Immunomodulatory Effect

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Immunomodulatory Effect of Isolated Triterpenes from *Alstonia scholaris* Linn. in Wistar Albino Rats

ABSTRACT

AIM: Immunomodulatory Effect of Isolated Triterpenes from Alstonia scholaris Linn. in Wistar Albino Rats. MATERIAL & METHODS: The flower part of Alstonia scholaris were extracted by using n-hexane, dichloromethane, methanol, and water. All the extracts were screened for the presence of various active compounds. DCM extract showed the occurrence of terpenoids and flavonoids. The methanolic extract showed the presence of flavonoids and other active components. DCM extract was then allowed to isolate the active compound by the means of column chromatography. N-hexane and dichloromethane solvents in various ratios were used for the isolation of active compounds. Then isolated compound was identified by the various spectral analysis techniques. The isolated compound was evaluated by cyclophosphamide and E. Coli model. RESULTS: In the case of cyclophosphamide induced myelosuppression, there was a decrease in the WBC reckoning in the control group. In treatment groups, the WBC count was found to be augmented with $p<0.01$ and $p< 0.001$ at 10 and 50 mg/kg dose respectively on the 11th day. In E. coli induced abdominal sepsis, mortality due to peritonitis was evaluated in control and treatment groups. There was 100% mortality in the control group. In the groups treated with α-amyrin 10 mg/kg body, wt 36 % mortality was observed respectively. CONCLUSION: The current discoveries are huge for the improvement of options, cheap and maybe safer strategies for the treatment of diseases. A detailed study is also required on structure determination of the compounds from bioactive fractions in order to find the structure-activity relationship.

KEYWORDS: *Triterpenes, Immunomodulation, NK Cells, Phytotoxins, Rasayana, Alstonia scholaris*

INTRODUCTION

The immune system is exposed to a myriad of substances intentionally and unintentionally viz. environmental pollutants, food, drugs and chemicals, radioisotopes, microbial agents, zootoxins, phytotoxins, mycotoxins, hormones, etc, in addition to various social and environmental factors. It is seen that intense pressure may improve safe reaction through persistent pressure may smother the resistant framework (Quinn, 1990; Kaminski et al., 2008).

Characteristic executioner cells frequently alluded to as NK cells are like the executioner T cell subset (CD8+ T cells). They work as effecter cells that straightforwardly execute certain tumors, for example, melanomas, lymphomas, and viral-contaminated cells, most remarkably herpes and cytomegalovirus-tainted cells. NK cells, in contrast to the CD8+ (executioner) T cells, murder their objectives without an earlier "gathering" in the lymphoid organs. Nonetheless, NK cells that have been enacted by emissions from CD4+ T cells will execute their tumor or viral-contaminated targets all the more viably (Yarchoan and Mitsuya 1993; Wood 1993; Francis and Meltzer 1992; Schroder et al., 2004)

Various Indian restorative plants and different 'Rasayana' have been professed to have Immunomodulatory movement. A portion of this planta is Withania somnifera, Tinospora cordifolia, and Mangifera indica (Davis and Kuttan, 2000; Dahanukar and Thatte, 1997; Makare et al., 2001). Much more are still to be investigated and offer extension for additional examination. Alstonia scholaris is quite a very much archived restorative plant and bloom some portion of this plant is generally revealed for Immunomodulatory movement. The bloom part is enhanced with Triterpenes so the principle of the current examination is to seclude the dependable Triterpenes for Immunomodulatory action.

MATERIALS & METHODS

Equipments: Digital Weighing Balance, Digital p^H meter, Incubator, Tissue Homogenizer, Laboratory Centrifuge, Hot air oven, UV- Visible spectrophotometer, FTIR, ^{1}H- NMR, ^{13}C-NMR, Mass spectrometer.

Glass wares: Borosil and ASGI make glasswares were used.

Chemicals: All chemicals used were of analytical grade.

Animals: Wistar albino rats of either sex (150 to 200 g) were purchased approved vendors for in-vivo Immunomodulatory activity.

Procurement and Identification of the plant:

The flower part of Alstonia scholaris was collected from the mountainous region of northern Iraq near Sulaymaniyah city and it shows white color with smooth surface. The plant flower was

washed well in tap water, dried in the shade, then powdered well, and used for extraction.

Extraction of Plant Material:

Extraction is the partition of therapeutically dynamic segments of plant tissues utilizing particular solvents through the standard methodology. The extraction was done by following the general procedure. Powdered material was packed in soxhlet apparatus. The drug was defatted with petroleum ether (60-80°C) for about 30-35 complete cycles. Defatted material was subjected to further extraction process by dichloromethane, methanol, and water. All the extracts were concentrated under a vacuum. After completion of the total, the extracted powder was discarded and the extracts so obtained were further processed. The excess solvent in the extracts were removed by distillation and the concentrated extracts so obtained were further dried at a temperature not exceeding 40° C in water bath. The extracts were then collected kept in a Petri dish and stored in desiccators at room temperature. The yield values and other physical properties were observed (Mukherjee, 2002).

Phytochemical Screening

Primer phytochemical screening was performed for all the concentrates (Kokate, 2003; Khandelwal, 2006).

Bioactivity Guided Isolation of Phytoconstituents

Preparation of the column

The slurry of adsorbent (silica gel; 60-120 work) was set up by blending the adsorbent in the n-hexane and utilized as the fixed stage. It was then poured into a glass column (90cm x 3cm) and allowed to settle. The air entrapped was removed by stirring with a glass rod. This method of column filling is called a wet filling method. A small amount of sand was kept at the top of the column. Excess of solvent was run off until the level of mobile phase fell to one cm just above the top of the sand layer (Shukla et al., 2012).

Preparation of sample and loading

Dichloromethane extract (10 g) was dissolved in a minimum volume of dichloromethane and adsorbed on silica gel (60-120 mesh), dried and applied on the column to separate possible phytoconstituents.

Selection of mobile phase for separation of phytoconstituents

Dynamic dichloromethane removal (10 g) was exposed to chromatographic division by stacking out on a glass section. The solvent system was used as n-hexane: dichloromethane. The gradient elusion was followed for the isolation. Initially, n-hexane was used as pure solvent, and then the column was eluted by increasing quantity (95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40, and finally 50:50) of dichloromethane. Total 250 fractions were collected of 25 ml each. All the fractions were monitored simultaneously on a TLC plate using n-hexane: ethyl acetate (75:25) as a solvent system. The fractions showing the same color and Rf on TLC were pooled together and finally 4 fractions (F1-F4) were obtained.

All the parts were exposed to pharmacological assessment to decide their phagocytic movement.

Characterization of Compound

The structure was characterized by means of IR, NMR, and MASS spectral analysis for the structure determination and their identity.

Acute Toxicity Studies of α-amyrin

Miller and Tainter (graphical) method were used to calculate LD50 of isolated compound. Overnight fasted adult Wistar albino rats were divided into 5 groups; 10 rats (5 male and 5 female) in each group. 5 dose levels were selected such that one dose with no mortality and a higher dose with 100% mortality; other 3 doses were in between. Creatures were noticed for an initial 2 h and afterward at sixth h for any poisonous manifestations. After 24 h the number of expired rodents was included in each gathering and % mortality was determined. For the evaluation of movement 1/10 of LD50 was chosen (Randhawa, 2009).

Doses Preparation

Portions equal to 200 mg and 400 mg of the rough medication per kilogram body weight were determined and suspended in 1% w/v Tween 80 answers for the trial.

Cyclophosphamide-induced myelosupression

Ziauddin et al., (1996) strategy was utilized for cyclophosphamide-incited myelosuppression. Pale skinned person rodents were partitioned into six gatherings assigned as control (I) and treatment (II–III, IV) gatherings, each gathering containing six rodents. The benchmark group got a saline arrangement. Gathering II was regulated with just cyclophosphamide at the portion of 30 mg/kg, i.p. while bunches III and IV rodents got cyclophosphamide with changed convergences of α-amyrin (5 and 10 mg/kg) for 10 days. On day 11, blood tests were gathered from the retro-orbital plexus of individual creatures and examined for hematological and serological boundaries.

Group 1: Served as control, treated by 10 ml/kg saline

Group 2: Received cyclophosphamide at the dose of 30 mg/kg, i.p

Group 3: Cyclophosphamide at the dose of 30 mg/kg, i.p+α-amyrin treated (5 mg/kg)

Group 4: Cyclophosphamide at the dose of 30 mg/kg, i.p+α-amyrin treated (10 mg/kg)

E. coli induced abdominal sepsis Model

Animals were isolated into three gatherings of six creatures in each gathering. The creatures of gathering I was controlled 1% sodium CMC as vehicle as it were. Animals of groups II, III were administered α-amyrin in doses 5 and 10 mg/kg body wt. p.o. All the animals were treated for15 days prior to bacterial challenge. On the 16th day, the animals were injected with 0.2 ml suspension of E. coli (1x 108 cells) i.p. Animals were then observed for 16 hours to find mortality if any. Blood samples were then withdrawn from retro-orbital plexuses using heparinized capillary tubes and by cardiac puncture in case of dead animals. Blood samples were analyzed for total and differential WBC count (Mugantiwar et al., 1997; Sagle et al., 2004).

The grouping schedules are as follows.

Group 1: Served as control, received 10 ml/kg saline

Group 2: α-amyrin treated (5 mg/kg)

Group 3: α-amyrin treated (10 mg/kg)

Statistical analysis

Information was communicated as the mean standard mistake of mean (S.E.M.) of the methods and measurable investigation was completed utilizing single direction ANOVA. Contrasts between the information were viewed as huge at $P < 0.05$.

RESULTS

PRELIMINARY PHYTOCHEMICAL SCREENING

The preliminary phytochemical analysis revealed that different active constituent present in different extracts such as carbohydrates, proteins, amino acids, fat, oils, steroids, terpenoids, glycosides, alkaloids, tannins, and other phenolics compounds.

Table 1: Qualitative chemical analysis of different extracts by chemical tests

S.No.	Test	n-hexane	Dichloromethane	Methanolic	Aqueous
1.	Carbohydrate ➢ Molish test ➢ Felling test	- -	- -	- -	+ +
2.	Glycosides ➢ Bronteger test	-	-	+	+
3.	Alkaloid ➢ Mayer test ➢ Hager test	- -	+ +	+ +	- -
4.	Phytosterol + Triterpinoids ➢ Salkowaski test	-	+++	-	-
5.	Protein + Amino acid ➢ Biuret test ➢ Ninhydrin test	- -	- -	- -	- -
6.	Phenolic test ➢ Ferric test ➢ Lead acetate test	- -	- -	+ +	+ +
7.	Flavonoids ➢ Alkaline test	-	-	+	+

8.	Saponin ➢ Foam test	-	-	-	-

Bioactivity Guided Isolation of active compounds

Acute toxicity studies of fractions

Toxicity studies of all the fractions obtained from column chromatography were performed. The fractions were given in the dose of 1000 mg/kg, however, all the fractions showed some hypersensitivity reactions. So doses of all fractions were reduced and given in a dose of 750 mg/kg. On this dose, all the fractions didn't show any toxic symptoms. Hence 1/10th portion of 500 mg/kg i.e. 50 mg/kg was selected for further activity.

Phagocytic response of Fractions (F1-F4)

The technique was depicted by Cheng et al., (2005). Oral organization of various divisions (50 mg/kg) for 7 days and 10 min preceding carbon infusion displayed a portion related expansion in the leeway pace of carbon by the cells of the RES. F4 portion indicated most noteworthy phagocytic file 6.24±0.99 separately. The phagocytic record of control (bunch I) was 3.87±0.16.

Table 2: Effect of different fractions on Phagocytic Index

S No.	Groups	Treatments	Dose	Phagocytic Index
1	Group I	Control (10 ml/kg vehicle control)	-------	3.87±0.16
2	Group II	Fraction 1	50 mg/kg	3.14±0.12
	Group III	Fraction 2	50 mg/kg	5.37±0.26*
	Group IV	Fraction 3	50 mg/kg	4.11±0.24*
	Group V	Fraction 4	50 mg/kg	6.24±0.99***

On TLC studies of fraction 4, this fraction showed a single spot on TLC plate. Since in phagocytic response, this fraction also showed maximum activity so structure determination of this fraction was performed by IR, NMR, and MASS to identify the structure.

Characterization and Identification of compound

α-amyrin

IR (KBr) 3442, 3056, 2860, 2693, 2237, 2159, 1963, 1731, 1643, 1512, 1483, 1419, 1359, 1344, 1279, 1220, 1147, 1049, 946, 842, 657, 537cm^{-1}

1H NMR (400 MHz, Chloroform) δ 5.40 (s, 7H), 3.47 (s, 7H), 2.80 (s, 6H), 2.23 (s, 7H), 2.02 (s, 5H), 1.96 (dd, J = 9.5, 1.7 Hz, 27H), 1.85 (d, J = 19.4 Hz, 13H), 1.77 (s, 5H), 1.68 (dd, J = 24.5, 5.8 Hz, 1H), 1.74 – 1.21 (m, 122H), 1.21 – 1.16 (m, 18H), 1.03 – 0.99 (m, 41H).

^{13}C NMR (100 MHz, Common NMR Solvents) δ 143.02 (s), 125.43 (s), 78.57 (s), 57.72 (s), 54.69(s), 46.52 (s), 42.14 (s), 40.20 (s), 39.67 (s), 38.71 (s), 38.39 (s), 38.04 (d, J = 8.4 Hz), 37.47 (s), 34.06 (s), 32.86 (s), 32.39 (s), 29.16 (s), 27.68 (d, J = 8.7 Hz), 25.88 (s), 24.13 (s), 23.89- 23.67 (m), 23.36 (s), 19.61 (s), 18.79 (s), 18.02 (d, J = 16.2 Hz), 17.05 (s).

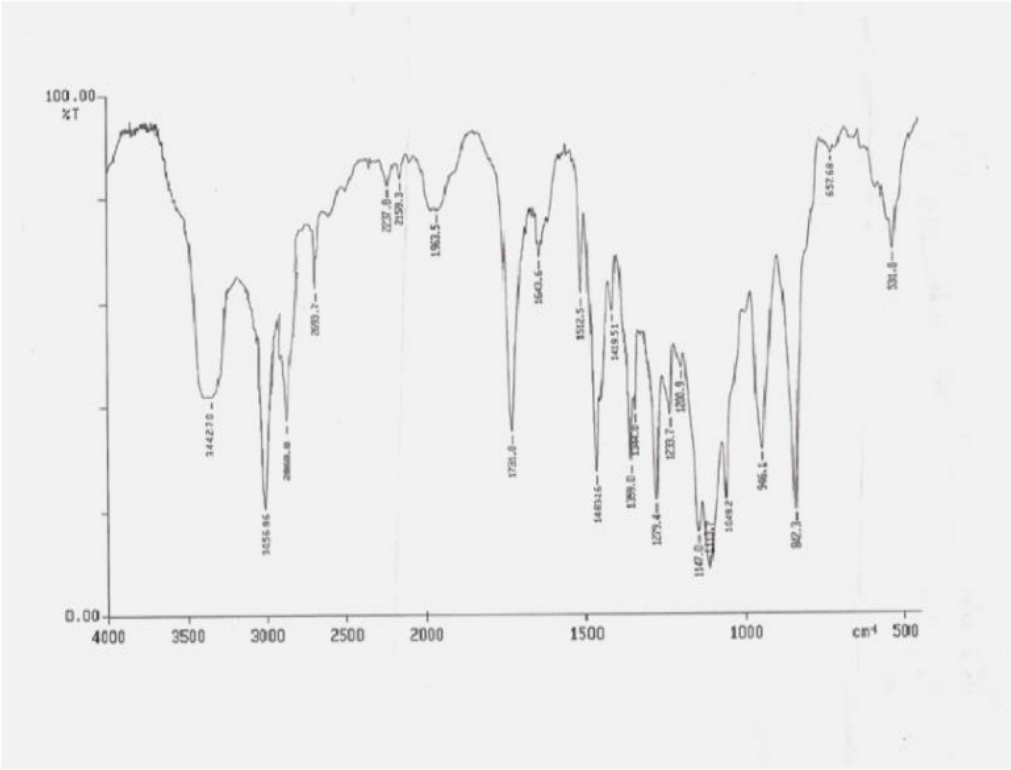

Figure 1: IR Spectra of α-amyrin

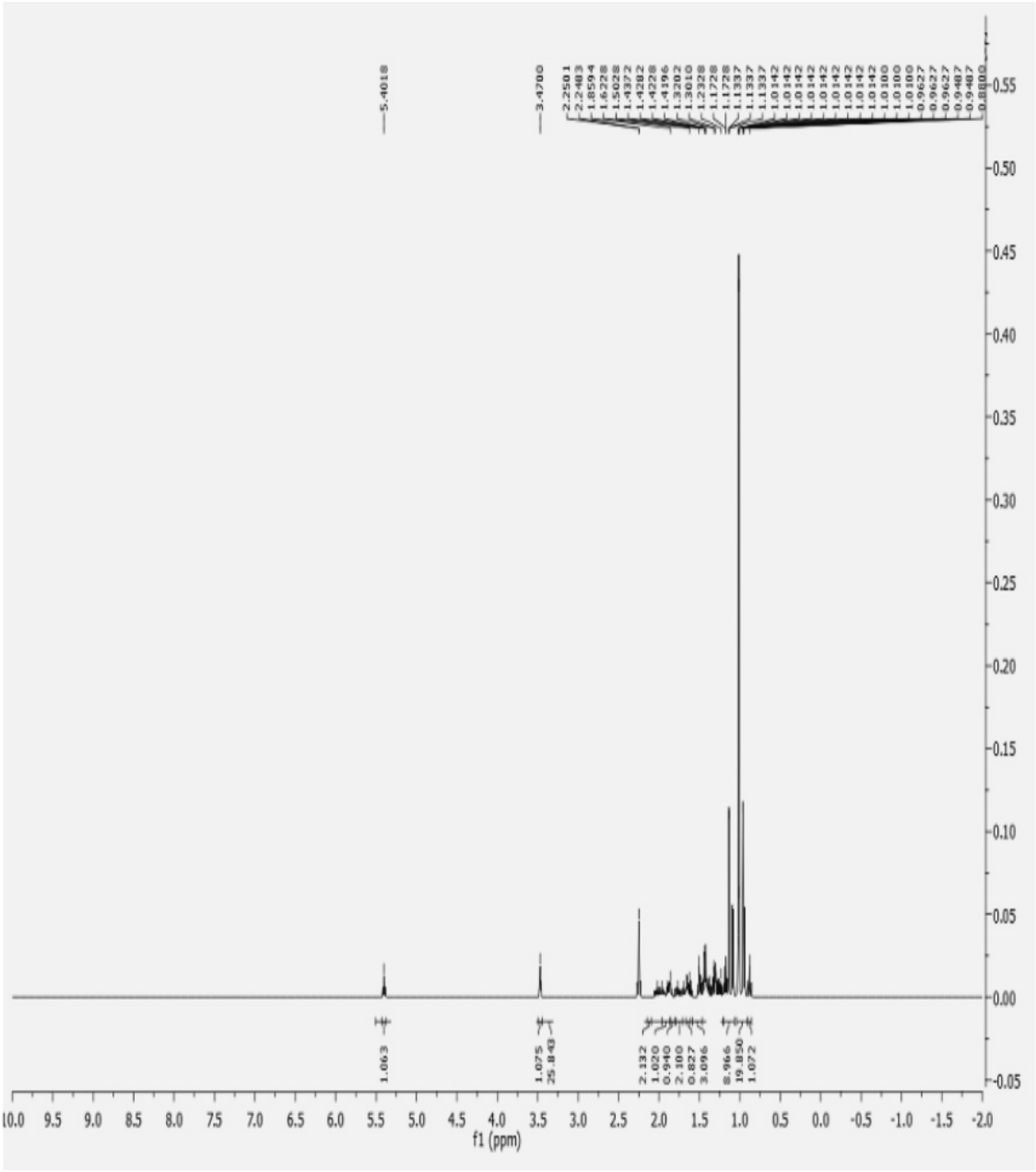

Figure 2: [1]H-NMR Spectra of α-amyrin

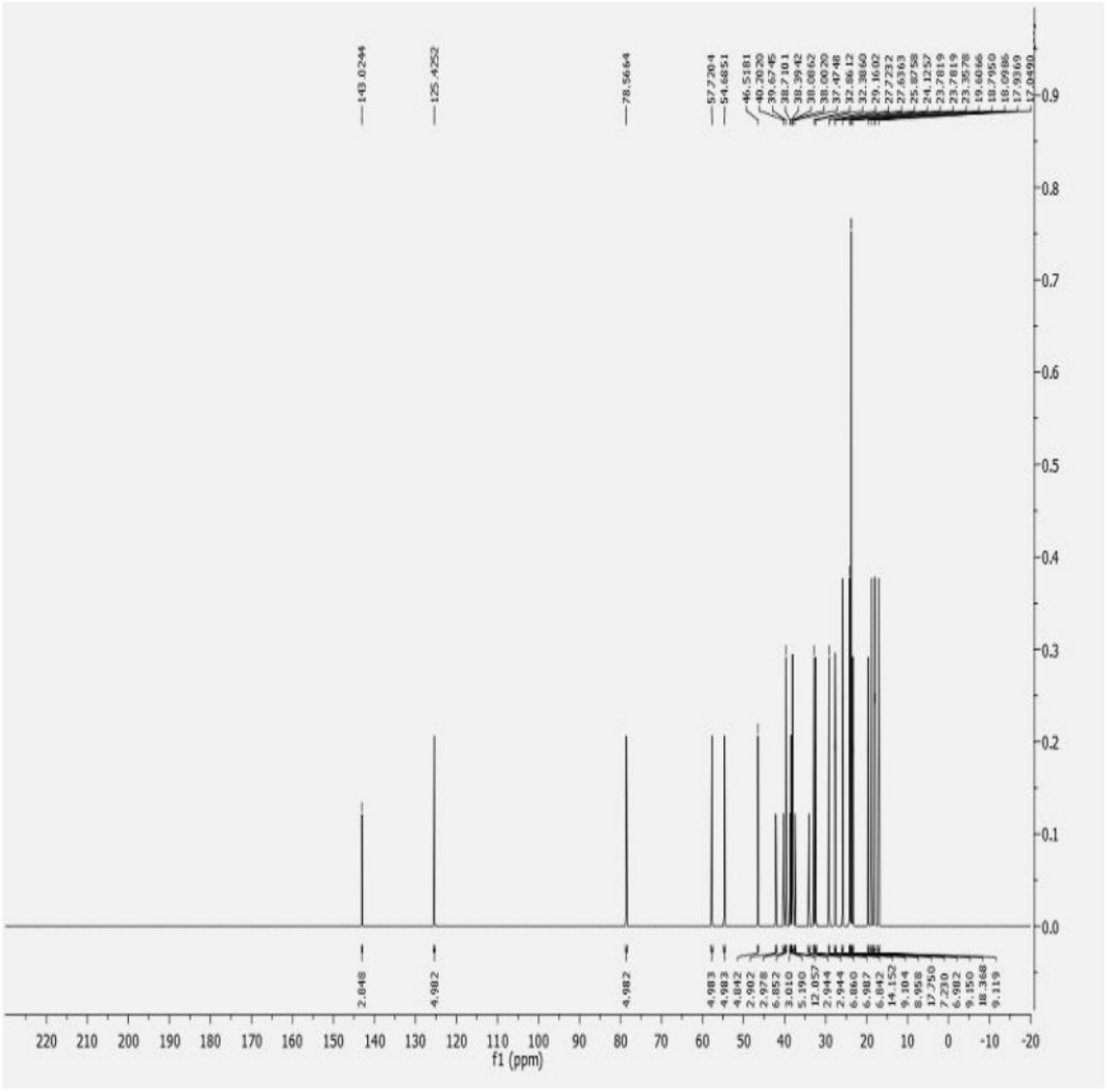

Figure 3: ^{13}C-NMR Spectra of α-amyrin

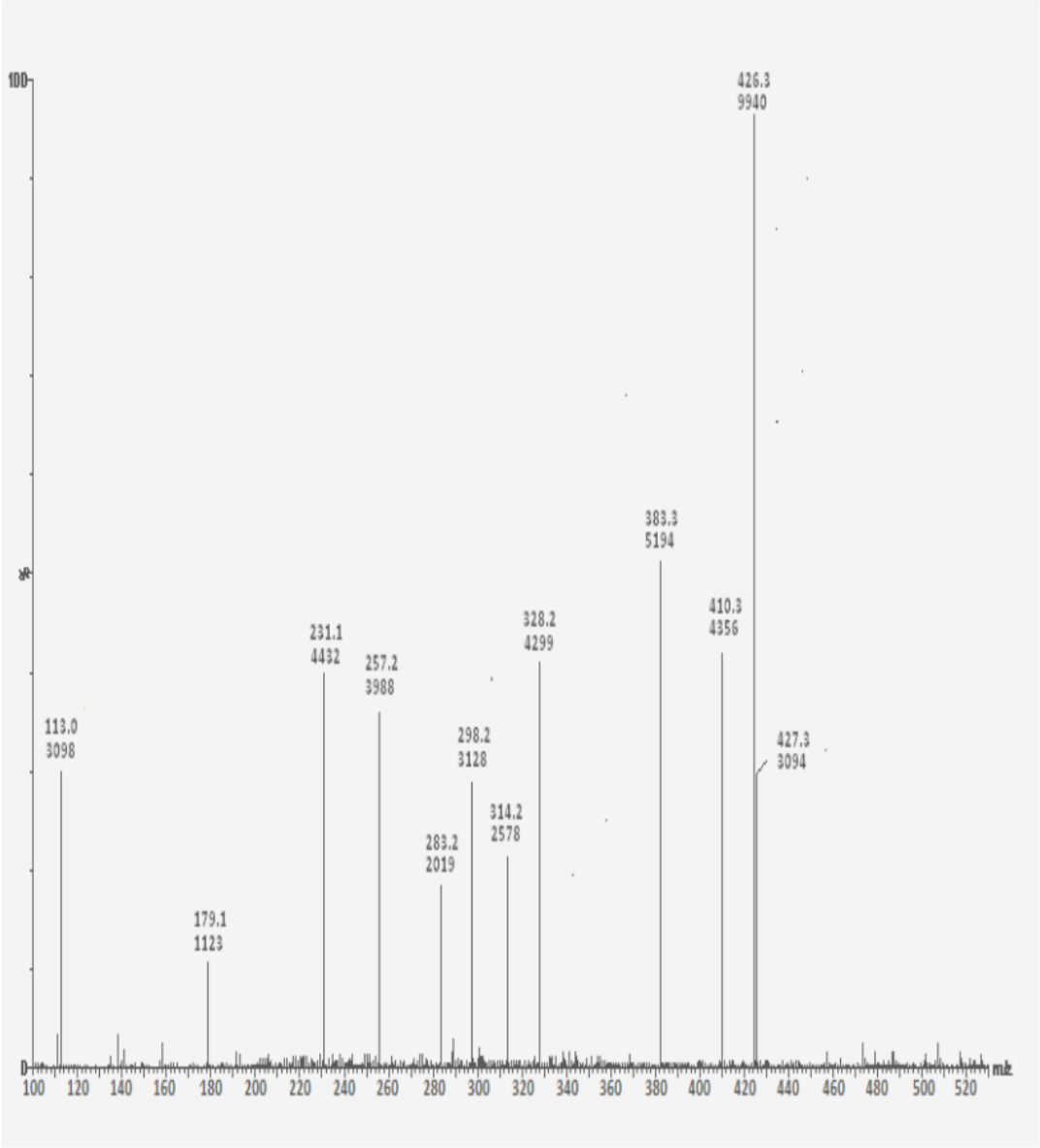

Figure 4: Mass Spectra of α-amyrin

In α-amyrin, IR absorption bands were appeared at 3442 cm^{-1} indicating the presence of hydroxyl group, 3056 cm^{-1} (C-H str. in CH_3), 2860 cm^{-1} (C-H str. in CH_2), 1731 cm^{-1} (C=O str.), 1483 cm^{-1} (C-H def. in CH_3), 1359 cm^{-1} (C-H deformation in gem dimethyl), 842 cm^{-1} (=C-H out plane bending). The ^{1}H- NMR spectrum shows that H-2 proton appeared at δ 3.47 as a multiplet and H-13 olefinic proton shows a singlet at δ 5.40. Also, eight methyl protons appeared as singlet as well as multiplet at δ1.13, δ 1.02, δ 1.01, δ 0.96, δ 0.94, 0.88 and δ 0.88 which were quite similar with α-amyrene as mentioned by Saha et al. The ^{13}C-NMR has shown recognizable

signals at 143.02 and 125.43ppm, which corresponds to double bond at C-12 and C-13. The δ value at 78.56 ppm is due to C-2 β- hydroxyl group. The peaks also showed that the isolated compound had eight methyl group, ten -CH_2 group and four –CH groups. The results were compared with the available literature and confirmed the presence of α-amyrin.

Acute toxicity studies of α-amyrin

α-amyrin didn't shown any toxic symptoms on 50 mg/kg, so 5 and 10 mg/kg was selected for further activity.

Cyclophosphamide-induced myelosupression

Cyclophosphamide at the portion of 30 mg/kg, i.p caused a huge decrease in the hemoglobin, RBCs, WBCs, and platelets check. In the case of cyclophosphamide-induced myelosuppression, there was decrease in the WBC count in the control group. In treatment groups, the WBC count was found to be increased with $p<0$ .01and $p< 0.001$ at 10 and 50 mg/kg dose respectively on the 11th day. There was no alteration in other hematological parameters like RBC, HB, HCT, and MCV.

Table 3: Effect of α-amyrin on WBCs and RBCs

S No.	Treatments	RBCs	WBCs	HB
		11th Day	11th Day	11th Day
1	Control	7.45±0.18	2.36±0.15	10.62±1.33
2	Cyclophosphamide	4.11±0.26**	0.76±0.24***	7.57±0.37**
3	α-amyrin (5 mg/kg)	5.42±0.32*	1.13±0.66**	8.65±0.61*
4	α-amyrin (10 mg/kg)	7.15±0.69**	1.98±0.72**	8.97±0.49*

Values are expressed as mean±SEM, n=6 in each group; * p <0.05, compared to control ** p<0.01, compared to control. *** p <0.001, compared to control.

E. coli induced abdominal sepsis Model

In E. coli induced abdominal sepsis, mortality due to peritonitis was evaluated in control and treatment groups. There was 100% mortality in the control group. In the groups treated with α-amyrin 10 mg/kg body, wt 36 % mortality was observed respectively. The effect of the compound on WBC and % neutrophils was evaluated. There was a dose-dependent increase in WBC and % neutrophils in treated groups.

Table 4: Effect of α-amyrin on *E. coli* induced abdominal sepsis

S. No.	Treatments	WBC 10^3 / mm3)	Neutrophils (%)
1	Control	2.85±0.64	10.12±2.13
2	α-amyrin (5 mg/kg)	3.27±0.65*	12.06±0.67*
3	α-amyrin (10 mg/kg)	3.72±0.19**	13.65±1.13**

Values are expressed as mean±SEM, n=6 in each group; * p <0.05, compared to control ** p<0.01, compared to control. *** p <0.001, compared to control.

DISCUSSION

An immunomodulator can be characterized as a substance, organic, or manufactured, which can animate or adjust any of the parts of the invulnerable framework including both inborn and versatile arms of the resistant reactions.

Triterpenoids have been read for their calming, hepatoprotective, pain-relieving, antimicrobial, antimycotic, virostatic, Immunomodulatory and tonic impacts. They are utilized in the counteraction and treatment of hepatitis, parasitic, and protozoa contaminations or more all, for their cytostatic impacts (Petr et al., 2006). The mitigating impacts of triterpenoids are to a great extent attributed to their capacity to hinder arachidonate 5-lipoxygenase (5-LO) and human leukocyte elastase (HLE) just as their potential for balancing the insusceptible reaction by influencing supplement and immunizer creation. Lipoxygenase is one of the main proteins that are engaged with the union of leukotrienes and this particle is engaged with different issues, for example, extreme touchiness, for example, asthma, joint pain, ulcerative colitis, and issues of the cardiovascular framework for example stun and ischemia of the myocardium (Wasserman, 1988). The supplement is another framework that might be affected by triterpenoids.

On the column chromatography, four fractions were obtained from flower extract. In the phagocytic index model, fraction 4 showed maximum activity. On phytochemical screening, fraction 4 showed presence of terpenoids. α-amyrin and rosmarinic acid were isolated from fraction 4.

α-amyrin:

IR (KBr) 3442, 3056, 2860, 2693, 2237, 2159, 1963, 1731, 1643, 1512, 1483, 1419, 1359, 1344, 1279, 1220, 1147, 1049, 946, 842, 657, 537cm^{-1}

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^{13}C NMR (100 MHz, Common NMR Solvents) δ 143.02 (s), 125.43 (s), 78.57 (s), 57.72 (s), 54.69(s), 46.52 (s), 42.14 (s), 40.20 (s), 39.67 (s), 38.71 (s), 38.39 (s), 38.04 (d, J = 8.4 Hz), 37.47 (s), 34.06 (s), 32.86 (s), 32.39 (s), 29.16 (s), 27.68 (d, J = 8.7 Hz), 25.88 (s), 24.13 (s), 23.89- 23.67 (m), 23.36 (s), 19.61 (s), 18.79 (s), 18.02 (d, J = 16.2 Hz), 17.05 (s).

Cyclophosphamide suppresses the humoral, cellular, non-specific, and specific cellular immune response. When the animal was treated with cyclophosphamide then hemoglobin (Hb), RBC counts, WBC count, Lymphocyte%, and Platelet count all are reduced significantly (Doherty, 1981, Gill and Liew, 1978, Habibullah et al., 1979). The suppressive effect of cyclophosphamide was protected by the administration of the isolated compounds. Flavonoids in biological systems tend to adhere to the molecules of cyclophosphamide this causes to increase in the size of the

molecules and prevent their entry to the stem cells. As already stated that such compounds are detected in the plant extract besides this some more compounds are there which are not only negating the effect of cyclophosphamide but also accelerating the total WBC and hemoglobin count. The amyrin significantly produces the changes in WBCs, RBCs, and hemoglobin levels. This suggests that the constituent of the plant preventing the access of cyclophosphamide to the stem cells so that the synthesis of hemoglobin, WBC, and RBC is not inhibited. Another point is that the compound is reutilizing this immunosuppressant before it could act upon hemopoietic and myeloid tissue.

Intense bacterial peritonitis is a dangerous condition portrayed by presence of microscopic organisms in the germ-free peritoneal pit. Host guard is an old-style space of the natural invulnerable framework as a fast reaction to microorganisms is basic for the host to endure. Treatment of this condition has focused on surgery, antibiotics, and nutritional support. But in spite of this, fatal complications have been reported. A factor that influences the recovery is the host defense mechanism. In E. coli induced abdominal sepsis, the protection offered by the isolated compound could be attributed to secretion of IL-1 and GM-CSF from activated macrophages. Activated macrophages secrete a number of cytokines like IL-1 and GM-CSF which in turn stimulates other immunocytes like neutrophils. The amyrin significantly protected the animals against peritonitis. Both also showed a significant increase in WBC and neutrophils thus it may have a humoral immune response potentiating effect.

CONCLUSION

Besides the obvious therapeutic importance, these components would be useful in understanding the mechanism of diseases with higher levels of cellular and molecular level. These components could serve as lead molecules for the development of prospective immunomodulator agents. Further detailed studies are required to elucidate the exact mechanism based on the molecular and genetic level responsible for the immunomodulatory activity. The current discoveries are huge for the advancement of option, reasonable, and maybe more secure techniques for the treatment of infections. A detailed study is also required on structure determination of the compounds from bioactive fractions in order to find the structure-activity relationship.

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ORIGINALITY REPORT

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