

## ***In vitro* Screening of *Ranunculus muricatus* for Potential Cytotoxic and Antimicrobial Activities**

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**Abstract:** Antibacterial and antifungal activities of four fractions (n-hexane, chloroform, ethyl acetate and ethanol) of *Ranunculus muricatus* were tested against two human Gram positive (*Staphylococcus aureus*, *Micrococcus luteus*) and four Gram negative pathogen (*Escherichia coli*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Klebsiella pneumoniae*). The antimicrobial activities were performed by agar well diffusion method. MIC and MBC were carried out by agar dilution method and viable cell count method, respectively. Cytotoxic activities of the crude and fractions were determined by using brine-shrimp (*Artemiasalina*) lethality bioassay by Mayer. The Ampicillin, Ofloxacin and Itraconazole were used as standard agents. Nearly all fractions exhibited more or less antimicrobial activity. The Ethyl acetate fraction illustrated maximum antimicrobial activity, MIC was 0.119 µg/ml against *Staphylococcus aureus*. Likewise n-hexane fraction exhibited highest antifungal activity against *Aspergillus niger* while all the other fractions also showed good antifungal activity. Cytotoxic activity of ethyl acetate fraction was high among all the other fractions.

**Key words:** *Ranunculus muricatus* • Cytotoxic • Antimicrobial Activities • Mic and Mbc

### **INTRODUCTION**

The genus *Ranunculus* belongs to the family *Ranunculaceae*, which comprise 50 genera and 2000 species, distributed throughout the northern hemisphere. It is also found in southern temperate regions, in the tropic where they are usually confined to higher altitude. In Pakistan, it is represented by 22 genera and about 114 species. Several genera were used for ornamental purpose, while other are toxic and are used for medicinal purposes [1]. The *ranunculus* is a genus of about 600 species of annual or perennial herbs widely distributed in the northern temperate region. About 23 to 25 species occur in Pakistan. The members of the genus *ranunculus* are reported to contain anemonin [2] flavones glycosides [3] and ranucosides. The most common use of *Ranunculus* species is for the treatment of antirheumatism, rubifacient and Intermittent fever.

For this use the plant is commonly prepared as decoction. It is also indicated as a remedy for antihemorrhagic (*Ranunculus repens*) [4] neuralgia pains, anti-spasmodic, diaphoretic (*Ranunculus bulbosus*) [5], vermifacient, anthelmintic (*Ranunculus hirtellus*) [6], tympany, conjunctivitis of an eye (*Ranunculus laetus*) [7, 8], cure internal abscess, malaria, scrofula, snake or scorpion venom and acute icteric hepatitis (*Ranunculus sceleratus*) [9].

There are 400 species of this genus in temperate and cold region and on tropical mountains. All the plants of this genus are sharp, bitter in taste and blistered the tongue. An ointment of the leaves or flowers would produce a blister on the skin. *Ranunculus* is a wide spread genera having unique toxicological and pharmacological activities. The different extracts from the plants exhibited anti-inflammatory and analgesic activities [10].

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The toxicological studies on *Ranunculus* have revealed the hepatotoxicity and photosensitization reaction with some species of the genus [11]. Some compounds isolated from *Ranunculus* have shown strong antibacterial and antifungal activities [12-14].

The present study was conducted to calculate Antioxidant, antimicrobial and antifungal activity of *Ranunculus muricatus* used locally as traditional medicine against numerous infections. The MIC and MBC was also carried out.

## MATERIAL AND METHODS

**Plant Material:** The plant was collected on the basis of information given by local residents during follow-up of ethnomedical and traditional uses of plants against infectious diseases used locally [15]. Plant was identified at GPG College Bannu by Prof; AbdurRahmankpk, Pakistan. The voucher specimen (NO: 120A) was deposited in the herbarium of Government Post Graduate College Bannu.

**Preparation of Crude Extracts:** Ninety grams of each powdered plant material were extracted with 80% methanol by maceration for 48 h with repeated agitation and the resulting liquid was filtered (Whatman No. 3 filter paper, Whatman Ltd., England). Extraction was repeated several times and the filtrates of all portions were combined with each other in one vessel. The solvent was removed by Rota-vapor (BU<sup>™</sup> CHI Rota-vapor R-205, Switzerland) at nearly 40°C. The aqueous filtrate was then placed in an oven at 40°C for approximately 48 h to remove the water. The resulting dehydrated mass was then powdered, packed into a glass vial and stored in a desiccator using silica gel [16].

**Preparation of Fractions:** About 90 g of *Ranunculus muricatus* were successively extracted with n-hexane, chloroform, ethyl acetate and ethanol using Soxhlet apparatus. The solvent was evaporated under reduced pressure and the fractions were then placed in a

vacuum oven at 40°C for about 24 h to remove any remaining solvent. The resulting semisolid mass of each fraction was stored in a desiccator using silica gel [16].

**Microorganisms:** Six bacterial species, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* (Clinical strain/PIMS), *Enterobacter cloacae* (Clinical strain/PIMS), *Staphylococcus aureus* (MRSA, clinical strain/PIMS) and *Micrococcus luteus* (Clinical strain/PIMS) were used in antimicrobial test. Strains were obtained from Microbiology Research Lab (MRL) School of life science Beijing University of chemical technology, china where these were identified and characterized. These strains were retained on agar slants at 4°C for antimicrobial tests. Microorganisms were incubated overnight at 37°C in Mueller-Hinton Broth (Oxoid) at pH 7.3. The reference antibiotics used were ofloxacin (10µg) and Ampicillin (10µg) (Oxoid) (Table 1).

### Anti-microbial Screening

**Screening for Antibacterial Activity:** The antibacterial activity was carried out by means of agar well diffusion method [17], All bacterial cultures were first grown up in nutrient broth at 37°C for 18-24 h incubated till turbidity became comparable to McFarland 0.5 turbidity standard was obtained. The inocula of the relevant bacteria were streaked on to the Muller Hinton agar (Oxoid) plates using a sterile swab in order to make sure a homogeneous thick lawn of growth following incubation. Wells of 6 mm in diameter were produced on to nutrient agar plates using a disinfected cork borer. The wells were filled with the given test agents (100 µl) and the plates were then allowed to keep on for 1-2 hours at 25°C. At last, the plates were incubated at 37°C for 18-24 h and the resulting diameters of zones of inhibition were measured.

**Determination of Minimum Inhibitory Concentration (MIC):** Minimum inhibitory concentration (MIC) of the crude extracts was found out by agar dilution method [18-20]. The sterilized Muller Hinton Agar (Oxoid) n was

Table 1: Zone of Inhibitions of reference antibiotics

Reference Antibiotic	Microorganism/mm					
	Ec	Kp	Ent	Ps	MI	Sta
Ampicilline	14.1(±0.05)	12.3(±0.5)	14(±0.11)	11(±0)	13(±0.05)	14(±0.05)
ofloxacin	15.1(±0.2)	14(±0.1)	13(±0)	11.9(±0.1)	10(±0.11)	9.06(±0.05)

Ec - *E. coli*, Kp - *Klebsiella pneumoniae*, Ent-*Enterobacter cloacae*, Ps - *Pseudomonas aeruginosa*, MI - *Micrococcus luteus*, Sta -*Staphylococcus aureus* (Methicilline resistant). na -not active, mm –millimetre

allowed to cool to 50°C and about 19 ml of this was added to sterilized test tubes which contained 1ml of dissimilar concentration of crude extract. This mixture was thoroughly mixed and poured into pre-labelled disinfected Petri-Dishes. Petri dishes having only growth media were prepared in the identical way so as to serve for comparison with Petriplate including crude extract. The concentrations of the extracts used in this test ranged from 3000µg/ml to 0.119µg/ml. The suspensions of the respective microorganisms having density adjusted to 0.5 McFarland turbidity standard were inoculated onto the chain of agar plates using standard loop. The plates were then incubated at 37°C for 24 hours. The lowest possible concentration which inhibited the growth of the respective organisms was taken as MIC. All tests were carried out in triplicate.

**Minimum Bactericidal Concentration (MBC):** Minimum Bactericidal Concentration (MBC) of the *Ranunculus muricatus* was calculated by the viable cell count method [21, 22] and the results were expressed as number of viable cells as a percentage of the control.

**Screening for Antifungal Activity:** The required amounts of each fungal strain were placed in 2ml of Sabouraud dextrose broth. This suspension was consistently spread on Petri plates containing Sabouraud dextrose agar media using disinfected swabs. Samples were applied into wells using same technique for bacteria and incubated at room temperature for 3 days. The plates were checked for the presence of zones of inhibition and the results were recorded. Itraconazole was used as a positive control.

**Cytotoxic Activity of Crude and Fractions:** Cytotoxic activities of the crude and fractions were determined by using brine-shrimp (*Artemiasalina*) lethality bioassay [23]. Artificial sea water was prepared by dissolving 3.7 g of sea salt per liter of double distilled water and filtered. "Sea" water was placed in small tank, added brine shrimp

eggs (1mg) and was darkened by covering with aluminum foil. Twenty mg of concentrated sample was dissolved in 2 mL of CHCl<sub>3</sub> (20 mg/2mL) and transferred to 500, 50 and 5 µL vials corresponding to 1000, 100 and 10 µg/mL respectively. Then three replicates were prepared for each concentration making a total of nine vials. The vials containing the sample were concentrated, dissolved in DMSO (50µL) and 5 mL "sea water" was added to each. Then ten shrimps were added per vial. All the vials were incubated at 37°C for 24 h and the brine shrimps that survived were counted. The activities of these extract and fractions were compared with standard drugs Ampicillin [24, 25]. The data were analyzed with a Finney computer program to determine LD<sub>50</sub> values with 95% confidence interval.

## RESULTS

Almost all fractions of *Ranunculus muricatus* presented hopeful activity against both Gram positive and negative bacterial pathogens.

The fraction A3 (Ethyl acetate) was reported as most dynamic fraction against all bacterial strains especially, against *Micrococcus luteus* and *Pseudomonas aeruginosa*. Like-wise n-hexane, ethanol and chloroform fractions showed reportable inhibitory effects against bacterial pathogens (Table 2). As a result of significantly high antibacterial activity, the A3 fraction was more processed for determination of MIC (Minimum inhibitory concentration) and MBC (Minimum bactericidal concentration) respectively. The MIC values ranged from 0.119 to >10µg/ml for all tested strains while the MBC values accounted were two times higher than MIC (Table 3).

Almost comparable pattern of defencelessness was reported against fungal strain *Aspergillus niger*. The widest zones of inhibition (Maximum antifungal activity) was given by A1 (n-hexane) and A3 (Ethyl acetate) fractions. The A2 (Ethyl acetate) and A4 (Ethanol) fractions illustrated comparatively lesser zone of inhibitions (Table 4).

Table 2: Inhibition zones of *Ranunculus muricatus* plant extracts

Fractions	Zone of Inhibition (mm)					
	Ec	Kp	Ent	Ps	MI	Sta
A1 (n-Hexane)	3 (±0.1)	2(0)	4(±0.11)	2(±0.1)	7(±0.6)	3(±0.3)
A2 (chloroform)	8 (±0.9)	8(±0.2)	4(±0.12)	6(±0.12)	3(±.03)	4 (±0.05)
A3 (ethyl acetate)	11(0)	13(±0.11)	12(±0.05)	14(±0.6)	14(±0.12)	8(±0.2)
A4 (ethanol)	6(±0.03)	3(±0.02)	N.d	2(±0.13)	N.d	5(±0.15)

Ec - *E. coli*, Kp - *Klebsiella pneumoniae*, Ent - *Enterobacter cloacae*, Ps - *Pseudomonas aeruginosa*, MI - *Micrococcus luteus*, Sta - *Staphylococcus aureus* (Methicillin resistant), na - not active, mm - millimetre

Table 3: MIC and MBC *Ranunculusmuricatus* (A3 Ethyl acetate) plant extract

A3(ethyl acetate) Fraction	MIC ( $\mu\text{g/ml}$ )	MBC ( $\mu\text{g/ml}$ )
<i>E.Coli</i>	> 10	N.d
<i>K. Pneumoniae</i>	> 10	N.d
<i>P.Aeruginosa</i>	> 10	N.d
<i>Enterobacter</i>	5	15
<i>M. luteus</i>	0.625	1.875
<i>S.Aureus</i>	0.133	1.24

N.d - not determined,  $\mu\text{g/ml}$  - microgram per millilitre

Table 4: Antifungal activities of *Ranunculusmuricatus* crude extracts

Fraction	Zone of Inhibition (mm)
A1 (n-Hexane)	6 ( $\pm 0.13$ )
A2(chloroform)	3 ( $\pm 0.16$ )
A3(ethyl acetate)	6 ( $\pm 0.14$ )
A4(ethanol)	2(0)
Standard (itraconazole)	8(0)

Table 5: Cytotoxic activities of crude extract and fractions

Concentration ( $\mu\text{g/ml}$ )	Fractions and crude extract % Deaths at doses					
	n-hexane (A1)	$\text{CHCl}_3$ (A2)	EtOAc (A3)	Ethanol (A4)	Ethanollic crude	Standard drug (Ampicillin)
10	40	15	50	30	20	40
100	50	25	60	35	30	80
1000	55	30	80	60	40	120
ED <sub>50</sub>	320.60	650.55	120.25	450.40	540.65	70.50

The crude ethanolic extract was successively extracted with n-hexane (A1), chloroform (A2), ethyl acetate (A3) and ethanol (A4). All these fractions and the crude extract were tested for their cytotoxic activities (Brine shrimp lethality assay). The A3 was found to be more effective and showed high activities, A1 showed optimum while A2 and A4 showed low lethality in brine shrimp assay (Table 5).

## DISCUSSIONS

The antibacterial and antifungal activities of four crude extracts (n-hexane, chloroform, ethyl acetate and ethanol) were tested against six bacterial species *Via Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Staphylococcus aureus* and *Micrococcus luteus*. Almost all fractions showed more or less antimicrobial activity against the test strains. The A3 (Ethyl acetate) fraction showed good antimicrobial activity against *Pseudomonas aeruginosa* and *Micrococcus luteus* with considerable MIC values. The MIC values are too high to be considered in susceptible ranges [26]. The MBC values of fraction A3 (Ethyl acetate) are 2 times the MIC. The n-hexane fraction exhibited highest antifungal activity against *Aspergillus niger* while all the other

fractions also showed good antifungal activity. Cytotoxic activity of ethyl acetate fraction was high among all the other fractions.

This report is possibly the first to investigate the antimicrobial activities of *Ranunculus muricatus*, as a wide-ranging literature review provide no information about the antimicrobial activities of this plant.

## CONCLUSION

*Ranunculus muricatus* have supreme antioxidant and antimicrobial activities. The fraction A3 (Ethyl acetate) showed highest antioxidant and antimicrobial activities. Fraction A1 have highest antifungal activity. This species is as one of the ingredient of the predictable medicine in some part of the world. More investigations are mandatory to exploit the hidden medicinal importance of this plant.

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