

Antimicrobial, antitumor and brine shrimp lethality assay of *Ranunculus arvensis* L. extracts

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Abstract: To investigate the antitumor activity, brine shrimp lethality assay, antibacterial and antifungal activity of Methanol Extract (ME), Water Extract (WE), Acetone Extract (AE), Chloroform Extract (CE), Methanol-Water Extract (MWE), Methanol-Acetone Extract (MAE), Methanol-Chloroform Extract (MCE) of *Ranunculus arvensis* (L.). Antitumor activity was evaluated with *Agrobacterium tumefaciens* (At10) induced potato disc assay. Cytotoxicity was evaluated with brine shrimp lethality assay. Antibacterial activity was evaluated with six bacterial strains including *Escherichia coli*, *Enterobacter aerogenes*, *Bordetella bronchiseptica*, *Klebsiella pneumoniae*, *Micrococcus luteus* and *Streptococcus anginosus* and antifungal screening was done against five fungal strains including *Aspergillus niger*, *A. flavus*, *A. fumigates*, *Fusarium solani* and *Mucor* species by using disc diffusion method. Best antitumor activity was obtained with ME and WE, having highest IC₅₀ values 20.27±1.62 and 93.01±1.33µg/disc. Brine shrimp lethality assay showed LC₅₀ values of AE, MAE and ME were obtained as 384.66±9.42µg/ml, 724.11±8.01µg/ml and 978.7±8.01µg/ml respectively. WE of *R. arvensis* revealed weak antimicrobial result against the tested microorganisms. On the other hand, the antifungal activity of the plant extracts was found to be insignificant. These findings demonstrate that extracts of *R. arvensis* possesses significant antitumor activity. Further extensive study is necessary to assess the therapeutic potential of the plant.

Keywords: *Ranunculus arvensis*; antitumor activity; potato disc assay; brine shrimp lethality assay; antimicrobial.

INTRODUCTION

Plant materials remain a vital source to conflict serious diseases in the world. In developing countries, people use traditional medicine when they suffering from diseases (Yildirim *et al.*, 2013). The medicinal values of the plants occur due to the presence of chemical active substances, which produce a specific physiological action on the body of human being (Shihabudeen *et al.*, 2010). They are helpful to cure many infectious diseases, use of synthetic antimicrobial compounds contain side effects that are associated with these synthetic drugs (Islam *et al.*, 2011). To cope with these challenges, the demand of better results oriented new antimicrobial agents has increased (Karakas *et al.*, 2012). Plants have host bioactive molecules, which most likely developed as chemical resistance against infections (Pierangeli *et al.*, 2011). Natural products obtain from these plants have potential for the treatment of cancer (Conforti *et al.*, 2008). The mortality rate of cancer increased 22% up until 1990, and ten million new cases were reported in 2000, over six million deaths were occur worldwide (Karaskas *et al.*, 2012; Parkin *et al.*, 2001). To promote the use of plants as a source of antitumor and antimicrobial drugs, it is

necessary to examine the activity, composition and also confirm their way of use (Nair and Chanda, 2007). Thus, plants are considered as most important and interesting candidate for the development of new and safe drugs for aliments (Parkin *et al.*, 2000). In many cases, the bio-active compounds of these plants extracts remain anonymous, and their occurrence is only detected by biological assays (Karaskas *et al.*, 2012). These bioassays are use for screening and evaluation of plant extracts possesses antitumor, antimicrobial activity, cytotoxicity etc. (Dzhambazov *et al.*, 2002).

Ranunculus arvensis (L.), commonly known as corn buttercup, belong to family Ranunculaceae. It is native to Europe, but mostly found in high mountains of the Mediterranean Region, Southeastern, Eastern Regions of Anatolia and also in Pakistan (Sayhan *et al.*, 2009; Kosa *et al.*, 2008). Ranunculaceae is a large family comprising around 2500 species distributed all over the world (Fostok *et al.*, 2009). *R. arvensis* is folk remedy for asthma, gout, arthritis, high fever and psoriasis in the Far East (Akbulut *et al.*, 2011). Since this plant has important medicinal properties the present study has been undertaken and we herein, report the antitumor activity, brine shrimp lethality assay and antimicrobial activity of various crude extracts of *R. arvensis* for the first time.

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MATERIALS AND METHODS

Selection and collection of plant material

Whole plant of *Ranunculus arvensis* (L.) was collected from Frontier Region Bannu, Pakistan. The plant was taxonomically identified by the experts of Herbarium; Quaid-i-Azam University and also deposited there. The whole plant was shed dried and powdered with grinder. The powdered whole plant was extracted in 1:10 of methanol (MeOH), water (H₂O), acetone (ACTN), chloroform (CHCl₃), methanol-water (MeOH-H₂O), methanol-acetone (MeOH-ACTN), methanol-chloroform (MeOH-CHCl₃) were placed in shaking incubator at room temperature for overnight. The extracts were filtered through Whatman filter paper No. 1. The filtrate was dried using rotary evaporator.

Potato disc antitumor assay

The potato disc assay was used to assess the antitumor activity of the *R. arvensis* with the method of (Ahmad *et al.*, 2008) with some as modifications. *Agrobacterium tumefaciens* (At10) was grown for 48 hours in Lauria broth containing 20µg/ml rifampicine. Red skinned potatoes (*Solanum tuberosum*) were washed and scrubbed with brush under running water and surface sterilized by immersion in 0.1% mercuric chloride for 5-7 minutes, followed by washing with autoclaved distilled water. Borer made potato discs (6x8mm) were placed on agar gel (1.5%) surface to fix the discs in petri plate and 50µl of inoculum of At10 containing different concentrations of extract (10µg/ml, 100µg/ml and 1000µg/ml) was applied separately on the surface of each disc. The plates were incubated at 28°C in incubator for 21 days. After 21 days incubation followed by 15 minutes staining with Lugol's solution (10% KI and 5% I₂) and tumors on each plate were counted under dissecting microscope (Celestron Model No. 44202) with side illumination. Lugol's reagent stains the starch in potato tissue to dark blue to dark brown color, but the tumors do not take up the stain and appear creamy to orange. Experiment was performed in triplicate. Percentage inhibition of tumors was calculated using the formula;

$$\text{Percentage inhibition} = 100 - \frac{\text{Average No. of tumors of sample}}{\text{Average No. of tumors of control}} \times 100$$

Brine shrimp lethality assay

Cytotoxic activity of *R. arvensis* extracts was determine by brine shrimp lethality assay reported by (McLaughlin and Rogers, 1998). Brine shrimp (*Artemia salina*) eggs (Ocean star, USA) were hatched in artificial seawater (34g sea salt/1 L distilled water) at 37°C. After 48 hours of incubation nauplii (brine shrimp larvae) were transferred with Pasteur pipette in the glass vials containing 2ml of seawater. Fifteen shrimps were transferred to each vial and volume was raised up to 5ml with seawater. Tested concentrations were 10µg/ml, 100 µg/ml and 1000µg/ml. A vial containing 50µl DMSO

instead of extract was used as control. After 24 hours of incubation the number of survived napulii in each vial was counted with the help of 3X magnifying glass (3572, Konus Optima). LC₅₀ was calculated using probit analysis (Finney, 1971).

Antibacterial activity

The screening of antibacterial activity of plant extracts was carried out using disc diffusion method as described (Bauer *et al.*, 1966). Antibacterial activity was carried out against four Gram-negative [*Escherichia coli* (ATCC 15224), *Enterobacter aerogenes* (ATCC 13048), *Bordetella bronchiseptica* (ATCC 4617), *Klebsiella pneumoniae* (ATCC 13883)] and two Gram-positive [*Micrococcus luteus* (ATCC 10240), *Streptococcus anginosus* (ATCC 33397)] bacterial strains. Sample discs were prepared by sterilized filter paper (6 mm diameter) were placed over bacterial lawn and loaded with 20µl to give a concentration of 400µg/ml of crude extract. These plates were incubated for 24 hours at 37°C. Standard drugs (roxithromycin and cefixime) of 10µg/ml concentration were used as positive control. After 24 hours, the antibacterial activity was determined by measuring the diameter of disc (6 mm), with the help of vernier caliper. The antibacterial activity was screen out against all the tested microorganisms in triplicates.

Antifungal assay

Antifungal activity of various extracts was carried by the disc diffusion method of (Rubio *et al.*, 2003). The screening was done against five fungal strains including *Aspergillus niger*, *A. flavus*, *A. fumigates*, *Fusarium solani* and *Mucor* species. In this method, each petri plate was prepared by adding 25ml of Sabouraud Dextrose Agar (SDA) and 250µl of suspension of spores in 1% tween-20. Sterilized filter paper discs of 6 mm diameter were placed on agar plate and loaded with 20µl (400 µg/disc) of crude extract and incubated for 72 hours at 28°C. A standard drug, terbinafine (2.5µg/disc) was used as positive control. The experiment was performed in triplicate and the diameter of zone of inhibition was recorded in mm.

STATISTICAL ANALYSIS

Three replicates of each sample were used for statistical analysis and the values are reported as mean ± Standard Deviation (S.D.).

RESULTS

The plant material was subjected to an extraction process with solvent like MeOH, H₂O, ACTN, CHCl₃, MeOH-H₂O, MeOH-ACTN and MeOH-CHCl₃. The crude ME of *R. arvensis* showed remarkably inhabitation of gall tumor growth caused by the *Agrobacterium tumefaciens* with IC₅₀ value 20.27±1.62µg/disc (table 1). In the Brine

Table 1: Anti-tumor activity of the extracts of *Ranunculus arvensis*

Percentage (%) inhibition of tumor growth at concentrations (µg/disc)				
Test extract	10	100	1000	IC ₅₀ (µg/disc)
ME	44.99	59.99	70.55	20.27±1.62
WE	32.77	51.11	67.77	93.01±1.33
AE	36.66	41.66	54.44	550.47±1.95
CE	24.44	29.99	37.22	>1000
MWE	17.22	46.66	60.55	150.73±1.94
MAE	22.77	47.77	59.44	140.98±2.42
MCE	27.22	29.44	35.55	>1000

Table 2: The results of brine shrimp lethality assay of the crude extracts of *Ranunculus arvensis*

Percentage (%) mortality after 24 hours incubation (µg/ml)				
Test extract	10	100	1000	LC ₅₀ (µg/ml)
ME	13.33%	30%	50%	978.7±8.01
WE	10%	16.66%	33.33%	>1000
AE	16%	30%	63.33%	384.66±9.42
CE	6.66%	23.33%	40%	>1000
MWE	16.66%	26.66%	43.33%	>1000
MAE	23.33%	30%	60%	724.11±8.01
MCE	6.66%	13.33%	36.66%	>1000

The average values of three calculations are presented as mean ± S.D. (Standard Deviation)

shrimp lethality assay AE, CE, MAE and ME exhibited potent cytotoxic behaviors with LC₅₀ of 384.66±9.42 µg/ml, 724.1±8.01µg/ml and 978.7±8.01µg/ml respectively (table 2). While remaining extracts were found to be inactive having LC₅₀>1000. The results indicated that all the extracts possessed weak antibacterial activity with zone of inhibition 7 mm. The WE inhibited the growth of all the tested gram-positive and gram-negative bacterial strains. On the other hand, insignificant antifungal activity was exhibited at 400µg/disc.

DISCUSSION

A broad and novel antitumor effect was detected by the potato disc assay using *A. tumefaciens*. The validity of potato disc assay is expected and may be observation of many tumorigenic mechanisms similar in plants and animals. Literature review suggested that inhibition of crown gall tumor on potato disc assay show a correlation with plant extracts and compounds are known to be active in 3PS antitumor assay. According to the report of (Ferrigini *et al.*, 1982) crown gall tumor on potato discs is usually in use as relatively fast, safe, inexpensive and reliable prescreen for antitumor activity. In our study, considerable level of antitumor activities were obtained with ME and WE while CE and MCE are the least active and had an IC₅₀ >1000µg/disc. Similar results were

reported by Arican, (2009) in *R. ficaria*, he found 62.6% inhibitory effects of the root extract.

Brine shrimp lethality assay after 24 hours of exposure to the crude extracts and positive control were investigated. AE, CE, MAE and ME exhibited potent cytotoxic behaviors. The remaining extracts were found to be inactive having LC₅₀>1000. Morshed *et al.*, (2011) while working on *Terminalia arjuna* ethanolic extract found LC₅₀ value of 50.11µg/ml at concentration of 50µg/ml in brine shrimp cytotoxicity assay. Similarly D'Souza *et al.*, (2002) reported that ethanolic extracts of *Bacopa monnieri* showed potent activity against brine shrimps. He found that Bacoside A, an active agent in the species showed the significant activity with a LD₅₀ of 38.3µg/ml. So this plant can be considered as a strong candidate processing cytotoxic potential.

The crude extracts from plants are always a mixture of active and non-active compounds. The WE showed very minor antibacterial activity against the inhibition of growth of bacteria at concentration of 400µg/disc. Similar work has been reported by Coban and Biyik (2010) on species of *Ranunculus* by using the disc diffusion method against various bacterial strains and found higher antibacterial activity. Kaya *et al.* (2010) reported antibacterial activity of *R. marginatus* and *R.*

sprunerianus, evaluated by the similar method and found inhibition zones ranged between 7-12mm and MIC values between 128µg/ml and 256µg/ml. So, this date of *R. arvensis* is not in accordance with the other species of *Ranunculus*.

With reference to antifungal activity, none of the extract was found to be active against any fungal organisms. Similar work has been reported on *R. asiaticu* and found that fresh shoot extract prevented growth of *Fusarium oxysporum* having inhibition zone of 2.5, 3.7 15.9 mm on 4th, 8th and 16th days of incubation respectively by (Qasem, 1996).

The result of the present investigations is quite encouraging and significant explores in-vitro antitumor activity of *R. arvensis* L. extracts, probably because of its direct cytotoxic effects. This plant could be used as a source of potent antitumor agents for antitumor drug development. Moreover, no significant results were obtained in antibacterial and antifungal activities. *In-vitro* phytochemicals effects have already been reported from *R. arvensis* extracts (Hussain *et al.*, 2011). Thus, the result prompted us for further isolation and identification of bioactive compounds. The future studies will be focused on characterization of active compound on all the extracts.

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