Antimicrobial activity of five constituents isolated from *Ranunculus muricatus*

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Antimicrobial activity of five compounds isolated from *Ranunculus muricatus*, namely, Tricin7-O-β-D-lucopyranoside (1), protocatechuyl aldehyde (2), isoscopoletin (3), anemonin (4), B-sitosterol (5) were tested against two human Gram-positive bacteria (*Staphylococcus aureus*, *Micrococcus luteus*) and four Gram-negative ones (*Escherichia coli*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Klebsiella Pneumoniae*). All the five compounds isolated, were tested against the fungal strain *Aspergillus niger*. The structures of the compounds have been confirmed by EIS, HR-EIMS and ¹H-¹³C-NMR spectroscopic methods. 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. Compound 1 showed maximum antimicrobial activities while the other compounds also showed superior antimicrobial activities. These compounds were isolated first time from *R. muricatus* which are persuasive as standard antimicrobial drugs.

**Key words:** *Ranunculus muricatus*, isolation, antimicrobial activity, minimum inhibitory concentration (MIC), minimum bactericidal concentration (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 others are toxic and are used for medicinal purposes (Riedl and Nasir, 1991). 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 occurs in Pakistan. The members of the genus *Ranunculus* are reported to contain anemonin (Ruijgrok et al., 1963), flavones glycosides (Litvinenko et al., 1969) 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 anhemorrhagic (*Ranunculus repens*) (David et al., 2000), neuralgia pains, anti-spasmodic, diaphoretic (*Ranunculus bulbosus*) (Maria et al., 2009), vermifacient, anthelmintic (*Ranunculus hirtellus*) (Sanjay et al., 2006), tympany, conjunctivitis of an eye (*Ranunculus laetus*) (Pande et al.,

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2007; Dobriyal et al., 1997), cure internal abscess, malaria, scrofula, snake or scorpion venom, and acute icteric hepatitis (*Ranunculus sceleratus*) (Mei et al., 2012).

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 (Prieto et al., 2008).

The toxicological studies on *Ranunculus* have revealed the hepatotoxicity and photosensitization reaction with some species of the genus (Kelch et al., 1992). Some compounds isolated from *Ranunculus* have shown strong antimicrobial, antibacterial and antifungal activities (Mares et al., 1987; Misra and Dixit, 1978; Tocan and Baron, 1969).

Here, we report the isolation, separation and antimicrobial activities of the compounds which are first time isolated from *Ranunculus muricatus*, namely, tricin7-O-β-D-lucopyranoside (1), protocatechualdehyde (2), isoscopoletin (3), anemonin (4), and β-sitoseterol (5).

The isolated compounds were screened for antibacterial and antifungal activities. The MIC and MBC was also carried out.

**MATERIALS AND METHODS**

**General experimental procedure**

To check the purity of the compounds, thin layer chromatography (TLC) was carried out by using aluminium TLC plates (20 × 20, 0.5 mm thick) pre-coated with silica gel (E. Merck, Darmstadt, Germany). Column chromatography (CC) was carried out using silica gel of 230 to 400 mesh (E. Merck, Darmstadt, Germany). Ceric sulphate and potassium permanganate solutions were used as visualization reagents. The UV spectra (λmax nm) were recorded on Shimadzu UV-2700 spectrophotometer (Shimadzu, Japan) in EtOH. Mass spectra was recorded on Bruker TOF Mass spectrometers (Billerica, USA) using electrospray ionization (ESI). The 1H NMR and 13C NMR spectra were recorded on a Bruker DPX-400 NMR spectrometer (Billerica, USA) (400 MHz for 1H and 100 MHz for 13C-NMR), using CDCl3 as solvents.

**Plant**

The plant material of *R. muricatus* was collected from Lakki Marwat, and identified by Abdur Rahman, Professor in Botany, Government Post Graduate College Bannu. The voucher specimen (NO: 120A) was deposited in the herbarium of Government Post Graduate College Bannu.

**Extraction and isolation**

The shade dried whole plant of *R. muricatus* (19 kg) was ground and extracted with ethanol at room temperature (3 × 25 L). The combined ethanolic extract was evaporated under reduced pressure to obtain a thick blackish gummy material (crude). It was fractionated with n-hexane (161 g), chloroform (164 g), ethyl acetate (116 g), and n-butanol (68 g) soluble fractions, respectively. The ethyl acetate soluble fraction was subjected to column chromatography over silica gel (65 to 225 mesh) eluting with n-hexane (100%), n-hexane: EtOAc:EtOH (0.5:9.5 to 9.5:0.5), EtOAc (100%), EtOAc:EtOH (0.5:9 to 9.5:0.5), EtOH (100%), in increasing order of polarity to obtain 11 fractions A-K. A series of silica gel column chromatography was carried out of fraction C eluting with n-hexane, n-hexane-EtOAc and EtOAc in increasing order of polarity to get compound 1 to and a preparative TLC using n-hexane: EtOAc (2:3) as solvent system to get compounds 2 and 3. Fraction D (5 g) was re-introduced to column chromatography over silica gel washing with n-hexane, n-hexane-EtOAc and EtOAc in increasing order of polarity. Which was followed by preparative TLC washed with n-hexane: CH2Cl2 (1:1.5) and got compounds 4 and 5 (Figure 5).

**Microorganisms**

Six bacterial strains, *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 antibacterial assay. One fungal strain *Aspergillus niger* was used for antifungal assay. Strains were obtained from School of Life Science and Technology, Beijing University of Chemical Technology, China, where these were identified and characterized. These strains were maintained on agar slants at 4°C in School of Life Science and Technology, Beijing University of Chemical Technology for antimicrobial tests. Microorganisms were incubated overnight at 37°C in Mueller-Hinton Broth (Oxoid) at pH 7.4. The reference antibiotics used were Ofloxacin (10 μg) and Ampicilline (10 μg) (Oxoid) (Figure 1 and Table 1).

**Antimicrobial screening**

**Screening for antibacterial activity**

Agar well diffusion method: The agar well diffusion method (Hadacek et al., 2008) was carried out to determine the antibacterial activity. All bacterial cultures were first grown in nutrient broth at 37°C for 18 to 24 h incubated till turbidity became correspondent to McFarland 0.5 turbidity standard was obtained. The inocula of the respective bacteria were splashed onto the Muller Hinton agar (Oxoid) plates using a sterile swab in order to make sure a uniform thick lawn of growth following incubation. With the help of sterile cork borer, wells of 6 mm in diameter were formed on to nutrient agar plates. The test agents (100 μl) were put to the wells and the plates were then allowed to stay for 1 to 2 h at room temperature. Finally, the plates were incubated at 37°C for 18 to 24 h and the resulting diameters of zones of inhibition were measured as shown in Figure 2 and Table 2.

**Determination of minimum inhibitory concentration (MIC):** MIC of compound 1 was determined by agar dilution method (EUCAST Definitive Document, 2000; Mukherjee, 2002; Anon, 2000). The sterilized Muller Hinton Agar (oxoid) was allowed to cool to 50°C and about 19 ml of this was added to sterilized test tubes which contained 1 ml of different concentration of compounds. This mixture was thoroughly mixed and poured into pre-labeled sterile petri dishes. Petri dishes having only growth media were prepared in the same way so as to serve for comparison with petri plate containing compound. The concentrations of the compound used in this test ranged from 2000 to 0.156 μg/ml. The suspensions of the
Table 1. Zone of inhibition of reference antibiotics.

<table>
<thead>
<tr>
<th>Reference antibiotic</th>
<th>Micro organisms and their zone of inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ec</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>15.1 (±0.02)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>14.1 (±0.05)</td>
</tr>
</tbody>
</table>

Ec: Escherichia coli; Kp: Klebsiella pneumoniae; Ps: Pseudomonas aeruginosa; Ent: Enterobacter cloacae; Ml: Micrococcus luteus; Sta: Staphylococcus aureus (methycillin resistant); mm: millimeter.

Figure 1. Zone of inhibition of reference antibiotics. Ec: Escherichia coli; Kp: Klebsiella pneumoniae; Ps: Pseudomonas aeruginosa; Ent: Enterobacter cloacae; Ml: Micrococcus luteus; Sta: Staphylococcus aureus (methycillin resistant).

Table 2. Inhibition zones of compounds 1 to 5.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Zone of inhibition (mm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ec</td>
</tr>
<tr>
<td>1</td>
<td>5 (±0.5)</td>
</tr>
<tr>
<td>2</td>
<td>8 (±0.3)</td>
</tr>
<tr>
<td>3</td>
<td>10 (±0.2)</td>
</tr>
<tr>
<td>4</td>
<td>11 (±0.2)</td>
</tr>
<tr>
<td>5</td>
<td>6 (±0.5)</td>
</tr>
</tbody>
</table>

Ec: Escherichia coli; Kp: Klebsiella pneumoniae; Ps: Pseudomonas aeruginosa; Ent: Enterobacter cloacae; Ml: Micrococcus luteus; Sta: Staphylococcus aureus (methycillin resistant); mm: millimeter.

Determination of minimum bactericidal concentration (MBC): MBC of compound 1 was measured by the viable cell count method (Toda et al., 1989; Anon, 2003), and the results were expressed as the number of viable cells as a percentage of the control.

Screening for antifungal activity

The required amount of each fungal strain was suspended in 2 ml of Sabauraud Dextrose broth. This suspension was uniformly spread on Petri plates containing Sabauraud Dextrose agar media using sterile swabs. Samples were applied into wells using same technique for bacteria and incubated at 25°C for 3 days. The plates were then examined for the presence of zones of inhibition and the results were recorded. Itraconazole was used as a standard or positive control.

Compound 1 has high antibacterial activity, so it was further processed for determination of MIC and MBC, respectively. The MIC values ranged from 0.156 to >10 mg/ml for all tested strains, while the MBC values reported were many times higher than MIC (Table 3 and Figure 3). Likewise, nearly similar pattern of susceptibility was reported against fungal strain A. niger. The widest zones of inhibition (maximum antifungal activity) were presented by compounds 1 and 2. Compounds 3 and 4 are reasonable, while the compound 5 has the lowest zone of inhibition was represented in Table 4 and Figure 4.
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Figure 2. Inhibition zones of compounds 1 to 5. Ec: Escherichia coli; Kp: Klebsiella pneumoniae; Ps: Pseudomonas aeruginosa; Ent: Enterobacter cloacae; Ml: Micrococcus luteus; Sta: Staphylococcus aureus (methicillin resistant).

Figure 3. MIC and MBC of Ranunculus muricatus compound 1.

Table 3. MIC and MBC of Ranunculus muricatus compound 1.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>MIC (mg/ml)</th>
<th>MBC (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>&gt;10</td>
<td>N.d</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>&gt;10</td>
<td>N.d</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>&gt;10</td>
<td>N.d</td>
</tr>
<tr>
<td>E. cloacae</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>M. luteus</td>
<td>0.625</td>
<td>1.877</td>
</tr>
<tr>
<td>S. aureus</td>
<td>0.156</td>
<td>1.260</td>
</tr>
</tbody>
</table>

N.d: Not detected.

RESULTS

The antimicrobial activities of five compounds isolated from ethyl acetate fraction were tested against six bacteria species E. coli, K. pneumoniae, P. aeruginosa, E. cloacae, S. aureus, and M. luteus. Nearly all compounds exhibited more or less antimicrobial activity against the test strains. Compound 1 exhibited the finest activity against these bacteria. Besides that, compounds 2 and 3 had moderate and compounds 4 and 5 had also sensible activity against these bacteria. Nearly all the constituents
from *R. muricatus* were primarily reported as active against the *A. niger* in spite of the fact that the inhibitory zones observed were not significantly wide. Compounds 1 and 2 showed the highest antifungal activities, while compound 5 showed the least antifungal activity.

**DISCUSSION**

All the compounds were isolated from the ethyl acetate fraction of *R. muricatus*, that is, 1 to 5 having antibacterial activity, but compound 1 had the utmost antibacterial activity. The MIC of compound 1 was taken as 0.156 mg/ml. It is important that the MIC value is too high to be taken in susceptible ranges (Paul et al., 2006). The MBC value of compound 1 is many times higher than MIC. The antibacterial and antifungal assays were carried out by Agar well diffusion method. The MIC was carried out using Agar well dilution method, while MBC was performed by viable cell count method.

Nearly all the compounds isolated from the ethyl acetate fraction of *R. muricatus* are polar and were primarily as antimicrobial reagents. Compounds tricin7-O-β-D-lucopyranoside (1), protocatechuyl aldehyde (2), isocopoletin (3) and compounds anemonin (4), β-sitoseterol (5) previously isolated from *R. sceleratus* and *R. bulbosus*, respectively (Louaar et al., 2012).

This report is probably the first to explore the antimicrobial activities of constituent of *R. muricatus*, as a comprehensive literature review to the best of our knowledge; there is no information about the antimicrobial activities of these isolated constituents from this
plant.

Conclusion

It is accomplished from these studies of five isolated compounds from ethyl acetate fraction and antimicrobial activities of fractions that the plant *R. muricatus* species has potential antimicrobial activities. Consequently, the other three fractions n-hexan, chloroform and ethanol are further recommended for investigations to explore the hidden medicinal value of the plant.

REFERENCES


