1 hour at room temperature after mixing. Finally, the plates were examined under a microscope for a haemagglutination reaction. The reciprocal of the highest dilution of the serum giving the agglutination was taken as antibody titer.

Chemical characterization of Juniperussquamata

Leaves of Juniperussquamata(1kg) were cleaned, shade dried and then powdered. The powdered material was finally extracted with methanol. Combined extracts were filtered and concentrated in vacuum to obtain a crude extract (100g), which

was then subjected to silica gel column chromatography with a gradient elution system of CHCl3:MeOH (100:0 to 50:50, v/v) to attain 9 fractions (F1 to F9). Fraction F3 was separated by a silica gel column using 100% chloroform to afford compound 1 (10mg). Fraction 6 was eluted using a gradient: CHCl3: MeOH (9:1v/v) to give compound 2 (8mg). Fraction 7 was purified on a silica gel column using a gradient CHCl3: MeOH (9.5:0.5v/v) to get two compounds 3 (7mg) [29].

Structure elucidation of isolated compounds

Isolation and purification of compounds from the active fractions were done by thin-layer chromatography and column chromatography. Characterization of the isolated compounds was carried out using IR, NMR, DEPT, and MS.FTIR was carried by KBr pellet technique as described previously(Fredericks et al., 1985). The individual samples (2mg) were mixed uniformly with potassium bromide (KBr, 200mg) and compressed under a pressure of 10Ton/nm2 to prepare circular transparent discs.Then, the samples were dried in a hot oven for 30 minutes at 37°C to avoid the interference of any leftover moisture. Each analysis included 45 scans, at a resolution of 4cm-1 in the wavelength range of 4000 to 400cm-1. The data was examined using FTIR control software (version: 1.10) coupled with the instrument.1H-NMR (400 MHz), 13C-NMR (100 MHz), and DEPT-135 spectra were measured in CDCl3 or CD3OD depending on the solubility profile of the compound. Chemical shifts (δ) were depicted in ppm relative to tetramethylsilane(TMS). TMS serves as an internal standard. The splitting patterns are reported as s(singlet), d(doublet), t (triplet), m(multiplet), and dd (doublet of doublets).

Determination of melting points of isolated compounds

A little quantity of isolated compounds was filled in a narrow capillary tube (0.5 mm), sealed at one end, and placed into the melting point apparatus fitted with a thermometer (0-360o C range). The capillary observed through the lighted magnifying glass showed the temperatures at which the compounds melted or the range within which they melted [30].

Phagocytosis test for isolated compounds

Peripheral venous blood, 7ml, was obtained in sterile heparinized tubes from the volunteers.FicollHypaque density gradient sedimentation was used to isolate the neutrophils. RBC-PMN pellet was subjected to dextran sedimentation. Then, the supernatant was collected and the cell density was adjusted to a concentration of 1x105 cells/ml using the MEM medium.The Candida albican cells (cell density adjusted to 1x106cells/ml) were used as test microorganismstogether with PMN cells. The mixture was then incubated at 37˚C in 5% CO2for 1 hour in presence of the isolated compounds (100µM each). After incubation cytosmears were prepared. Fixing of smears was done with methanol and was then stained withGiemsa. Finally, they were observed under 100x oil immersion objectiveto find out the activity of PMNcells (phagocytic activity)

Statistical analysis

Results obtained in the study were expressed as mean ±standard deviation(SD). The level of statistical significance was assessed usinga one-way analysis of variance (ANOVA). Evaluation of the results was done by using the Origin 8.1 version and SPSS (version 12.0) software. P values less than 0.05 were considered statistically significant.

Results

Preliminary toxicity and effect of JSME on lymphocyte proliferation

JSME was found to be non- toxic at a dose of 1500mg/kg body weight.

Our results showed that JSME enhanced lymphocyte proliferation in a dose-dependent manner when compared to control as depicted from increased optical density values. Greater the value of absorbance, the greater is the proliferating activity. At concentration of 20µg/ml, 40µg/ml, 80µg/ml, and 100µg/ml, optical density values of 0.45, 0.54, 0.57 and 0.60nm were observed, respectively. Cells treated with concanavalin A only, showed an optical density of 0.3nm while the control group showed an optical density of 0.11nm (Figure 1).These results reinforce that extract is nontoxic to immune cells, however, modulating the cellular response by increasing the cell viability.

Effect on phagocytosis

The study revealed that JSME significantly enhanced the phagocytic activity in a dose-dependent manner in polymorphonuclear cells. In untreated control,the phagocytic index of 1.6±0.11 was observed. However, in cells treated with plant extract, phagocytic index of 1.69±0.13, 1.74±0.10, 1.77±0.13, and 1.81±0.12 was observed at a concentration of 20, 40, 80 and 100µg/ml, respectively (Figure 2). Thus, methanolic extract enhances the efficacy of PMN cells to engulf more Candida cells when compared to control. This indicates JSME has the potential to enhance the non-specific immune response.

Effect on cyclophosphamide-induced myelosuppression

Cyclophosphamide at a dose of 25mg/kg body weight resulted in a noteworthydecline in the hemoglobin, WBCs, RBCs, and platelet count. Pretreatment of rats with JSME resulted in the restoration of bone marrowactivity in a dose-dependent manner (Table 1). At a higher dose of 200mg/kg body weight methanolic extract significantly augmented the levels of hemoglobin, RBCs, WBCs, and platelets count in rats. Thus, the administration of JSME helps in combating myelosuppression caused by cyclophosphamide.

Effect on carbon clearance

It was observed that animals that were given cyclophosphamide showed a decrease in the phagocytic index (0.0132±0.0087) when compared to control (0.0570±0.0051). Pretreatment of rats with JSME resulted in the restoration of phagocytic activity. Methanolic extract of Juniperussquamata at a concentration of 200mg/kg body weight showed a phagocytic index of 0.0687±0.0029 whereas the phagocytic index of 0.0497±0.0063 was observed at a concentration of 100mg/kg body weight (Table 2). Thus, the administration of JSME the colloidal carbon particles clearance from the blood as depicted

by a noteworthy increase in the phagocytic index compared to cyclophosphamide treated group.

Effect onhaemagglutination antibody (HA)titer

The HA titer is used to evaluate the humoral immune response. Administration ofmethanolic extract produced a significant dose-related enhancement in HA titer value when compared to the control. While in the negative control group, cyclophosphamide treatment produced a noteworthy decrease in titer value versus control. The administration of JSME resulted in a significant recovery of the immunosuppressive effect of cyclophosphamide (CP). At a concentration of 100mg/kg body weight, the HA titer value of 8.73 ±0.75 was observed as compared to control which showed HA titer value of 10.32±0.73. However, at a higher dose of 200mg/kg body weight, there was a considerable increase in HA titer (11.01±0.73) when compared to cyclophosphamide treated group which showed HA titer value of 4.210.32 ±0.35 (Table 3). Hence, the plant extract showed a protective effect over humoral immunity [31].

Effect of isolated compounds on phagocytic activity

The study revealed the isolated compounds enhanced the phagocytic activity in polymorphonuclear cells as compared to control. In untreated control,the phagocytic index of 1.61±0.11 was observed. However, in cells treated with isolated compounds, the phagocytic index of 1.98±0.13, 2.4±0.10, and 2.91 ±0.09were observed, respectively, (Table 4). However, among all the tested samples, JS3 showed the best phagocytic activity. The above study depicts that test samples increase the phagocytic activity of PMN cells by causing increased engulfment of Candida cells versus the control. This experiment depicts that JSME enhances the non-specific immune response.

Chemical characterization of JSME

Compound 1 (JS1):

Compound 1 was obtained as white needles (Figure 3). The ESI mass spectrum showed a molecular peak m/z 413 [M+H]+. The melting point was found in the range of 167-168˚C. The 1H NMR spectrum showed resonance signals owing to the presence of two quaternary methyl groups at δH 0.73 (3H, s, H-18) and 0.90 (3H, s, H-19) and three secondary methyl groups at δH 0.93 (3H,d,J= 6.5 Hz, H-21) and 0.86 (6H, d, J =5.0, H-26, H-27). The signals due to primary methyl groups were observed at δH 0.71 (3H, m, H-29). Further, resonances in the 1HNMR spectra displayed at δH 5.05 and 4.86 (1H each, dd, J =15.5 Hz, 8.2 Hz, H-22, H-23), confirmed a disubstituted double bond (Figure 4). In 13C NMR the signals observed at 140.7668 ppm and 121.7274 ppm represents double bond at C-5 and C-6. Besides, the δ values at 71.8260 ppm are due to observed at 71.8260 c-3 β-hydroxyl group. Signals observed at 21.225 ppm and 12.0547 ppm correspond to angular carbons at C-19 and C-18, respectively (Figure 5)

In the IR spectrum,the absorption band found at 3354.91cm-1 isa typical feature of O-H stretching.Absorption at 2934cm-1 and 2866cm-1indicates the presence of aliphatic or C-H stretching (CH3). A band at 1667 cm-1 appears due to the existence of an olefinic group in this compound. A search in the literature of the above spectral data of JS1 confirmed it is stigmasterol.

Compound 2 (JS2):

Compound 2 was obtained as a yellow powder and its molecular formula was recognized as C15H10O5(Figure 6) due to its ESI mass spectral data which showed molecular ion peak at m/z 271.1 [M+H]+. The melting point of the compound was found to be in the range of 340-346˚C. Further, JS2 gave a yellow spot on a silica gel thin-layer chromatogram when sprayed withanisaldehyde reagent [Rfof 0.6; petroleum ether: ethyl acetate (3:7)]. 1H NMRspectrum of the compound revealed the occurrence of two meta coupled aromatic doublets at δH 6.33 and 5.98 which correspond to H-6 and H-8 protons. Two doublets at δH 6.79 and 7.21 correspond to H-3′/ H-5′ and H-2′/ H-6′protons of ring B as shown in Figure 7. A singlet at δH 5.71 corresponds to the H-3 proton. This data depicts the features of 5,7,4′-tri-substituted flavones. The 13C NMR and DEPT 135 depict twelve aromatic carbons as shown in Figure 8.

In FTIR spectrum intensive bands between 1600cm-1 and 1580cm-1 were observed which correspond to aromatic ring vibrations. The bands at 3287cm-1 and 3097cm-1 are probably the result of hydrogen-bonded O-H stretch and C-H vibrations. The bands between 1300cm-1 and 1000 cm-1 correspond to C-O stretch vibrations. The intensive bands between 1440cm-1 and 1500cm-1depict C-H vibrations and the peak at 2697cm-1 corresponds to the C-H symmetric stretching. A comparison of the above spectral data of JS2, with the reported data in the literature, suggested that it is 4′,5,7-tri-hydroxyflavone, commonly called apigenin(Ersoz).

Compound 3 (JS3)

Compound 3 was obtained in the form of yellow powder and its molecular formula was established as C15H10O6from it's mass spectrum data which showed a molecular peak at ion [M +H]+(Figure 9).The melting point of the compound was found in the range 345-350˚C. The 1H- NMR of the compound showed the occurrence of three meta coupled aromatic doublets atδ 6.29,6.45, and 7.35 and an ortho coupled doublet at δ 6.86 one, one doublet of doublets at δ 7.56 representing an ortho and meta couplet aromatic species, and a singlet singletat 6,75; characteristic for 5,7,3',4' tetra-substituted flavones (Figure 10). The13C NMR and DEPT 135 spectral data revealed the presence of 8 quaternary carbons, 12 aromatic carbons,6 methine carbons and an unsaturated carbonyl carbon (Figure 11)

In the FTIR spectrum, bands at 3396.3cm-1 correspond to stretching vibrations of phenolic groups having hydrogen bonds. The weak band at 2935.15cm-1represents the stretching vibrationof the aromatic C-H group. A medium band at 2038.65cm-1corresponds to bending vibration of C-H aliphatic group. A sharp and strong band at 1654.81sharp cm-1indicates vibrations of the aromatic (C=O) group. Further, a medium band at 1535.23cm-1represents stretching vibration of aromatic (C=C) group. A search in the literature revealed the above to be consistent with that of luteolin (3′,4′, 5, 7-trahydroxyflavone).

Discussion

Natural products provide a valuable source of potential drugs from which mankind has recognized several phytomedicines and herbal remedies. Most of our current anticancer and antibiotics

drugs are derived from nature. Herbal drugs are employed worldwide for the prevention of various diseases and have gained huge popularity. These drugs are often considered harmless as they are natural and easily available. Besides, they can be used for self-medication without supervision(Mates JM; Tyler VE).The current study was intended to examine the immunomodulatory potential of the methanolic extract of Juniperussquamataleaf (JSME).The extract was further subjected to column chromatography for the isolation of bioactive compounds. Juniperussquamatais used ethnopharmacologically for the treatment of various complaints.

The in vitroimmunomodulatory activity of JSME was evaluated on lymphocytes via proliferation assay (MTT assay) and phagocytosis of PMN cells. Lymphocytes are the chief cells of the immune system associated with health and disease. They participate in both innate and acquired immune defenses The effect of medical plants on host defense against pathogenic agents and certain tumors were directly correlated with their capacity to stimulate lymphocyte proliferation

In the present study methanolic extract of Juniperussquamata was found to stimulate the concanavalin A-induced splenocyte proliferation. Therefore, this is quite obvious that the methanolic extract is not potentially toxic to immune cells but is modulating the cellular arm of the immune system by augmenting the cell viability. Similar results were reported while studying the in vitro immunomodulating effect of Allium hookeri.

In the defense system, phagocytosis plays an important role. The functional activation of phagocytes to strengthen systems of capture and elimination of pathogenic microorganisms is a primary feature of immunomodulation. Neutrophils are phagocytic granulocytes and are among the first cells to arrive at sites of inflammation. The chief targets of neutrophils are pathogenic microbes. Neutrophils arrive at the site of inflamed tissue and encounter microorganisms. Thereby phagocytize these microbes and fuse the phagosome with intracellular granules that are loaded with bactericidal proteins and acidic hydrolases. In this experiment, the administration of JSMEon the phagocytic potential of PMN cells was evaluated. The results showed noteworthy enhancement in the percentage of phagocytosis and phagocytic index. This indicates that JSME enhances the phagocytic efficacy of the PMN cells by causing increased engulfment of the Candida cells in comparison to control, thereby stimulating the immune response.

The in vivoimmunomodulatory activity of JSME leaf was assessed by cyclophosphamide-induced immunosuppression in rats and various parameters such as hematological changes, HA titer, phagocytic carbon clearance, and DTH response were studied. Cyclophosphamide is a well knownanticancer drug; however, it produces deadly side effects such as immunotoxicity, hematotoxicity, and mutagenicity due to its alkylating property. It is also reported that this drug has a potent immunosuppressive action, capable of inhibiting humoral and cell-mediated immune function.

In the current investigation, the effect of JSME was checked in cyclophosphamide-induced-myelosuppressionin animal models. The results showed that cyclophosphamide lowered the RBCs, platelets, and total WBCs count. Interestingly, pretreatment with JSME restored the changes and lead to an increase in hematological parameters. This prevention of myelosuppression by the plant extract indicates its protective effect on the immune system. Besides,a carbon clearance test was used to find out the effect of JSME on the reticuloendothelial system. This system mainly consists of phagocytic cells (macrophages), that help inthe removal of foreign substances from the bloodstream. In this assay, the colloidal particles as ink are directly injected into the systemic circulation. The rate of clearance of these particles from the blood is calculated using an exponential equation. Administration of JSME (100 and 200mg/kg body weight) enhanced the clearance of colloidal carbon from the blood in rats as depicted by enhancement of phagocytic index versus the control group. Therefore, it can be concluded that the reticuloendothelial system was activated by the extract. Similar results were observed while evaluating the immunomodulatory potential of Aeglemarmelosin experimental animals.

The assessment of cell-mediated and humoral immunity was carried by the measurement of HA titer. Cyclophosphamide treatment resulted in a decrease in HA titer values.Antibodies, products of B lymphocytes and plasma cells, are central to humoral immune response. They are involved in complement activation, opsonization, neutralization of toxins, etc. The results of the HA titertest showed that pretreatment with JSME significantly increased the circulating antibodies in animals compared to those which were treated with cyclophosphamide. So, from this model, it is obvious that the methanolic extract stimulates the humoral immune system which otherwise was suppressed by cyclophosphamide. This activity of JSME could be attributed to the occurrence of flavonoid that augments thehumoral response, by stimulating the macrophages and B lymphocytes involved in antibody production.

Based on the above results, JSME was subjected to isolation of compounds.Three main compounds were isolated from the extract using silica gel column chromatography.These were identified through various spectral procedures (NMR, FTIR, DEPT etc). All of these compounds, identified as Stigmasterol (JS1), Apigenin (JS2), and Luteolin (JS3) showed a significant enhancement in the phagocytic activity of PMN cells. JS1 being a steroid compound was isolated with 100% chloroform. JS2 and JS3 owing to their flavonoid (polar) structure were isolated with gradient: CHCl3: MeOH (9:1v/v) and CHCl3: MeOH (9.5:0.5v/v), respectively. Apigenin and Luteolin exhibited higher immunomodulatory potential which could be attributed to the presence of C-5 and C-7 hydroxyl groups in their structures (Bylka et al., 2004). Further, the presence of 2, 3- double bond conjugated with the 4-oxo group involved in electron delocalization in Apigenin may be responsible for its efficient immunomodulatory property.

Conclusion

From the above findings, it is concluded that JSME has potential for enhancing immune activities by modulating either the cellular or humoral immunity. Besides, among isolated compounds, apigenin and luteolin showed a potent

immunomodulating activity suggesting that these constituents may act synergistically. To our knowledge, the isolated compounds are being reported for the first time in the Juniperussquamata. To an extent, this work provides a pivot upon which future isolations and characterizations of the constituents of Juniperussquamatacan anchor. Furthermore, the potent constituents mayact as lead compounds for the development of agents with therapeutically useful immunogenic properties andthe possibility of the development of vaccine adjuvants.Further studies are needed for understanding the accuratemechanisms accountable for the immunomodulatory potential of JSME.

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