REPORT

Antibacterial, antifungal, phytotoxic, antioxidant and hemagglutination activities of organic fractions of *Arisaema tortuosum*

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Abstract: In the current study, the antimicrobial, phytotoxic, haemagglutination and antioxidant potential of crude methanolic extract (Crd. MeOH Ext.) and four organic fractions of *Arisaema tortuosum* was investigated. All fractions have been screened for antimicrobial properties against eight bacterial pathogens and six fungal pathogens using agar well diffusion and tube dilution method, respectively. Furthermore, the organic fractions were also screened for its phytotoxicity against *Lemna minor.* Haemagglutination was performed against all human blood groups while free radical scavenging activity was performed to investigate the antioxidant potential of *A. tortuosum.* Results obtained for antibacterial activity exhibited various degree of zone of inhibition and significant activity was observed for *Pseudomonas aeruginosa* (27.16±0.60) followed by *Bacillus cereus* (18.55±0.69) for Crd. MeOH Ext. and chloroform ($CHCl_3$) fraction, respectively while some strains showed resistant at same concentration. Similarly, non-significant antifungal activity was observed for the plant extracts. However, the highest activity among the strains was observed for *Alternaria alternata* (22±1.24%) and *Aspergillus niger* (20±1.00%) for ethyl acetate (EtOAc) fraction and Crd. MeOH Ext., respectively. The plant extracts showed good phytotoxic activity with 77.06% inhibition for *n*-hexane fraction at 1000µg/mL. The result of Nitric Oxide (NO) reducing assay revealed that the plant has less antioxidant activity with 46.06% inhibition for $CHCl_3$ fraction at 900µg/mL. For haemagglutination assay, the result displayed no agglutination in all the testing concentration. Based on the current results, it can be concluded that *A. tortuosum* has significant antimicrobial and moderate phytotoxic potential and therefore can leads to antibiotics and herbicide production.

Keywords: Antibacterial, antifungal, antioxidant, *Arisaema tortuosum*, haemagglutination, phytotoxicity.

INTRODUCTION

Plant based therapies for different diseases have gained massive attention over the years in order to find novel ways of treating a disease and therefore research on medicinal plants has gone at a pace that matches no parallel in the history (Newman *et al.,* 2000 and Ilahi *et al.,* 2012). The international market of herbal products is estimated to be US$ 62 billion per year and it is also noteworthy that the conventional medicines market is expanding at a rate of >20% annually (Newman *et al.,* 2000 and Patwardhan *et al.,* 2004). Larger extent of plants of the world is still unexplored for its therapeutic potential (Mahesh *et al.,* 2008 and Ali *et al.,* 2011). Pharmacological screening gives a foresight about the therapeutic potential of a plant and therefore considered inevitable (Khalil *et al.,* 2013). *A. tortuosum* is a member of *Araceae* and is widely known for its therapeutic properties (Verma *et al.,* 2012). It has a distinctive purple or green whip-like spadix arising from the mouth of its "jack-in the-pulpit" flower. It is widely distributed in rhododendron forest, scrub and alpine meadows in the Himalayas, Western China, Southern India and some parts of Myanmar. It grows in forests, shrubberies and open slopes and even in moist places at elevation of 1500-2200m in Nepal. They grow within woodland garden and dappled shade. They are suited to deeply shaded location. Their natural habitats are abundant in East Asia, Himalayas, Simla, Sikkim and Bhutan (Diwan *et al.,* 2012). The plant is commonly referred as whip-cord cobra lily because of its appearance while locally known as "Quxu nan xing" in Chinese, "Kiri Ki Kukri", "Samp Ki Kumb", "Bagh-Jandhra" in Hindi, "Sapkanda" in Marathi, "BirBango" in Nepalese, "KatuCeni" in Tamil. Traditionally *A. tortuosum* has been used for constipation, indigestion, abdominal pain, dysentery (Gangwar *et al.,* 2010), rheumatism and stomachache (Hussain *et al.,* 2006), contraceptive (Paulsamy *et al.,* 2007), snake-bite (Bhatt *et al.,* 2006) and piles (Suresh *et al.,* 2011). *A. tortuosum* is also screened for its anti-inflammatory and anti-proliferative potential (Nile *et al.,* 2013). It is also reported for Abscess and also used against nematodal infections (Choudhary *et al.,* 2008). The plant is also claimed for its effectiveness in the dog bite and liver complaints (Jain *et al.,* 2005).

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In the present study, four different fractions (Crd. MeOH Ext., *n*-hexane, EtOAc and $CHCl_3$ fractions) of *A. tortuosum* were prepared and screened for its antibacterial potential against *Proteus mirabilis, Staphylococcus aureus, Escherichia coli, B. cereus, Salmonella typhosa, Klebsiella pneumonia, P. aeruginosa* and *Enterococcus faecalis*. The anti-fungal potential of the fractions were tested against; *A. alternata, Aspergillus. niger, A. flavus, Fusarium solani, F. oxysporum* and *Penicillium notatum* fungal species. Further, the haemagglutination, phytotoxic and antioxidant activity for all test samples were performed.

MATERIALS AND METHODS

Test bacterial and fungal strains

Both bacterial (*E. coli, P. aeruginosa, S. aureus, S. typhi, E. faecalis, B. cereus, K. pneumonia* and *P. mirabilis*) and fungal species (*A. alternata, A. niger, A. flavus, F. solani, F. sporium* and *P. notatum)* were obtained from Center of Biotechnology and Microbiology, University of Peshawar (UOP)**.** Nutrient agar media (Merck 5450) was used in sub-culturing the bacterial strains while fungus strains were propagated in Sabouraud Dextrose Agar (SDA) (Oxoid CMOO41). The stock cultures were maintained at 4°C till further use.

Plant material

A. tortuosum rhizome and aerial parts were collected from Baragali, Khyber Pakhtunkhwa, Pakistan and were taxonomically authenticated by Prof. Dr. Farrukh Hussain, Department of Botany, UOP. Plant material was washed with distilled water and allowed for shade drying. After drying the plant material-weighing 1kg was ground to fine powder using electric grinder and soaked in methanol for 15 days. After soaking for 15 days, part soluble in methanol was removed by filteration. The filtrate was concentrated at 40ºC using rotary evaporator. Plant extracts (yellowish appearance) 120g was obtained.

Fractionation

The 100g of Crd. MeOH Ext. of *A. tortuosum* was suspended in distilled water (400 ml) and partitioned with *n*-hexane (3x400ml), $CHCl_3$ (3x400ml), EtOAc (3x 400ml) to obtain; *n*-hexane (5g), $CHCl_3$ (8g) and EtOAc (40g). About 20g of Crd. MeOH Ext. was kept to use for pharmacological/biological screenings.

Standardization of microbial cultures

Cultures were standardized as described by Baker and Thomas berry (1983). Briefly the turbidity of the microbial cultures was adjusted visually with 0.5 McFarland solution with the help of sterilized deionized water. 0.5 McFarland solution corresponds to the *E. coli* suspension of 1.5×10^8 cells/ml.

In vitro antibacterial assay

Agar well diffusion method as described by Khalil *et al.,* (2014) was used to determine the antibacterial activity. Briefly nutrient agar plats were prepared and incubated overnight for sterility test. Standardized inoculum was introduced to the plats and uniform lawns were prepared using sterilized bent glass rod. 6mm borer was used to make wells in the plates. 100µl of the test sample (3mg/ml) was introduced in each well. Imipenem disc (10µg) was used positive control while Dimethyle sulfoxide (DMSO) as negative control. Zones of inhibition (mm) were measured after overnight incubation.

In vitro antifungal assay

Tube dilution assay as described by Ahmad *et al.,* (2009) was used to determine the percent linear inhibition in fungal growth. Stock solutions (24mg/ml) of the test samples were prepared in DMSO (<1%). SDA medium was prepared for the growth of fungal specie. 4ml aliquots transferred to screw test tubes that were sterilized using moist steam sterilization method. After sterilization 66.6µl from the stock were transferred to the test tubes. The test tubes were allowed to cool in slanting position. The final concentration became 400µg/ml of SDA. Fresh culture of each fungal species was inoculated to test tubes. Other test tubes supplemented with DMSO and standard drugs, used as negative and positive control, respectively. All the test tubes were incubated for 5-7 days at 27±1ºC. On day 7^{th}, the visible non-mycelia linear growth inhibitions of fungal species were calculated by comparison with reference drug.

Antioxidant activity

All fractions were examined for their antioxidant potential. The NO free radical scavenging activity was performed by as per reported procedure with slight modification (Gupta *et al.,* 2007). Stock solution (3 mg/ml) of the test samples was prepared in DMSO. From stock solution, by dilution, different concentration of the test samples; 0.3, 0.6, 0.9, 1.2 and 1.5mg/ml were prepared. 10µl from each concentration of the test sample were taken in micro titer plate and add 20µl of phosphate buffer. The micro titer plate is then incubated at 20-25ºC for one and half hour. The plate was well shacked after incubation and adds 50µl of sulphanilic acid. Take the absorbance (pre-absorbance) at 570 nm. After pre-reading add 50µl of [N-(1-Napthyl) Ethylene-diamine-di-hydorchloride], shake well and take the final reading. Vitamin C and DMSO were run as positive control and blank, respectively.

Phytotoxic activity

The test samples (Crd. MeOH Ext. and fractions) were tested for phytotoxic bioassay against *L. minor* according to McLaughlin protocol (McLaughlin *et al.,* 1991). Stock solutions (20mg/ml) of the test samples were prepared in

methanol and E-medium was prepared for the growth of *L. minor.* From the stock solution, 10, 100 and 1000 µg/ml test sample were poured to flasks and left at room temperature to evaporate the organic solvent. After evaporation, 20ml of the E-media was added to each flask. Sixteen healthy *L. minor* plants were selected, with a rosette of three fronds and put in respective flasks. The flasks containing *L. minor* and E-media were incubated at 27±1ºC in growth chamber for seven days. Results were noted after seven days of incubation.

Haemagglutination activity

Haemagglutination activity of test samples was performed according to procedure of Bashir *et al.,* (2009). Phosphate buffer (pH 7.0) was prepared by dissolving 0.453g of KH_2PO_4 and 0.47g of Na_2HPO_4 each in 50 ml of distilled water. The dissolved KH_2PO_4 and Na_2HPO_4 were mixed in ratio of 3:7 (V/V). Stock solution (1mg/ml of DMSO) was prepared and diluted (1:2, 1:4, 1:8 and 1:16) in phosphate buffer. Fresh blood samples from healthy persons were collected on the same day of the experiment and centrifuged. The plasma was discarded and red blood cells (RBC's) were collected for the next steps. 2% RBC's suspension was prepared in the phosphate buffer. From each dilution 1ml of sample was taken in a test tube and then adds 1ml of the RBC's suspension to the sample. Incubate the test tubes for 30 min at 37ºC. After incubation the test tubes were examined for the button formation, rough button formation indicates positive results and vice versa

STATISTICAL ANALYSIS

The *in-vitro* antibacterial, antifungal, antioxidant and phytotoxic activity of *A. tortuosum* were performed in triplicate. The data obtained were statistically analyzed using SPSS Ver. 21 software. Post Hoc Multiple Comparison test through one-way ANOVA was applied for comparison with in column. All the data were represented by mean value (MV) ± standard deviation (SD). A probability value P<0.05 was taken as significant at 95% confidence interval.

RESULTS

Raw products obtained from plants and animals are playing a key role for various ailments and diseases from ancient time (Koehn *et al.,* 2005). The side effect associated with allopathic medicines leads to increased interest for natural remedies consumption (Verma *et al.,* 2008). *A. tortuosum* is a wild herbal plant, has been used for both as food and ethano-medico practices by tribes. The plant (rhizome part) is also used as anthelmentic while its tuber part is used traditionally as anti-nematodal and wound healing (Verma *et al.,* 2012). Therefore, the present study was performed to evaluate the hidden potential of Crd. MeOH Ext. along with various fractions of *A. tortuosum* against different human pathogens for possible antibacterial activity. The results are summarized in table 1. The percent inhibition of the test sample is given with respect to positive control in fig. 1. At 3mg/ml concentration all the strains showed various degree of zone of inhibition, ranging from 3.43±0.49 to 21.54±0.67mm. The highest activity was observed for *P. aeruginosa* (27.16±0.60) followed by *B. cereus* (18.55±0.69) for Crd. MeOH Ext. and $CHCl_3$ fraction, respectively. When the effect of fractions upon strains were compared a significant difference (P<0.05) between the strains was observed for Crd. MeOH Ext., *n*-hexane, $CHCl_3$ and EtOAc fractions except *S. aureus* and *P. aeruginosa* (P>0.05).

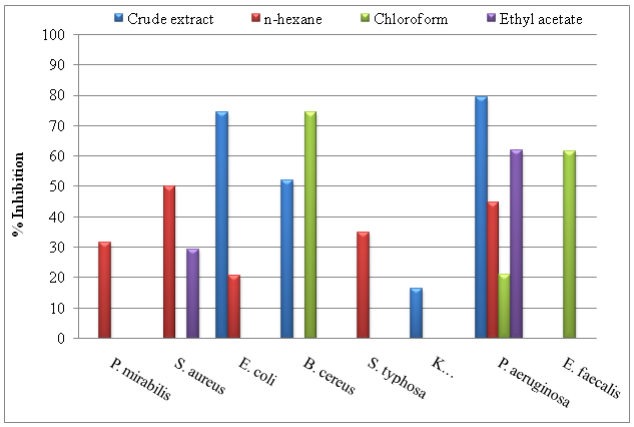

Fig. 1: Percent antibacterial activity relative to Gentamycin (standard drug).

Similarly, the plant materials were checked for antifungal activity. The results were recorded as percent growth inhibition, compared with positive control. Amphotericin B was used as standard drug (table 2). All the strains were resistant to the test sample compared to the positive control. The highest activity among the strains was observed for *A. alternate* (22±1.24%), *A. niger* (20±1.00%) followed by *F. solani* (18±1.48%) for EtOAc, Crd. MeOH Ext. and *n*-hexane fraction, respectively. When the data was compared within rows there were no significant differences observed (P>0.05) except for *A. alternate* and *A. niger* (P<0.05).

Allelopathy is an important element of plant interference in managed and natural ecosystem. This phenomenon is generally based on chemical interfere with germination, development of growth of other plant species (Weston *et al.,* 2003). The Crd. MeOH Ext. and various fractions of the plant showed phytotoxic activity (Table 3). At highest concentration (1000µg/ml) the test sample showed moderate phytotoxic activity in the following order $CHCl_3$ (12.33±0.57) > Crd. MeOH Ext. (8.66±0.57) < *n*-hexane (8.66±1.15) < EtOAc (5.00±0.00). The potent extracts can be used as herbicide (Dzoyem *et al.,* 2011). At lowest concentration the activity was reduced. When the effect of fractions at 1000µg/ml concentrations was

Table 1: Antibacterial activity of crude methanolic and various fractions of *A. tortuosum* against different bacterial strains.

Bacteria	Drug 10µg disc	Crude extract	*n*- hexane	$CHCl_3$	EtOAc
P. merabulus	25.23±0.49[ab]	--	7.97±0.96	-	-
S. aureus	26.06±0.40[a]	--	13.03±0.67[a]	-	7.66±0.62
E. coli	22.83±0.20	16.99±0.83	4.73±0.38	-	-
B. cerus	24.96±0.17[b]	12.97±0.44	-	18.55±0.69	-
S. typhi	26.70±0.42[ac]	--	9.33±0.40	-	-
K. pneumonia	21.06±0.23	3.43±0.49	-	-	-
P aeroginosa	27.16±0.60[c]	21.54±0.67	12.15±0.33[a]	5.73±0.38	16.79±0.32
E. faecalis	23.89±0.17	-	-	14.68±0.40	-
P. mirabilis	26.78±0.25	-	08.50±0.46	-	-

Table 2: Antifungal activity of crude methanolic and various fractions of *A. tortuosum* against different fungal strains.

Test sample	% inhibition					
	-ive Control	+ive Control	Crude Extract	*n*-hexane	$CHCl_3$	EtOAc
A. alternata	--	100	--	08±1.49[a]	--	22±1.24[b]
A. niger	--	100	20±1.00	--	--	--
A. flavus	--	100	--	16±1.47[a]	16±0.77[a]	--
F. solani	--	100	--	18±1.48[a]	18±1.42[a]	--
F. sporium	--	100	--	--	--	--
P. notatum	--	100	12±1.93[b]	10±0.88[ab]	10±1.09[ab]	08±2.1[a]

Table 3: Phytotoxic activity of crude methanolic extract and various fractions of *A. tortuosum*.

Sample Name	Con. (µg/mL)	Number of fronds					+ive Control
		Crude Extract	*n*-hexane	$CHCl_3$	EtOAc	-ive Control	
L. minor	1000	8.66±0.57[a]	8.66±1.15[a]	12.33±0.57	5.00±0.00	16.00±0.00	0.015
	100	5.66±0.50[a]	4.66±1.52[a]	9.33±1.52	2.66±1.15	16.00±0.00	
	10	2.33±0.57[a]	0.66±0.57	2.66±0.57[a]	--	16.00±0.00	

*Lower case letters shows significance difference within column; Values are means and standard deviation of three replicates; Means sharing a letter in common are not significantly different at P>0.05; Means sharing no letter in common are significantly different at P<0.05

compared, there was a significant difference (P<0.05) observed for $CHCl_3$ and EtOAc fractions while Crd. MeOH Ext and *n*-hexane fraction was not significantly different (p>0.05). Similarly, at 100µg/ml of concentration same pattern was observed. While at 10µg/ml; Crd. MeOH Ext and $CHCl_3$fraction exhibited no significant difference (P>0.05).

Nitric oxide is a strong pleiotropic inhibitor of various physiological processes like smooth muscle relaxation, regulation of cell-mediated toxicity, inhibition of platelet aggregation and neural signaling. NO is a free radical play major role as an effectors molecule in a vasodilation, antitumor, neural messenger and antimicrobial activities (Hagerman *et al.,* 1998). In the present study we evaluated the plant for its NO reducing potential as summarized in table 4. The result revealed that the plant has very little antioxidant potential when compared to ascorbic acid. The highest percent inhibition was recorded for $CHCl_3$ fraction (46.06%) and the lowest was observed for EtOAc fraction (22.76%) at 900µg/ml. Our result-support the finding of Nile and Park (2013), who reported the IC_{50} value (852µg/ml) for DPPH free radical scavenging potential of the tuber methanolic extract. Lectins are glycoprotein that precipitate complex carbohydrate and agglutinate cells (Goldstein *et al.,* 1980). Lectins can be found everywhere in biosphere and traditionally found in dicotyledonous plants but for the last few years it is also isolated and characterized from monocotyledonous families; alliaceae (Sharon *et al.,* 1990), amaryllidaceae (Damme *et al.,* 1993), araceae (Van *et al.,* 1995), orchiaceae (Damme *et al.,* 1994) and liliaceae (Damme *et al.,* 1996). In this study the plant extract was screened for haemagglutination activity as shown in Table 5. The result divulged that the plant extract did not induce any agglutination of human erythrocytes. It has been previously shown that lectins isolated from *A. tortuosum* did not agglutinate ABO blood groups of human, which is the agreement of our finding (Dhuna *et al.,* 2005).

Table 4: Haemagglutination activity of crude methanolic extract and various fractions of *A. tortuosum*

Blood groups	Crude extract				*n*-hexane				$CHCl_3$				EtOAc			
	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16
A^+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B^+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AB^+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O^+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A^-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B^-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AB^-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O^-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 5: Antioxidant activity of crude methanolic extract and various fractions of *A. tortuosum*

Concentration µg/ml	% Inhibition			
	Crude Methanolic Extract	*n*-hexane	$CHCl_3$	EtOAc
300	29.02	21.07	30.40	11.83
600	32.67	25.33	39.49	17.46
900	39.24	27.93	46.06	22.76

Standard: Ascorbic acid was used as standard (47.5µg/mL)

The pharmacological/biological investigations revealed that *A. tortuosum* possesses significant antibacterial and moderate phytotoxic activity. It showed less antifungal, antioxidant and no haemagglutination activity. Hence, the plant extracts contains active ingredients, which could provide as an alternative agents for different diseases. Further studies are needed to check its *In-vivo* clinical response and toxicities.

DISCUSSION

Mycotoxins are secondary metabolites produced by a number of fungi, which have been found to be toxic to human and animals for example Malformins produced by *A. niger* (Smedsgaard *et al*., 1997). As reported, the essential oils, extracted from the *n*-hexane fraction of *Acacia modesta* were screened against various fungal species; *C. albicans, A. flavus, M. canis, F. solani* and *C. glabrata*. The essential oils showed moderate (40%) antifungal activity against *M. canis*, low (25%) activity was recorded against *F. solani* (*Bashir et al*., 2012). The present study revealed that the Crd. MeOH Ext. and various fractions were found inactive against most of the fungal strains however; the highest antifungal activity (22±1.24 and 20±1.00%) against *A. alternate* and *A. niger* was recorded for EtOAc fraction and Crd. MeOH Ext. extract, respectively. These results indicate that *A. tortuosum* lack antifungal potential.

The Crd. MeOH Ext. and various fractions of the aerial parts of *Vitex agnus castus* were evaluated for possible antibacterial effect against *E. coli*, *P. aeruginosa*, *S. aureus*, *S. epidermidis*, *S. typhi*, *B. pumalis*, *K. pneumoniae*, *S. pneumoniae* and *E. aerogenes*. The results revealed that the $CHCl_3$ fraction of the plant exhibit significant antibacterial activity (81%) against *K. pneumonia* (*Bashir et al., 2010*). In the present study the Crd. MeOH Ext. and $CHCl_3$ fraction showed good antibacterial activity against *E. coli*, *P. aeruginosa*, *B. cereus* and *E. faecalis*. The results revealed that the selected plant may contains some active constituents which need to be isolated and purified for the treatment of infectious diseases caused by these pathogenic bacteria.

The aerial parts of *A. modesta* were screened for phytotoxic effect against *L. minor.* The results revealed that Crd. MeOH Ext. and various fractions showed low phytotoxic activity (Bashir *et al.*, 2011). In the present study the *n*-hexane fraction of the selected plant showed good (77.06%) phytotoxic activity at higher concentration, which revealed that *A. tortuosum* may be effective for control of weeds.

The Crd. MeOH Ext. and various fractions of *Hedera nepalensis* possess a concentration dependent antioxidant activity (*Bashir et al., 2012*). In the present study we also observed a concentration dependent antioxidant activity for the Crd. MeOH Ext. and various fractions.

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