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Evaluation of Antioxidant and Anti-Inflammatory Activity of Methanolic and Aqueous Extract of *Arisaema propinquum* Schott Rhizomes

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Abstract

Objective: The present study was carried out to evaluate the antioxidant and *in-vitro* anti-inflammatory activity of *Arisaema propinquum* Schott rhizomes.

Methods: The alcoholic and aqueous extracts were first tested for phytochemical screening. *In-vitro* antioxidant activity of extracts was evaluated by DPPH free radical (1, 1-diphenyl-2-picryl-hydrazyl) scavenging assay, reducing power method and hydrogen peroxide (H_2O_2) scavenging assays. To investigate the *in-vitro* anti-inflammatory activity of the extracts HRBC (Human Red Blood Cell) membrane stabilization method and percentage protein denaturation inhibition method was used.

Results: Phytochemical screening of the extracts reveals the presence of alkaloids, carbohydrates, cardiac glycosides, coumarins, proteins, amino acids, phenols, tannins, flavonoids, saponins, steroids and terpenoids. The total phenolic and flavonoid content in methanolic and aqueous extract was found to be 554.06 ± 131.60 and 425.21 ± 101 mg/g Gallic Acid Equivalents (GAE) and 324.71 ± 99.84 and 225.56 ± 42 mg/g Rutin Equivalents (RE) respectively. The methanolic extract showed potent percentage inhibition of DPPH and H_2O_2 scavenging activity as compared to standards rutin and ascorbic acid. Both the extracts showed good membrane stabilizing as well as protein denaturation activity when compared with the standard Indomethacin.

Conclusion: The results of the present study demonstrate the antioxidant and anti-inflammatory potential of the methanolic and aqueous extracts of *Arisaema propinquum* Schott rhizomes. However, it is necessary to isolate and purify the active principles responsible for pharmacological activity to determine its mechanism of action.

Keywords: *Arisaema propinquum*; Antioxidant; Phytochemical screening; Anti-inflammatory

Introduction

Free radicals or Reactive Oxygen Species (ROS) produced in our body as a result of biological oxidation and metabolism normally scavenged by endogenous defence systems such as superoxide dismutase, peroxidase-glutathione and catalase system [1-3]. The excess production of free radicals cause damage to the body and contribute to oxidative stress which leads to degenerative diseases including cancer, coronary artery disease, hypertension, ageing, diabetes immunosuppression, neurodegenerative diseases and others [4,5]. Antioxidants are the substance that significantly prevent or delay the oxidation of other molecules by scavenging the free radicals and inhibiting the process of oxidizing chain reactions, and therefore they are very important [6]. Inflammation is a defensive response involving immune cells, blood vessels, and molecular mediators against any infection or stimuli that initiates tissue damage [7]. The main utility of this process is to initiate tissue repair by eliminating the initial cause of cell injury and clear out necrotic cells [8]. Inflammation, a natural healing process, when it becomes excessive and prolonged, leads to extensive tissue damage, organ dysfunction, and mortality [9]. The oxygen free radicals generated inside the body play a vital role in the progression of inflammation in distant organs by the activation of nuclear factors, NFκB or AP-1, that induce the synthesis of cytokines. By the synergistic action between free radicals and cytokines, endothelial cells are activated, which promote the synthesis of inflammatory mediators and adhesion molecules. Finally, free radicals exert their toxic effects at the site of inflammation by reacting with different cell components, inducing loss of function and cell death [10].

Arisaema propinquum (Wallich's cobra lily or cobra lily) is a tuberous plant which belongs to the family of Araceae and locally (Kashmiri) known as *Hapat-makei* or *Hapat-mundh*. The plant grows at an altitude of 2400-3600 m found in the Himalayas, from Kashmir to SE Tibet. The plant is a flowering species grows under shady sites with soil rich in humus, up to 5-6 feet in height, with three large yellowish green glossy leaves covering the flower or cobra hood (spathe). The spathe or hood is dark purple in colour striped with white bands which is thick at the base and ends in a pointy beak comprised 8-20 cm wipe like long tongue emerging out from inside, hanging down from the front of the spathe. At the curvature of the hood there is a white netting effect which presents the side edges like a stained window. Leaf stalk and stem are often brown-spotted. Flowering occurs in May-June. The plant is used as food material and in ethano-medico practice by tribals [11].

Materials and Methods

Collection of plant material and preparation extracts:

The rhizomes of *Arisaema propinquum* Schott was collected in July 2013 from Doodhpathri, Dist. Budgam, of Kashmir Valley. The plant was authenticated by the Centre of Plant taxonomy, Department of Botany, University of Kashmir, Hazratbal, and specimen was kept in KASH

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herbarium under specific voucher number 1895-KASH. The plant material was dried under shade and then crushed to coarse powder. The powdered drug material (500 g) was taken in a Soxhlet apparatus for (hot extraction) using methanol as solvent for 12 hours. The crude extract was filtered through Whatman No-2 filter paper. After filtration the organic phase was independently concentrated under vacuum by evaporating to dryness. The preliminary phytochemical analysis of the various fractions was carried out as per the methods of [12-14].

Source of chemicals:

All the chemicals used were of analytical grade and were procured from registered dealers like HiMedia Laboratories Pvt. Ltd .Mumbai, Central Drug House Ltd. New Delhi, India.

Antioxidant activity:

Determination of DPPH free radical scavenging:

The free radical scavenging capacity of aqueous and alcoholic extracts of *Arisaema propinquum* was determined using [15] method with slight modification. Different concentrations of extracts (20 µg/mL to 100 µg/mL) were added to freshly prepared DPPH (2,2-diphenyl-1-picrylhydrazyl), solution in test tubes. The reaction mixture was incubated at room temperature for 30 min. After incubation the absorbance was read at 517 nm using a spectrophotometer. Ascorbic acid was used as standard. Control sample was prepared containing the same volume without any extract or standard. Methanol served as blank [16].

Determination of reducing power: The reductive capability of the extracts was quantified by the method of [17]. Different concentrations of *A. propinquum* extracts (100-500 µg) were prepared in distilled water and were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. Similar concentrations of standard ascorbic acid were used as standard. The mixtures were incubated at 50°C for 20 min. After incubation 2.5 mL of 10% trichloroacetic acid was added to the mixture. The whole reaction mixture was then centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and 0.5 mL of 0.1% $FeCl_3$. Blank reagent was prepared in the earlier mentioned manner without adding extract. The absorbance was measured spectrophotometrically at 700 nm against a blank sample. Increased absorbance of the reaction mixture indicated greater reducing power [18].

Scavenging of hydrogen peroxide: The ability of the *A. propinquum* extracts to scavenge hydrogen peroxide was determined according to [19] with slight modification. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4).Different dilutions of extract 250-1250 µg/mL prepared in distilled water were added to a hydrogen peroxide solution (0.6 mL, 40 mM). The absorbance of mixture was determined at 230 nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenged by the extracts and standard compounds were calculated as follows:

$$\% \text{ Scavenged}\left[H_2O_2\right] = \left[\left(Ao - A1\right)/Ao\right] \times 100$$

Where Ao is the absorbance of the control and A1 is the absorbance in presence of sample of extract and standard [20].

Total phenolic content: The concentration of total phenolics in alcoholic and aqueous extract of *Arisaema propinquum* was determined by Folin–Ciocalteu method [21]. Crude alcoholic and aqueous extracts of *Arisaema propinquum* were dissolved in distilled water at a concentration of 1 mg/mL. The reaction mixture was prepared by mixing 0.5 mL of extract, 2.5 mL of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 mL of 7.5% $NaHCO_3$. Blank was concomitantly prepared, containing 0.5 mL methanol, 2.5 mL of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 mL of 7.5% $NaHCO_3$. The samples were then incubated for 45 min at 45°C. Absorbance was measured at 765 nm. The samples were prepared in triplicates for each analysis and the mean value of absorbance was obtained. Gallic acid was used as standard. The total phenolic content was calculated from the calibration curve, and the results were expressed as mg of gallic acid equivalent per g dry weight.

Total flavonoid content: The total content of flavonoids in plant extracts was determined using [22] method. The sample was prepared in methanol in the concentration of 1 mg/mL. The reaction mixture contained 1 mL of alcoholic solution and 1 mL of 2% $AlCl_3$ solution dissolved in methanol. The samples were incubated for an hour at room temperature. After incubation absorbance was determined at 415 nm using spectrophotometer. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of rutin. Based on the measured absorbance, the concentration of flavonoids was read (mg/mL) on the calibration line; and was expressed in terms of rutin equivalent (mg of RE/g of extract) [23].

In-vitro anti-inflammatory activity:

The human red blood cell (HRBC) membrane stabilization method: Fresh Human blood (10 mL) was collected from the volunteers who had not taken any anti-inflammatory drugs for two weeks prior to the experimental plan and the blood is transferred to the heparinised centrifuged tubes, centrifuged at 3000 rpm for 10 min. The supernatant was carefully removed with a sterile pipette. The packed cells were resuspended in an equal volume of normal saline and centrifuged again. The washing was continued for 3 to 4 times until the supernatant was clear. A 10% Red Blood Cell (RBC) suspension was then prepared with normal saline and kept at 40°C undisturbed before use. The test sample consists of 0.5 mL of HRBC suspension, 1 mL of Phosphate buffer, pH 7.4, different concentrations of the drug (500, 1000, 1500, 2000, 2500 µg/mL and 2 mL hypotonic or hypo saline (0.25%, w/v NaOH). Indomethacin at varying concentrations is used as standard. All the assay mixtures were incubated at 37°C for 30 min and then centrifuged at 3000 rpm. The supernatant liquid was decanted and the hemoglobin content was estimated by a spectrophotometer at 560 nm. The percentage membrane-stabilizing activity was determined using following equation [24,25].

$$\% \text{ Stabilizing activity} = 100\left[\frac{\text{Optical density of drug}}{\text{Optical density Control}}\right]\times 100$$

Inhibition of protein denaturation: Protein denaturation was carried according to method described by [26,27] followed with minor modifications. The reaction mixture (5 mL) consists of 0.2 mL of egg albumin from fresh hen's egg. 2.8 mL of phosphate buffered saline pH 6.4 and various concentration of drug. Final volume was made with double distilled water. The reaction mixture is then incubated at 37 ± 2°C in a BOD incubator for 15 min and then heated to 51°C for 20 min, after cooling the samples the turbidity was measured at 660 nm using vehicle as blank. The percentage inhibition of protein denaturation was calculated by following equation.

$$\text{Percentage inhibition} = \left[\frac{\text{Abs control-Abs sample}}{\text{Abs control}}\right]\times 100$$

Results and Discussions

DPPH free radical scavenging activity:

The ability of alcoholic and aqueous extract of *A. propinquum* to scavenge DPPH free radical was calculated as percentage inhibition which was 92.07% and 52.42% respectively at concentration of 100 µg/mL, whereas percentage inhibition of rutin at the same concentration was 98.99% Figure 1 and Table 1.

Reducing power:

Both alcoholic and aqueous extract of *A. propinquum* showed good reducing power when compared with ascorbic acid Figure 2. The alcoholic and aqueous extracts showed reducing power of 1.078 and 1.009 respectively at 500 µg/mL. Standard ascorbic acid showed reducing power of 1.252 at the same concentration Table 2.

Scavenging of hydrogen peroxide:

The scavenging of hydrogen peroxide by alcoholic and aqueous extracts of *A. propinquum* was expressed as percentage inhibition of scavenging. The alcoholic and aqueous extracts showed 87.14% and 79.52 % inhibition respectively at concentration of 1250 µg as compared to standard ascorbic acid which showed 99.32% at same concentration Figure 3 and Table 3.

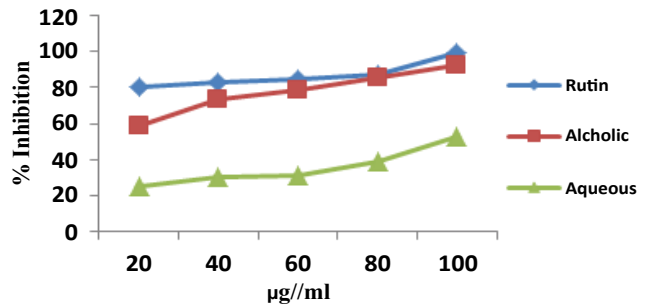

Figure 1: DPPH scavenging activity of aqueous and alcoholic extract of *A. propinquum* Reducing power

Tests	Alcoholic	Aqueous
Alkaloid	+	+
Carbohydrates	+	+
Coumarins	+	+
Cardiac glycoside	+	+
Proteins	+	+
Terpenoids	+	+
Phenolic	+	+
Flavonoid	+	+
Saponins	+	+
Tannin	+	+
Amino acids	+	+
+ Present, - absent.		

Table 1: Phytochemical analysis of aqueous and alcoholic extract of *A. propinquum*

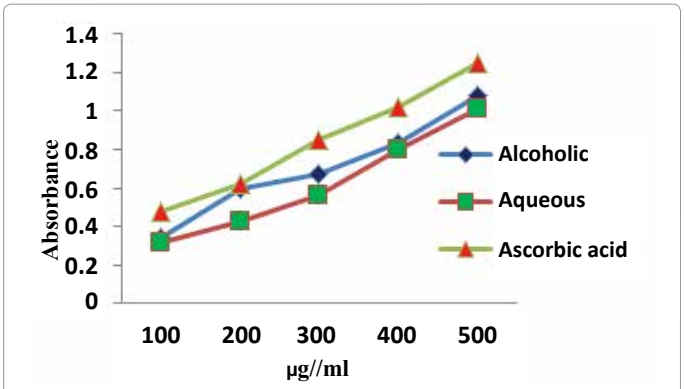

Figure 2: Reducing power of aqueous and alcoholic extract of *A. propinquum* Scavenging of Hydrogen Peroxide

	% Inhibition of DPPH free radical of extracts		
Conc. (µg/mL)	Standard	Alcoholic	Aqueous
20	80 ± 0.81	58.98 ± 0.70	25.11 ± 0.35
40	82.98 ± 0.56	73.44 ± 1.00	30.04 ± 0.70
60	84.89 ± 1.07	78.91 ± 1.00	31.63 ± 0.37
80	87.28 ± 0.67	85.22 ±0.32	38.79 ± 0.63
100	98.99 ± 0.26	92.07 ± 0.70	52.42 ± 0.63

Table 2: DPPH scavenging activity of aqueous and alcoholic extract of *A. propinquum*

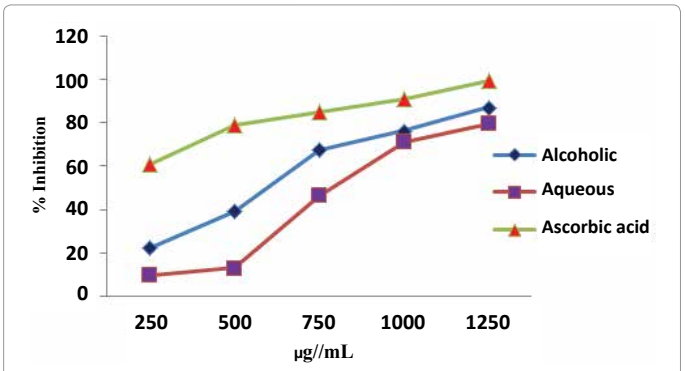

Figure 3: Hydrogen peroxide scavenging ability of alcoholic and aqueous extract of *A. Propinquum*

	Absorbance		
Conc. (µg/mL)	Standard	Alcoholic	Aqueous
100	0.479 ± 0.05	0.345 ± 0.01	0.321 ± 0.03
200	0.623 ± 0.06	0.598 ± 0.01	0.432 ± 0.01
300	0.852 ± 0.08	0.675 ± 0.2	0.567 ± 0.04
400	1.025 ± 0.07	0.832 ± 0.01	0.802 ± 0.03
500	1.252 ± 0.02	1.078 ± 0.03	1.009 ± 0.01

Table 3: Reducing power of aqueous and alcoholic extract of *A. propinquum*

Total phenolic and flavonoid content:

The results of total phenolic and flavonoid content of alcoholic and aqueous rhizome extracts of *A. propinquum* are given in Figure 4. The total content of phenolic compounds in alcoholic and aqueous extracts (mg/g) in Gallic acid equivalent was 554.06 and 425.21 mg/g respectively and total flavonoid content in alcoholic and aqueous extracts was 324.71 and 225.56 (mg/g) in Rutin Equivalents (RE) Table 4.

Membrane stabilization:

The percentage membrane stabilizing activity shown by methanolic and aqueous extracts of *A. propinquum* was 76.09% and 46.57% respectively at concentration of 125 µg/mL, whereas indomethacin showed 95.56% of membrane stabilizing activity at the same concentration Table 5 and Figure 5.

Protein denaturation:

The percentage membrane stabilizing activity shown by methanolic and aqueous extracts of *A. propinquum* was 65.70% and 41.67% respectively at concentration of 500 µg/mL, whereas indomethacin showed 88.26% of membrane stabilizing activity at the same concentration Tables 6,7 and Figure 6.

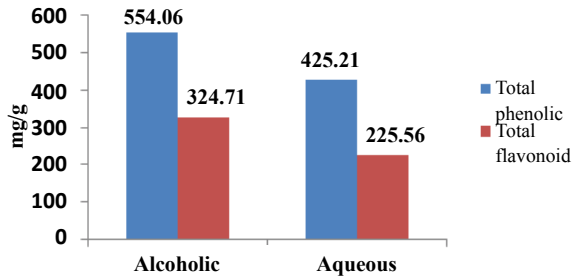

Figure 4: Total phenolic and flavonoid content of alcoholic and aqueous extract of *A. propinquum*

	% Inhibition		
Conc. (µg/mL)	Standard	Alcoholic	Aqueous
250	60.76 ± 0.28	22.47 ± 0.16	9.73 ± 0.67
500	78.73 ± 0.27	39.2 ± 0.32	12.85 ± 0.41
750	84.97 ± 0.31	67.79 ± 0.32	46.44 ± 0.34
1000	91.32 ± 1.61	76.27 ± 0.38	71.28 ± 0.41
1250	99.32 ± 0.10	87.14 ± 0.63	79.52 ± 0.32

Table 4: Hydrogen peroxide scavenging ability of alcoholic and aqueous extract of *A. Propinquum*

Extract	Total phenolic mg/g (in GAE)	Total flavonoid mg/g (in RE)
Alcoholic	554.06 ± 131.60	324.71 ± 99.84
Aqueous	425.21 ± 101.00	225.56 ± 42.00
(a): average of three determinations		

Table 5: Total amount of phenolic and flavonoid content of alcoholic and aqueous rhizome extracts of *A. propinquum*. (Mean ± S.E.M.)

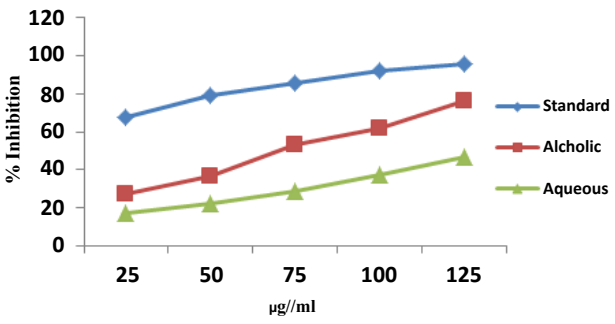

Figure 5: *In vitro* membrane stabilization activity of methanolic and aqueous extracts of *A. propinquum*

	% Membrane Stabilization		
Conc. (µg/mL)	Standard	Alcoholic	Aqueous
25	67.82 ± 0.63	27.41 ± 0.38	16.98 ± 0.18
50	79.34 ± 0.43	36.64 ± 0.42	22.16 ± 0.32
75	85.59 ± 0.17	52.93 ± 0.33	28.87 ± 0.35
100	91.81 ± 0.10	61.77 ± 0.18	37.38 ± 0.63
125	95.56 ± 0.10	76.09 ± 1.00	46.57 ± 0.70

Table 6: *In vitro* membrane stabilization activity of methanolic and aqueous extracts of *A. propinquum*

	% Inhibition of protein denaturation		
Conc. (µg/mL)	Standard	Alcoholic	Aqueous
100	44.28 ± 0.90	18.20 ± 0.38	07.44 ± 0.39
200	59.43 ± 0.90	29.46 ± 0.38	17.65 ± 0.82
300	71.20 ± 0.32	37.93 ± 0.35	24.68 ± 0.60
400	82.01 ± 0.70	51.22 ± 0.39	33.58 ± 0.24
500	88.26 ± 0.38	65.70 ± 0.38	41.67 ± 0.24

Table 7: *In vitro* protein denaturation activity of methanolic and aqueous extracts of *A. propinquum*

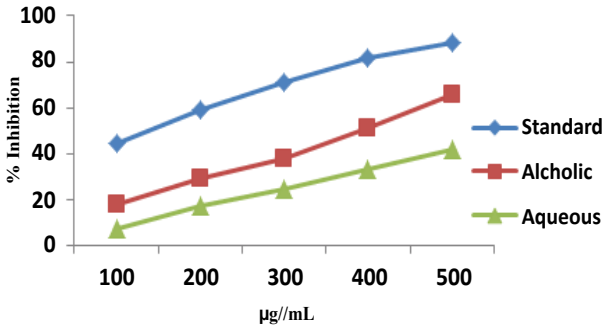

Figure 6: *In vitro* protein denaturation activity of methanolic and aqueous extracts of *A. propinquum*

Conclusion

It is concluded from the data that both alcoholic and aqueous extracts of the rhizomes of *A. propinquum* Schott possess potent antioxidant activity when compared with ascorbic acid and rutin. But among the two alcoholic extract showed statically significant as compared to aqueous extract. Further studies to isolate, identify and characterize the active principle(s) are in the progress to substantiate the present findings.

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