Global Journal of Pharmacology 8 (1): 53-59, 2014
ISSN 1992-0075

DOI: 10.5829/idosi.gjp.2014.8.1.81247

In vitro Antimicrobial Activity of the Chemical Ingredients of *Ranunculus hirtellus*

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Abstract: The phytochemical study on the n-butanol fraction of *Ranunculus hirtellus* led to the first time isolation of four known constituents (1-4).The antimicrobial and enzyme inhibition activities of these four constituents isolated from *Ranunculus hirtellus* are presented in this study. These constituents were screened against two human Gram-positive bacteria (*Staphylococcus aureus*, *Micrococcus luteus*) and four Gram-negative ones (*Escherichia coli*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Klebsiella Pneumoniae*) by using Agar well diffusion method for both antibacterial and antifungal activities. MIC was determined by Agar well dilution method. MBC was performed by viable cell count method. Compound (1) showed maximum antimicrobial activity while the other compounds showed least antimicrobial activities. In addition these four isolated compounds were screened for their Acetyl cholinesterase (AChE) and Butyryl cholinesterase (BChE) inhibitory activities. Moreover, the IC_{50} (50% inhibitory effect) values of compounds 2 and 3 against AChE were determined to be 3.14 and 1.53 µM while the values obtained against BChE were 5.55 and 6.11 µM respectively.

Key words: Ranunculus Hirtellus · Antimicrobial Activity · Cholinesterase Inhibition · MIC · MBC

INTRODUCTION

The *Ranunculus* is a genus of *Ranunculaceae*, which have 50 genera and 2000 species, founded in the northern hemisphere and southern temperate regions. There are present about 22 genera and 114 species of *Ranunculaceae*. Some genera are used for decorative purpose, while some are toxic and are used for therapeutic purposes [1]. The *Ranunculus* has about 600 species of annual or permanent herbs generally distributed in the northern temperate region.

The plants of *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 indicated as a therapy for antihemorrhagic (*Ranunculus repens*) [4]. The general use of *Ranunculus* species is for the treatment of antirheumatism, rubifacient 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 venom and acute icteric hepatitis (*Ranunculus sceleratus*) [9], *Ranunculus diffuses* is used for the treatment of Rheumatism [10].

About 400 species of this genus *Ranunculus* found in temperate and cold region and on mountains. All the species of this genus *Ranunculus* are sharp, bitter in taste and sore the tongue. An ointment of the leaves or flowers would create a blister on the skin. *Ranunculus* has unique toxicological and pharmacological activities. The different extracts from the plants of *Ranunculus* have anti inflammatory and analgesic activities [11].

In the present study, we investigated the four known constituents first time isolated from n-butanol fraction of *Ranunculus hirtellus* namely, *R (+)-Delbergi phenol (1), 5-hydroxy traptamine* (2), *Hexadecanoic acid* (3),

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B-amyrin (4), [12-15] were screened for antimicrobial and cholinesterase inhibitory activities. The MIC and MBC of compound 1 were also takes place.

MATERIALS AND METHODS

Al-TLC plates (20 × 20, 0.6 mm thick) deposited with silica gel 60 F_{254} (20x20 cm, 0.2 mm thick; E. Merck, Darmstadt, Germany) were used to check the purity of the compounds. Column chromatography (CC) was done with silica gel of 230-400 mesh (E. Merck, Darmstadt, Germany). Ceric sulphate and potassium permanganate solutions were used as visualization reagents. The UV spectra (λ_{max} nm) were recorded on Shimadzu UV-2700 spectrophotometer (Shimadzu, Japan) in EtOH. Mass Spectra was took on Bruker TOF Mass spectrometers (Billerica, USA) using electrospray ionisation (ESI). The ^{1}H NMR and ^{13}C NMR spectra of the compounds were recorded on a Bruker DPX-400 NMR spectrometer (Billerica, USA) (400 MHz for ^{1}H and 100 MHz for ^{13}C-NMR), using CDCl3 as solvents.

Plant Material: The Plant materials of *Ranunculus hirtellus* were collected from Azad Kashmir Pakistan in May 2011. The plant was identified by Professor Dr. Manzoor Ahmad Plant Taxonomist on the basis of its morphological features in Government Degree College Abbotabad, Pakistan, where a voucher specimen was deposited in herbarium (Accession No. C-065).

Extraction and Isolation: The powdered material of *Ranunculus hirtellus* (6 kg) was extracted with methanol (60 L) at room temperature for 12 days (3 ×60 L). Then the resulting extract was filtered and evaporated under high pressure with the help of rotary evaporator to obtain dark greenish gummy crude (88 g). Four fractions were formed from the methanolic crude i.e *n*-hexane (K1, 15 g), chloroform (K2, 16 g), ethyl acetate (K3, 18 g) and *n*-butanol (K4, 10.3 g).

The *n*-butanol soluble fraction was subjected to column chromatography uploaded over silica gel (70-230 mesh) eluting with n-hexane (100 %), n-hexane: n-butanol (1:19?19:1), n-butanol (100%), n-butanol: MeOH (1:19?19:1), MeOH (100 %), with increasing order of polarity to obtain 10 fractions A-J.

Fraction B (6 g) of *n*-butanol fraction was again uploaded to a sequence of silica gel column chromatography eluting with *n*-hexane, n-hexane-*n*-butanol and *n*-butanol in increasing order of polarity give compound 1 with 100% *n*-butanol and to a preparative TLC using n-hexane: *n*-butanol (1:4) as solvent system to afford compounds 2 and 3 respectively.

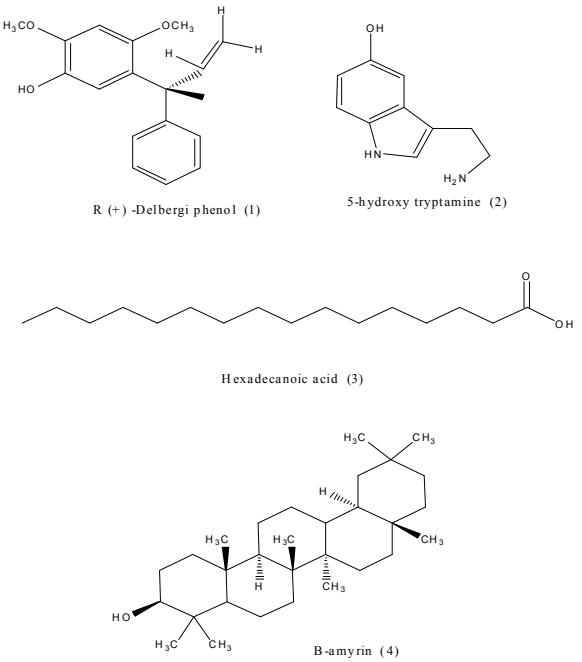

Fig. 1: Structures of compounds 1-4

Fraction F (6 g) was subjected again to column chromatography over silica gel eluting with *n*-hexane, n-hexane: *n*-butanol and *n*-butanol in increasing order of polarity followed by preparative TLC eluted with *n*-hexane: *n*-butanol (4:1) and isolated compound 4.

Microorganisms: Six bacterial species two Gram-positive and two Gram-negative i.e. *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 screened for antimicrobial test. Strains were obtained from School of biological science Beijing University of chemical technology, china. Their identification and characterization were take place in School of biological science Beijing University of chemical technology. These strains were kept on agar slants at 4°C for antimicrobial screening. Microorganisms were placed in incubator overnight at 37°C in Mueller-Hinton Broth (Oxoid) at pH 7.1. The ofloxacin (10µg) and Ampicillin (10µg) (Oxoid) were used as reference antibiotics (Table 1).

Table 1: Inhibition zone (mm) of reference antibiotics (Mean±SD)

Reference	Micro organisms and their zone of inhibition(mm)					
Antibiotics	Ec	Kp	Ps	Ent	Ml	Sta
Ofloxacin	15.1±0.02	14±0.01	11.9±0.01	13±0	10±0.11	9.6±0.05
Ampicillin	14.1±0.05	12.3±0.5	11±0	14±0.11	13±0.05	14±0.05

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

Antimicrobial Screening

Screening for Antibacterial Activity *(Agar well diffusion method):* In order to check the antibacterial assay of these four isolated compounds from the plant *Ranunculus hirtellus,* the agar well diffusion method [16] was used. All bacterial cultures were grown in nutrient broth at 37°C for 24 hrs placed in incubator till turbidity became correspondent to McFarland 0.5 turbidity standard was obtained. The inocula of the respective bacteria were sprayed on to the Muller Hinton agar (Oxoid) plates using a sterile swab in order to confirm a consistent thick lawn of growth following incubation. With the help of antiseptic cork borer wells of six millimetres in width were shaped on to nutrient agar plates. The screening agents (100 µl) were put to the wells and the plates were then allowed to keep on for 1:30 h at 25°C. Finally, the plates were kept in incubator at 37°C for 24 hrs and the resulting with of inhibition zones was measured. The compound 1 showed maximum antibacterial activity while all the other compounds also showed least antibacterial activity.

Determination of Minimum Inhibitory Concentration

Agar Dilution Method: Agar well diffusion method was used to determine the Minimum inhibitory concentration (MIC) of the compound 1 [17-19]. The Muller Hinton Agar (oxoid) was unadulterated and was allowed to cool to 50°C. About nineteen millilitre of this was mixed with one millilitre of different concentrations of compound 1 in cleaned test tubes. This mixture was poured into pre-labelled sterile Petri dishes. Petri dishes having only growth media were prepared in the same way so as to serve for assessment with petri plate containing compound. 2000 µg/ml to 0.312 µg/ml concentrations of the compound were used in this assay. The density of the suspensions of the individual microorganisms was adjusted to 0.5 McFarland turbidity standards. By using unadulterated standard loop these were inoculated onto the successive agar plates. The incubation of these plates was taken place at 37°C for 24 hours. The lowest concentration of the compound which stops the growth of the respective organisms was considered as MIC. All assays were carried out in triplicate.

Determination of Minimum Bactericidal Concentration

Viable Cell Count Method: Minimum Bactericidal Concentration (MBC) of the compound 1 was determined by viable cell count method [20, 21] and the results were expressed as number of viable cells as a percentage of the control.

Screening for Antifungal Activity: The required quantity of the fungal strain was suspended in 2 ml of Sabauraud dextrose broth. By using cleaned cotton pads, the suspension was homogeneously kept on Petri dishes having sabauraud dextrose agar media. Samples were poured into wells using same technique for bacteria but the fungus was placed in incubator at 25°C for 72 hours. The plates were then checked for the presence of inhibition zones and the results were recorded. An effective antifungal "Itraconazole" was used as a standard.

Cholinesterase Inhibition Activity and Determination of IC_{50} values: Acetyl cholinesterase (EC 3.1.1.7) acetylthiocholine iodide, butyrylthiocholine chloride, butyrylcholinesterase (horse-serum E.C 3.1.1.8), galanthamine and DTNB (5, 5-dithiobis [2-nitrobenzoic-acid]) were purchased from Sigma. The Acetyl cholinesterase (AChE) and butyrylcholinesterase (BChE) inhibiting activities were performed by the spectrophotometric assay used by Ellman [22], Protocol and activity conditions were similar to that of Rocha [23].

Butyrylthiocholine chloride and Acetylthiocholine iodide were used as reactant to determined acetyl cholinesterase and butyrylcholinesterase activity respectively. 5, 5´-Dithiobis [2-nitrobenzoic-acid] (DTNB) was applied for the measurement of cholinesterase activity. 0.2 mM DTNB in 62 mM sodium phosphate buffer (pH 8.0, 880 µL), test compound solution (40 µL) and acetyl cholinesterase or butyrylcholinesterase solution (40 µL) were mixed and placed in incubator for 15 minutes (25º C). The reaction was initiated by the addition of acetyl thiocholine or butyrylthiocholine (40 µL) respectively. The hydrolysis of acetylthiocholine and butyrylthiocholine were observed by the formation of

yellow 5-thio-2-nitrobenzoate anion by the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine and butyrylthiocholine, respectively, at a wavelength of 412 nm (15 min). Reactions were performed in triplicate in a BMS spectrophotometer (USA). The concentrations of test compounds that inhibited the hydrolysis of substrates (acetylthiocholine and butyrylthiocholine) by 50% (IC_{50}) were determined by observed the effect of increasing amount of these compounds in the activity on the inhibition values. The final DMSO quantity in the reaction mixture was 6%.

RESULTS

Compound 1 has high antibacterial activity isolated from *Ranunculus hirtellus* which is shown in (Table 2) and (Figure 2). As Compound 1 has high antibacterial activity so it was further proceed for determination of MIC (Minimum inhibitory concentration) and MBC (Minimum bactericidal concentration) respectively. The MIC values ranged from 0.312 µg/ml to > 10 µg/ml for all tested strains while the MBC values reported were much higher than MIC which is shown in (Table 3, Figure 3).The MBC values for *Enterobacter cloacae* was higher than all.

Nearly similar pattern of vulnerability was reported against fungal strain *Aspergillus niger*. The widest inhibition zones (Maximum antifungal activity) were presented by Compounds 1. Compound 4 has lowest inhibition zone which is represented in (Table 4, Figure 4).

Our concentration was to identify AChE and BChE inhibiting small molecules from herbal medicinal species, so performed bioassay-guided search for acetyl cholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitors from *Ranunculus hirtellus.* Compounds 1-4 isolated from *Ranunculus hirtellus* were tested against AChE and BChE, which show a good result for drug treatment of neurone design and Alzheimer's disease [24]. The percentage of inhibition was first checked at 0.1 mM. Those compounds which were having enzyme inhibition greater than 50% were consequently assayed for IC_{50} (50% inhibitory effect) determination. Among the isolated compounds, 2 and 3 showed most effective inhibition activity against AChE and BChE as compared to standard drugs; allanzanthane and galanthamine in a dose dependent manner. The IC_{50} values of compounds 2 and 3 against AChE were determined to be 3.14 and 1.53 µM, while against BChE, were measured as 5.55 and 6.11 µM respectively. Compound 4 showed weak inhibition profile against AChE and BChE (Table 5).

Table 2: Antibacterial activity of compounds 1-4

	Zone of inhibition (mm)					
Compounds	Ec	Kp	Ent	Ps	Ml	Sta
1	16±0.4	16±0.4	15±0	9±0.4	14±0.5	8 ±0.4
2	7±0.6	7±0.5	5±0.4	6±0.4	7±0.5	7±0.4
3	6±0.3	9±0.4	7±0.3	5±0.2	5±0.4	5±0.5
4	3±0.2	3±0.2	4±0.4	4±0.5	5±0.3	3±0.5

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

Table 3: MIC and MBC of *Ranunculus hirtellus* compound 1

Microorganisms	MIC µg/ml	MBC µg/ml
E. coli	>10	14
K. pneumoniae	>10	13
P. aeruginosa	>10	13
E. cloacae	5	15
M. luteus	0.625	1.877
S. aureus	0.312	1.260

Table 4: Antifungal activities of compounds 1-4

Compounds	Zone of inhibition (mm)
1	7±0.5
2	4±0.6
3	4±0.9
4	3±0.9
Standard	8.0±0.0

Table 5: AChE and BChE inhibitory activities of compound 1-4 from *Ranunculus hirtellus* (IC_{50}, µM)

S.No	Compounds	AChE ± SEM[a]	BChE ± SEM[a]
1	1	4.11±0.04	12.13±0.06
2	2	3.14 ± 0.07	5.55 ± 0.079
3	3	1.53 ± 0.03	6.11 ± 0.079
4	4	5.27 ± 0.04	14.76 ± 0.087
5	Allanzanthane b	2.94± 0.45	12.96 ± 0.053
6	Galanthamine [b]	1.79 ±0.061	7.98 ± 0.01

[a] Standard error of mean of five assays

[b] Positive control used in the assays.

Note: Data showed are values from triplicate experiments.

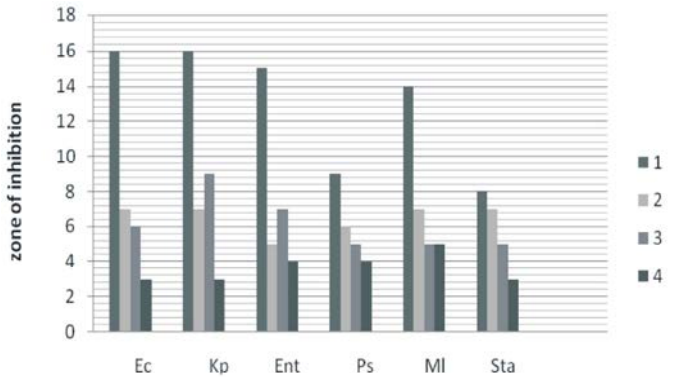

Fig. 2: Antibacterial Activity of Compounds 1-4

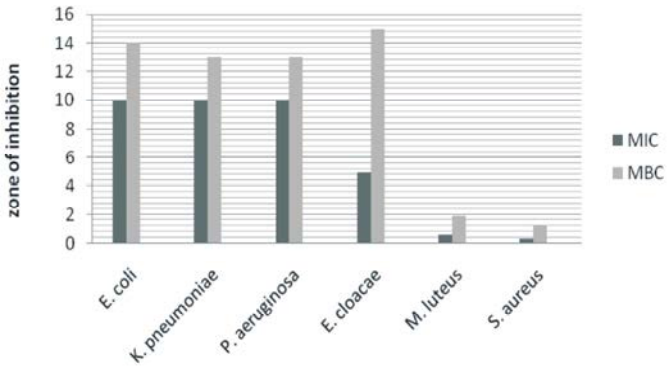

Fig. 3: MIC and MBC of Ranunculus hirtellus compond 1

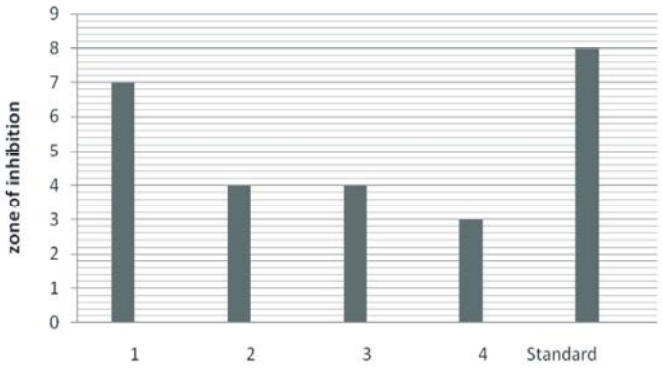

Fig. 4: Antifungal Activities of Compounds 1-4

DISCUSSION

The antimicrobial assay of four compounds isolated from n-butanol fraction of *Ranunculus hirtellus* were tested against two Gram-positive and four Gram-negative bacteria species *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterobacter cloacae, Staphylococcus aureus and Micrococcus luteus*. Nearly every compound has more or less antimicrobial activity against the tested cultures. Compound 1 was examined to have best activity against these bacterial species. Besides that, compounds 2, 3 and 4 have lowest activity against these bacterial species. Nearly all constituents from *Ranunculus hirtellus* were primarily investigated as active against the *Aspergillus niger*. Compounds 1 showed highest antifungal activity, while compound 4 showed least antifungal activity.

The MIC of the compound 1 was taken as 0.312µg/ml. It is significant that the MIC value is too high to be taken in susceptible ranges [25]. The MBC value of compound 1 is much higher than MIC. The Agar well diffusion method was used for determination of antibacterial and antifungal assay. The MIC was done by using Agar well dilution method while MBC was performed by viable cell count method.

All the four compounds isolated from the n-butanol fraction of *Ranunculus hirtellus* are polar and were primarily as antimicrobial reagents. Compounds 2 and 3 have highest enzyme inhibition activity i.e. Acetylcholinesterase and butyrylcholinesterase inhibitory effects.

CONCLUSION

From the present studies it is clear that four compounds were isolated from n-butanol fraction of *Ranunculus hirtellus,* which showed high antimicrobial and enzyme inhibitory assay. This report is almost certainly the first to explore the antimicrobial and enzyme inhibitory activities of compounds 1-4 of *Ranunculus hirtellus* as a comprehensive literature review to the best of our knowledge this work is novel and there is no information about the antimicrobial and enzyme inhibitory activities of these isolated constituents from the plant *Ranunculus hirtellus.* Consequently the other three fractions n-hexane, ethyl acetate and chloroform need further investigations to explore the higher medicinal compounds.

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