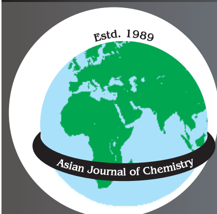

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Antimicrobial Efficacy and Cytotoxic Screening of Dichloromethane Sub-fractions of *Rumex obtusifolius*

ABDUL KHABIR[1], FARMAN KHAN[1], MASOOD AFZAL[1], ZIA UL HAQ[1], MALIK AMAN ULLAH[1], AMIR MIRAJ HUSSAIN SHAH[1], ABDUR RAUF[1], FAWAD ALI[2], MUHAMMAD QAISAR[3] and SHAFIULLAH KHAN[1,*]

[1]Institute of Chemical Sciences, Gomal University, Dera Ismail Khan, D.I. Khan 29050, Pakistan
[2]Institute of Chemical Sciences, University of Peshawar, Peshawar 25100, Pakistan
[3]Medicinal Botanic Centre, PCSIR Labs Complex, Peshawer 25120, Pakistan

*Corresponding author: E-mail: fermeon@gu.edu.pk

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The present study has been carried out to investigate the antibacterial, antifungal and cytotoxic screening of sub-fractions of dichloromethane fraction of *Rumex obtusifolius* at early vegetative stages for seeking bioactive sub-fractions. Its common name is broad-leaf dock. This plant is used as an antidote to nettle, astringent, depurative, tonic and laxative. It is also used for treatment of tumors, blisters, sores, burns and cancer. The bioactivity screening of F_1, F_2, F_3, F_4, F_5 and F_6 sub-fractions of dichloromethane of *R. obtusifolius* were assessed using conventional disc diffusion method, agar tube dilution method and the brine shrimp lethality assay. The most remarkable antibacterial activities are observed with the F_6 having zone of inhibition (22.5mm) against ampicillin-resistant *Escherichia coli*. The antifungal results indicate that F_3, F_4 and F_5 inhibited fungal growth more competently as compared to F_1, F_2 and F_6. Maximum inhibition was recorded by F_4 (69.4 %) against *Aspergillus flavas*. F_1 does not show any prominent inhibition (7.4 ± 2.51 to10.3 ± 2.21) against all fungal strains, b/c the probability of bioactive compounds in F_1 is less, as ethanolic crude was already extracted with *n*-hexane in solvent extraction process. LD_{50} values of cytotoxicity indicate that F_4 sub fraction of dichloromethane fraction is the most effective (LD_{50} 437.4), having 66.65 % maximum mortality of brine shrimp at 100 ppm,while least effective one is F_1.

Keywords: *Rumex obtusifolius*, Antimicrobial efficacy, Cytotoxic screening, Dichloromethane.

INTRODUCTION

Plants and plant-derived products are getting importance against many diseases due to their non-toxic and no or less side effective nature. Therefore, scientists have been continuously working on the identification, investigation, characterization and evaluation of medicinal plants [1,2]. About 400 million people at present time depend on traditional drugs of plant origin even in developed countries all over the world. About 25 % of standard drugs recommended by a physician originate from folk medicines even in present time [3]. People of developed and developing countries have turned back their interest towards plant based medicines for a substitutive health care purposes as they are an accessible and cheap as compared to synthetic medicines [4].

Many drugs have developed resistance due to the random use of commercial antimicrobial drugs regularly used for the treatment of infectious diseases that compelled scientists to seek for new antimicrobial substances [5] so investigation of traditional medicinal plant in order to identify and develop safe and effective remedies for ailments of both microbial and non-microbial origin is essential.

Plants of *Rumex* (Polygonaceae) are traditionally used as anti-inflammatory [6], bactericidal [7], diuretic, cholagogue, tonic and laxative agents [8] and antitumor, astringent and anti-dermatitis [9]. It is reported that the methanol extract of roots of *Rumexnepa lensis* L. has significant antibacterial potential [10]. *Rumex spp.* was used for the cure of skin diseases and tuberculosis [11].

Rumex obtusifolius L. (family name: Polygonaceae) is one of the most common wayside weeds. It occurs in silage fields, in ditches on river banks and on waste ground. It is inhabitant to Northern Ireland and a number of other countries in Africa, Europe and temperate Asia [12]. This species has long been used in traditional medicine. It is used as an antidote to nettle, astringent, depurative, tonic and laxative. It is also used for treatment of tumors, blisters, sores, burns and cancer [13].

Plants comprise numerous of biologically active compounds. For their analysis, necessary tools and methods are needed. These include suitable chemical screening methods and biological assays. To investigate for novel antimicrobial agents is necessary to see microbial resistance and occurrence of fetal opportunistic infections [14].

One of the important methods used to screen bioactive compounds from plant extracts is brine shrimp lethality test [15]. Anthraquinones and flavonoids *e.g.*, emodin and quercetin are good antibacterial agents against many human pathogenic bacteria (*Pseudomonas aeruginosa, Escherichia coli, Staplylococcus aureus, Streptococcus* sp*),* which cause many dangerous diseases for example, urinary infections, vomiting, gastroenteritis and diarrhea. Ears and eye diseases may also be caused by bacteria. Infections around nose and spreading over the face, piles, carbuncles may be also caused by bacteria; *Streptococci*. Impetigo is mainly caused by *Staphylococcus*, bacteria; *Klebsiella* may also cause urinary tract infections [16-21]. The aim of this work is to seek active sub-fractions of dichloromethane fraction of *Rumex obtusifolius* by performing antibacterial, antifungal and cytotoxic screening in early vegetative stages.

EXPERIMENTAL

The plant *R. obtusifolius* was collected from Bannu region, Khyber Pakhtoon Khawa (KPK) Pakistan during the month of December. It was identified by Prof. Abdur Rehman, Botany Department, GPGC Bannu. The whole plant excluded fruit and seed was washed with distilled water, shade dried and grinded into powders with the help of electrical grinder.

Extraction and fractionation: The powder plant was extracted with ethanol at room temperature for 15 days. Ethanol extract was evaporated under reduced pressure to obtain a thick gummy crude. The gummy crude was successively fractionated with *n*-hexane, dichloromethane, ethyl acetate on the basis of increasing polarity and then the dichloromethane fraction was sub-fractionated into F_1, F_2, F_3, F_4, F_5 and F_6 by column chromatography. All the fractions were evaporated by rotary evaporator. All experimental studies on the plant were carried out in Institute of Chemical Sciences, Gomal University, D.I. Khan and University of Science & Technology Bannu, KPK, Pakistan.

Antibacterial screening

Test bacteria: Antibacterial screening of different fractions was assessed against eight pathogenic bacterial strains, *Bacillus subtilis* (NCTC 10400), ampicillin-resistant *Escherichia coli* (NCTC 10418), *Bacillus cereus* (ATCC 11778), *Escherichia coli* (ATCC 8739), *Staphylococcus aureus* (NCTC 1803), *Pseudomonas aeruginosa* (ATCC 27853), *Streptococcus pneumoniae* (ATCC 49619), *Salmonella typhii* (NCTC 10203), obtained from Biotechnology Department, University of Science and Technology, Bannu, Pakistan.

Procedure: The antibacterial bioassay was carried out by conventional disc diffusion method [22,23]. In brief, the concentration was 500 × 10^{-6} g/disc in case of each extract. These discs and positive controls (cefotaxime, 10 × 10^{-6} g/disc) along with negative control discs were sited on Petri dishes having an appropriate agar medium seeded with the test microorganisms using sterile transfer loop and kept at 4 °C to assist maximum diffusion. The plates were incubated at 37 °C for bacterial growth. The diameter of clear area in the Petri dish which was devoid of bacterial growth was measured which indicated antibacterial activities of the test agents in terms of mm.

Antifungal screening

Test fungi: *Fusarium solani, Aspergillus flavas, Aspergillus niger, Mueor* species, *Alternaria alterata, Aspergillus fumigates, Fusarium moniliformes*.

Procedure: The conventional agar tube dilution method was performed for antifungal screening of the factions was determined according to the protocol reported by Choudhary *et al.* [24].

For the preparation of media for fungi 32.5 g savored dextrose agar was mixed with 500 mL double distilled H_2O. After that it was steamed to be dissolved and 5 mL was dispensed into screw cap tubes. These tubes were marked and autoclaved at 121 °C for 20 min.

For each fungus tubes were prepared in triplicate in order to get more accurate and precise results. Tubes were then allowed to cool. 100 × 10^{-6} L of extract (0.02 g/mL in DMSO) and 83 × 10^{-6} L of terbinafine (0.012 g/mL in DMSO) were added just before solidification, as positive control in tubes to get concentration of 0.4 and 0.2 × 10^{-3} g/mL, respectively. Pure 0.1 mg per tube DMSO was used as negative control. Each tube was inoculated with a four mm diameter piece of inoculum from a culture 7 days. All these tubes were incubated at 27 °C for 7 days. Fungal growth was determined by measuring linear growth in terms of mm. Percentage inhibition of fungal growth was determined by the following formula:

$$Y = \left(100 - \frac{A}{B}\right) \times 100$$

where; A= linear growth in test (mm), B= linear growth in control (mm), Y= Percentage inhibition of fungal growth.

Cytotoxic screening: Cytotoxic brine shrimp assay for various fractions were analyzed by performing brine shrimps hatched in saline method, as recommended by Meyer-Alber *et al.* [25], with slight modification.

In brief 20 mg each sample was dissolved in 2 mL of respective solvent. From this stock solution 5 × 10^{-6} L, 50 × 10^{-6} L and 500 × 10^{-6} L was poured separately into 5 mL; 20 mL 50 mL vials respectively. They were kept open in continuous air flow for evaporation. Artificial sea water (3 mL) was poured in each vial and then ten matured brine shrimp larvae were added. Finally, sea water was added up to the mark and 10, 100, 1000 ppm working solution were made by simple dilution method. They were kept under illumination and after 24 h of incubation survivors were counted with help of 3 × magnifying glass. LD_{50} value was determined by probit analysis in finny computer software.

RESULTS AND DISCUSSION

A common disc diffusion method was applied to evaluate the antibacterial screening of sub-fractions of dichloromethane fraction of *R. obtusifolius*. The F_1 and F_2 do not show any antibacterial activity, the F_6 is active against all bacterial strains, while F_3 is active only against *Escherichia coli* (Table-1). The most remarkable antibacterial activities are observed with the F_6. Highest zone of inhibition (22.5 mm) is noted for F_6 against ampicillin-resistant *Escherichia coli.* The antibacterial activity of DCM fraction of *R. obtusifolius* is largely due to average polarity compounds, *e.g.* (phenolics, flavonoids) present

TABLE-1
ANTIBACTERIAL SCREENING OF DICHLOROMETHANE SUB-FRACTION OF *Rumex obtusifolius* (mm)

Sub-fractions	*Bacillus cereus*	*Bacillus subtilis*	*Escherichia coli*	Ampicillin-resistant *Escherichia coli*	*Staphylococcus aureus*	*Salmonella typhii*	*Pseudomonas aeruginosa*	*Streptococcus pneumonia*
F_1	R	R	R	R	R	R	R	R
F_2	R	R	R	R	R	R	R	R
F_3	R	R	9	R	R	R	R	R
F_4	13	9	14	19	8	12.5	9.5	R
F_5	14	11	17.5	11	9	13	10.5	R
F_6	15	14	16	22.5	9	14.5	11	10
Cefotaxime	32	31	30	30	30	30	30.5	30

*Fractions (F_1, F_2, F_3, F_4, F_5, F_6) obtained from column chromatography of the dichloromethane fraction by a mixture of dichloromethane-*n*-hexane in the specified proportions as eluent; R = Resistant = Show no activity.

TABLE-2
PERCENTAGE INHIBITION OF FUNGI OF SUB-FRACTIONS OF DICHLOROMETHANE FRACTION OF *Rumex obtusifolius*

Fractions	*A. flavas*	*F. solani*	*Mueor* species	*A. niger*	*A. fumigatus*	*A. alterata*	*F. moniliformes*
F_1	9.4 ± 1.39	10.3 ± 2.21	8.2 ± 1.4	9.4 ± 2.85	8.4 ± 2.53	9.8 ± 1.39	7.4 ± 2.51
F_2	30.2 ± 1.23	15.3 ± 1.23	23.6 ± 1.32	14.2 ± 2.21	21.3 ± 1.43	37.4 ± 2.12	23.8 ± 2.13
F_3	25.4 ± 2.53	15.8 ± 1.39	14.1 ± 1.51	31.7 ± 2.49	31.7 ± 2.49	31.7 ± 2.49	34.2 ± 1.25
F_4	69.4 ± 3.18	36.4 ± 1.89	38.0 ± 1.34	36.0 ± 1.68	56.2 ± 2.77	37.6 ± 1.32	30.1 ± 1.31
F_5	66.2 ± 1.41	36.2 ± 2.14	41.3 ± 1.34	49.1 ± 1.23	50.2 ± 2.41	42.4 ± 2.51	35.1 ± 2.21
F_6	22.6 ± 2.32	27.0 ± 2.56	43.2 ± 1.80	51.3 ± 1.67	15.5 ± 2.34	40.5 ± 1.07	30.4 ± 1.34
Terbinafin	87.6 ± 2.70	84.5 ± 2.13	81.78 ± 2.12	88.4 ± 3.16	87.4 ± 3.17	84.4 ± 3.13	89.2 ± 1.23
DMSO	Nil	Nil	Nil	Nil	Nil	Nil	Nil

Mean ± SE (n = 3)

TABLE-3
LD_{50} VALUES FOR DIFFERENT CONCENTRATIONS OF SUB-FRACTIONS

Concentration (ppm)	Percentage					
	F_1	F_2	F_3	F_4	F_5	F_6
10	13.3	23.3	13.3	13.3	20.0	16.6
100	23.3	30.0	16.6	20.0	26.6	20.0
1000	30.0	43.3	56.6	66.6	50.0	50.0
LD_{50} values	53970.9	4753.8	867.8	437.4	1371.8	1552.1

in medium polar fractions F_4, F_5 and F_6. It is also indicated that the active agent accountable for antibacterial activity is more soluble in polar organic solvent.

The results of Table-2 indicate that F_3, F_4 and F_5 inhibited fungal growth more competently as compared to F_1, F_2 and F_6. Maximum inhibition was recorded by F_4 (69.4 %) against *Aspergillus flavas*. F_4 and F_5 show prominent inhibition ranging from (35.1 ± 2.21 to 69.4 ± 3.18) except F_4 against *Fusarium moniliformes*. F_1 does not show any prominent inhibition (7.4 ± 2.51 to10.3 ± 2.21) against all fungal strains, b/c the probability of bioactive compounds in F_1 is less, as ethanolic crude was already extracted with *n*-hexane in solvent extraction process. In short F_4 is the most active and F_1 least active fraction among the six fractions. Our results confirmed previous study in which fungi-toxic surface flavonoids (isoflavonoids) have been characterized on are reported to be extracted with non-polar and low polar solvents [26] like *n*-hexane and dichloromethane mixture.

Brine shrimp assay is recommended to be a suitable probe for the pharmacological activities in plant samples [27]. LD_{50} values (Table-3) indicated that F_4 sub-fraction of dichloromethane fraction is the most effective (LD_{50} 437.4), having 66.65 % maximum mortality of brine shrimp at 100 ppm, while least effective one is F_1. Our results revealed that none of the fractions are found highly effective ($P > 0.05$) however, at higher concentration, fractions were effective at probability level 0.05. Other fractions do not show any prominent results as higher value of LD_{50} lower will be % mortality of brine shrimp. It can be said that presence of a wide range of bioactive compounds with different structures and their synergistic effect may put into the overall activity of F_4.

Conclusion

This study showed that F_4, F_5 and F_6 sub-fractions of dichloromehtane fraction of *R. obtusifolius* appeared as an important source for the discovery of new antibacterial, cytotoxic and antifungal agents.

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