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Immunomodulatory, antileishmanial and phytotoxicity of *Arisaema Jacquemontii* Blume plant extracts

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ABSTRACT

ArisaemajacquemontiiBlume (Araceae) is reputed for its physiological properties in the folk medicine system and used in Chinese herbal medicine as anti-convulsant. Methanolic extracts of roots and fruits, the chloroform extracts of roots and shoots of ArisaemajacquemontiiBlume were investigated for immunomodulatory,antileishmanialand phytotixic activities. Immunomodulatory activitywas investigated using luminol based chemiluminescence assay. Present study demonstrated the effect of these extracts on ROS production of human whole blood and isolated polymorphoneutrophils after activation with Serum opsonized Zymosan. Methanol and chloroform extracts of roots of the plant possess significant inhibitory activity while methanol extract of fruits and chloroform extract of shoots of the plant possess moderate inhibitory activity.Antileishmanial activities of the chloroform and methanol extracts were evaluated by 96-well micro titer plate method. The chloroform extract of the roots of the plant have shown significant antileishmanial activity while the methanol extracts of the shoots and fruits have shown low antileishmanial activity. Phytotoxic activities of same extracts were tested against the Limnaminorusing themodified protocol of McLaughlin bioassay. Methanolicextracts of fruits and roots and chloroform extract of shoots and roots were studied for their phytotoxic activity. The chloroform extract of shoots of the plant showed 100 % inhibition at concentration of 1000µg/mL while chloroform extract of the roots and methanol extract of fruits of the plant showed moderate activity at the highest dose.

Key words: *Arisaemajacquemontii*, immunomodulation, antileishmanial and phytotoxicity.

INTRODUCTION

The genus *Arisaema* is known by its 150 species of which 14 are found in Pakistan [1] The plants of the genus are reputed for their physiological properties and are frequently used in folk medicine system as an anti-convulsants agent [2]. *Arisaemajacquemontii*Blume is an herb found in the northern regions of Pakistan and Kashmir. It is also found in upper forest and lower alpine zone in the drier areas of Himalayas in the range of 2400-4000 meters [3]. The plant is known as toxic plant as it resembles the snake, Cobra and is therefore locally named after the snake as "sap-ki-booti" (snake herb). The plant contain minute needle like crystals of calcium oxalate, which are intensely irritating when brought to contact with the mucus membrane of the mouth, nose and throat, or with tender skin. But they are easily neutralized by thoroughly drying or cooking the plant or by steeping it in water [4]. *A..jacquemontii* have immense medicinal importance. The juice from the tubers of the plant is applied to the skin by the Khasi and Garo tribes of Meghalaya in India for the treatment of ring worms and other skin diseases [5]. A tuber lectin having potent anti-insect and anti-proliferative activity was purified from the plant [6-7].Roots and fruits of the plant are used in psychic and nervous disorder. A chemical literature survey revealed the isolation of two tetra cyclic triterpenoid and substituted benzophenol (arisaemanone) from the plant [8]. Earlier we have reported the activity of

methyl ethyl ketone and methanol extracts of the plant in *rad 6* and *rad 52* yeast assays [9]. Keeping in view its important biological activities [10], we have been investigating the plant for the last twenty years [11]. Our recent investigations have resulted the immunomodulatory, antiliashmanial and phytotoxic activities of different extracts of plant.

MATERIALS AND METHODS

Collection, Authentication and Extraction of Plant Material

*Arisaemajacquemontii*Blume(Araceae), fresh whole plants were collected from Nathiagalli forests, Abbottabad Khyberpakhtunkhwa, Pakistan in September 2009 and authenticated by the plant taxonomist at the Department of Botany, University of Azad Jammu and Kashmir, Muzaffarabad (AJK) Pakistan. The voucher specimen NoXX-278has been kept in the herbarium of the department.The collected plant material, roots, shoots and fruits of the plant were washed thoroughly, dried in shade and milled separately. The plant material, the dried roots, shoots and fruits of 4 kg each were soaked in chloroform and methanol respectively for three days at room temperature. The process was repeated thrice in order to get maximum quantity of extracts. Pooled solvents were removed under reduced vacuum at 60 ^{0}C ± 1 ^{0}C. All extracts were kept in well stopper glass container for further study.Prelimnary phytochemical screening was carried out on the extracts using standards screeningmethods [12].

Phytochemical screening

Prelimnary phytochemical screening for various secondary metabolites such as cardiac glycosides, tannins,saponin, terpenes and long chain esters were carried out for methanol and chloroform extracts of roots, shoots and fruits of the plant, using the usual procedures [12].

Immunomodulatory activity

Reagents, Chemicals and Equipments

Luminol (3- aminophthalhydrazide) was purchase from Research Organics, while Hanks Balance Salts Solution (HBSS) were purchased from Sigma, Germany. Lymphocytes Separation Medium (LSM) was purchased from MP Biomedicals Inc. Germany. Zymosan-A was obtained from Fluka (Biochemika). Dimethylsulphoxide (DMSO), ethanol and ammonium chloride of analytical grades were purchased from Merck Chemicals, Darmstadt, Germany. The luminometer used was Luminoskan RS Finland.

Isolation of Humannpolymorphoneutrophils (PMNs)

Heparinized blood was obtained by vein puncture aseptically from healthy volunteers. Blood mixed with Hank's Balance salt solution [Ca and Mg free] ($HBSS^{--}$) and LSM. After 30 mints RBCs were settle down and plasma layer taken and layered on LSM. After centrifugation of 30 mints supernatant was discarded and palate was taken which is mixture of PMNs and RBCs. RBCs was lysed adding 1 mL Distilled water and this lysis is stoped by adding 1 mL $HBSS^{--}$ 2x and washed by adding $HBSS^{--}$. After centrifugation of 10 mints PMNs was setteled down in white pallet. Viability was cheked by Trypan Blue and cell counted by using Hemocytometer and microscope. Cells were adjusted to their required concentration using Hank's Balance salt solution containing Ca and Mg ($HBSS^{++}$) pH, 7.2.

Chemiluminescence Assay

Luminol-enhanced chemiluminescence assay were performed as described by Helfand et al. (1982) [13]. Briefly 25 µL diluted whole blood (1:50 dilution in sterile $HBSS^{++}$) or 25 µL of PMNs (1 x 10^6) cells were incubated with 25 µL of serially diluted plant extract with concentration ranges between 12.5 to 200 µg/mL. Control wells have received $HBSS^{++}$ and cells without extracts. Tests were performed in the white 96 well plates. Plates were incubated at 37 ^{0}C for 20 minutes in the thermostated chamber of the Luminometer. Serum Opsonized Zymosan 25 µL, followed by 25 µL luminal 7 x 10^5 M) $HBSS^{++}$ was added to each well to obtain a 100 µL volume / well. The Luminometer results were monitored as chemiluminescence RLU with peak and total integral value set with repeated scans at 60 seconds interval and one second points measuring time.

Statistical analysis

Student T-test was performed to compare the significance mean difference between the control and tested extracts for various chemiluminescence results. The differences considered to be significant at level of $P \leq 0.05$.

Chemiluminescence study of extracts with whole blood

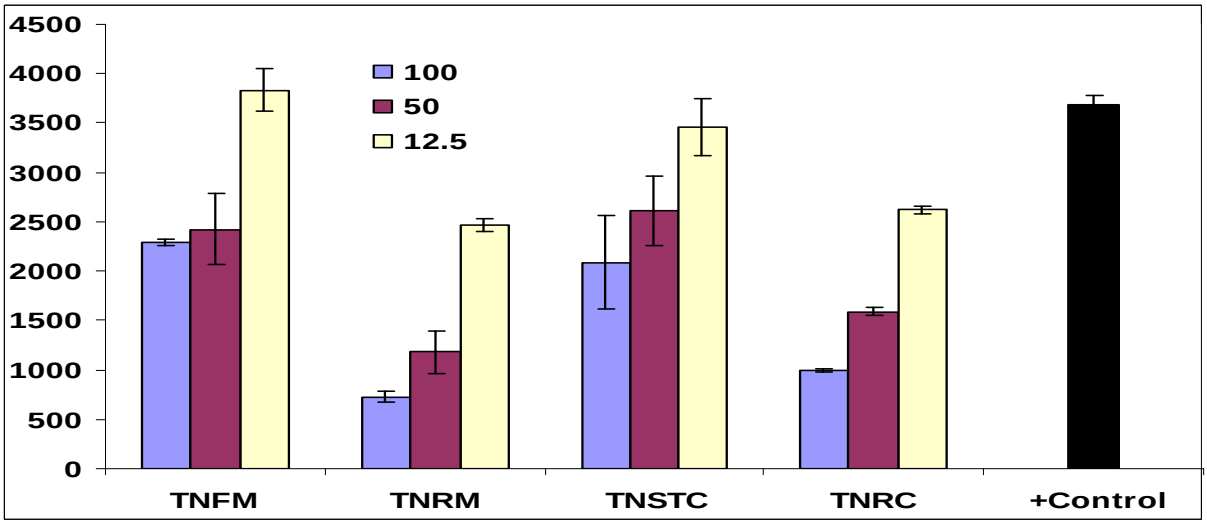

Figure-1: Effect of extracts TNFM, TNRM, and TNSTC & TNRCon oxidative burst of whole blood
TNFM = Methanol extract of fruits, TNRM = Methanol extract of roots. TNSTC = Chloroform extract of shoots. TNRC = Chloroform extract of roots.

Chemiluminescence study of extracts with PMNs by using luminol substrate

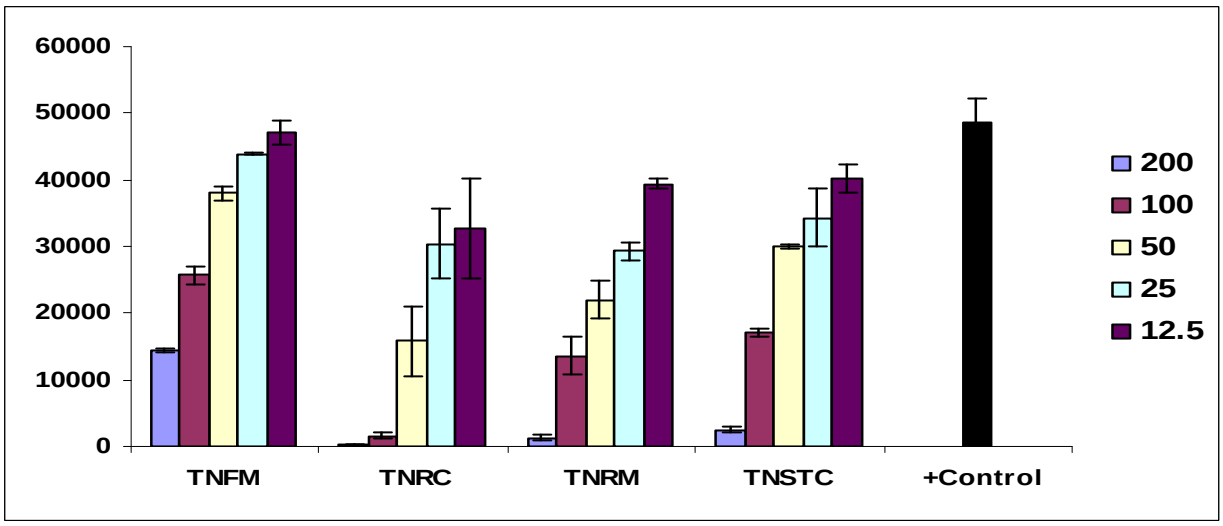

Figure-2: Effect of extracts: TNFM, TNRM, TNSTC & TNRC on oxidative burst of Activated PMNs.
TNFM = Methanolic extract of fruits.TNRM = Methanolic extract of roots.
TNSTC = Chloroform extract of shoots.TNRC = Chloroform extract of roots.

***Antialishmanial*activity**

Leishmania Promastigotes are grown in bulk early in modified NNN biphasic medium using normal physiological saline. leishmania parasite Promastigotes were cultured with RPMI 1640 medium supplemented with 10% heat inactivated foetal bovine serum (FBS). Parasites at log phase are centrifuged at 2000 rpm for 10 minutes and washed three times with saline at same speed and time. Parasites are diluted with fresh culture medium to a final density of 10^6 cell / m L.In a 96-well micro titer plate, [14] 180 µL of medium was added in different wells. 20µL of the extract of the plant was added in medium and serially diluted. 100µL of parasite culture was added in all wells. Two rows were left for negative and positive control. Negative control received medium while the positive control contains varying concentration of standard antileishmanial compounds e. g. Amphotericin B and Pantamidine. The plate was incubated between 21-22^0 C for 72 hours. The culture was examined microscopically on an improved neubauer counting and IC_{50} values of the extracts possessing antileishmanial activity were calculated by Software Ezfit 5.03 perella Scientific. All assays were run in duplicate. Percent mortality was observed by following formula.

Percent mortality = 100 -No. of parasites ($_{test}$)x 100
No. of parasites $_{(negative\ control)}$

Table-I:*Antileishmanial* activity of chloroform extract of roots of *A.jacquemontii.*

S. No.	Leishmanicidal Activity	IC_{50} (µg / mL) ± S.D
1	Test sample	20.92 ± 0.28
	Standard drugs used	
2	Amphotericin B	0.50 ± 0.02
3	Pentamidine	2.56 ± 0.09

Test organism Leishmania major (DESTO). Incubation period was 72 hours and incubation temperature was 22 ^{0}C. Sample showed significant antileishmanialactivity.

Table-2:Antileishmanial activity of mathanol extract of shoots of A. *jacquemontii.*

S. No.	*Leishmanicidal* Activity	IC_{50} (µg / mL) ± S.D
1	Test sample	81.59 ± 1.32
	Standard drugs used	
2	Amphotericin B	0.50 ± 0.02
3	Pentamidine	2.56 ± 0.09

Test organism *Leishmania* major (DESTO). Incubation period was 72 hours and incubation temperature was 22 ^{0}C. Sample showed low *antileishmanial* activity.

Table-3:Antileishmanial activity of methanolic extract of fruits of *A.jacquemontii*.

S. No.	Leishmanicidal Activity	IC_{50} (µg / mL) ± S.D
1	Test sample	83.33 ± 0.37
	Standard drug used	
2	Amphotericin B	0.50 ± 0.02
3	Pentamidine	2.56 ± 0.09

Test organism Leishmania major (DESTO). Incubation period was 72 hours and incubation temperature was 22 ^{0}C. Sample showed low antileishmanial activity.

Phytotoxic Assay Protocol

The *Limna*bioassay was carried out the using the modified protocol of Mclaughlin [15.-16]. The limna minor (duckweed) were cultivated under optimum condition for 1 and 2 days, briefly washed in water and transferred into the E-medium. This E-Medium was arranged by addition of a variety of constituents in 1L distilled water. The KOH pellets were added to maintainthe pH between5.5 to 7.0. Stock solution 100 mL was dissolved in 900mL of distilled water to obtain working E. medium. The crude extract 30 mg was dissolved in 1.5 mL of methanol, from which 10, 100 and 1000µL of solution was transferred to three flasks and the solvent was allowed to evaporate. The working E-medium 20 mL and 20 fronds of lemna minor were added in each flask. These flasks as well as positive (standard drug of plant growth inhibitor + 20 fronds of *Lemna minor*) and negative (solvent + 20 fronds of *lemna minor*) control were placed in growth chamber for seven days and results were recorded. Formula that was used to work out the percent growth inhibition is

Percent growth inhibition = 100 -No. of fronds in testx 100
No. of fronds in control

RESULTS AND DISCUSSION

The results of phytochemical screening revealed the presence of terpenes. saponins and glycosides in the extracts. The methanolic extracts of roots, fruits and chloroform extracts of roots, shoots with ethno medicinal uses, were tested for their *in vitro*immunomodulatory activities. In the current study we used two probes (luminol and lucigenin) that can detect the reactive oxygen species (ROS). Luminol is characterized by its ability to enter the cell and react with the intercellular ROS [17]. This inhibitory activity could be due to the ability of the extracts to block the complement receptor, consequently inhibiting NADPH oxidase, a cell membrane associated enzyme. As zymosan-A activates phagocytes by binding to complement receptor type 3 on the cell surface; this however, differs from the PMA which acts directly on the intercellular messengers, activating the production of O_2^- through the NADPH oxidase [17]. Immunomodulatory activity studies results on the whole blood showed that methanol and chloroform extracts of roots of the plantexhibited significant inhibitory activity at the initial screening doses (12.5, 50 and 100 µg / mL). While methanol extracts of fruits and chloroform extract of shoots of *A. jacquemontii* showed remarkable increase in cell oxidative burst response. All the four extracts were further investigated for their effect on zymosan-A activated polymorphoneutrophils (PMNs). All the extracts showed a dose dependant effect with > 90 % inhibition at the highest dose concentration (200 µg/mL) except the methanol extract of fruits of the plant which showed 50 % inhibitory activity.Theantileishmanial activities of roots, shoots and fruits extracts of the plant and standarddrugs used as reference compoundsare shown in tables I, II and III. The test organism wasLeishmania major (DESTO). Incubation period was 72 hours and incubation temperature was 22 ^{0}C.The chloroform extract of the roots of *Arisaemajacquemontii*Blume have shown significant antileishmanialactivity. ItsIC_{50} value was 20.92 ± 0.28 µg / m L. Whereas methanolic extracts of shoots and fruits of the plant have shown low antileishmanial activities. Its IC $_{50}$ value were 81.59 ± 1.32 µg / mL± S.D and 83.33 ± 0.37 µg / mL± S.D respectively. Amphotericin B IC_{50} = (0.50 ± 0.02 µg / m L) and PentamidineIC_{50} = (2.56 ± 0.02 µg / mL) were used as standard drugs.. Its IC_{50} value indicates the effective concentration of a compound in µg / mL necessary to achieve 50 % growth inhibition. The activity spectrum of the extracts showed that the plant as a whole possesses significant antileishmanial activity. Herbicides, Paraquate (0.075µg/mL) causes a significant phytotoxic effect on *lemna minor* and used as standard phytotoxic agent in the assay. Methanolicextracts of fruits and roots and chloroform extract of shoots and roots of *Arisaemajacquemontii* were studied for their phytotoxic activity. The chloroform extract of shoots of the plant showed 100 % inhibition at concentration of 1000µg/mL while chloroform extract of the roots and methanol extract of fruits of the plant showed moderate activity at the highest dose.

CONCLUSION

To the best of our knowledge there is no earlier report on immunomodulatory. Antileishmanial and phytotoxicity of the plant. The results of these studies indicate that extract are relatively non toxic. Chloroform extract of roots of the plant showed significant immunomodulatory and antileishmanial activities. While the chloroform extract of shoots of the plant showed 100 % phytotoxic activities at concentration of 1000µg/mL. Further investigations are progressing for isolation of secondary metabolites responsible for observed activities in the extracts and these will be reported at latter date..

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