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ANTIMICROBIAL AND SYNERGISTIC STUDIES OF *RANUNCULUS MURICATUS* L. AGAINST SOME INDIGENOUS BACTERIA

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Abstract

In the present study, antibacterial activity of the whole plant methanolic extract of *Ranunculus muricatus* L., was analyzed against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Bacillus pumilus, Salmonella typhimurium* and *Pseudomonas aeroginosa*. Methanol was regarded as an excellent solvent for antimicrobial activity. It was observed as best bactericidal at a minimal inhibitory concentration (MIC) of 1-10 µg/ml against all the bacterial cultures viz. *B. pumilus, B. subtilis, S. aureus, E. coli, P. aeroginosa* and *S. typhimurium*. Synergistic antibacterial activity of methanolic extracts was tested with respect to solvent extract of leaves of *Ricinus communis, Nerium oleander, Withania somnifera,* whole plant of *Heliotropiaum curassavicum* and fruits of *Citrullus colocynthis*. Synergistical study revealed the best antibacterial activity against *B. subtilis* and *B. pumilus* at a level of 1 µg/ml except *E. coli* and *S. aureus*.

Introduction

It has been recorded in the history that plants have been utilized as medicines since long time. These medicines are used in terms of crude drugs such as tincture, teas, poultices, powders and other herbal formulations (Balunus & Kinghom, 2005). The plants used to cure the specific diseases and the procedure of their application is passed from generation to generation. Herbal pharmacopoeia is the main source of this type of information related to medicinal plants (Hiremath *et al*., 1997; Tipu *et al*., 2006). As the climate of Pakistan is diverse, so the medicinal herbs are found over a wide area. Uptill now 600 herbs are found to exhibit medicinal properties (Shah *et al*., 2013). The curative properties of medicinal plants along with vast herbal treatment have motivated the pharmaceutical industries to commercially exploit the extraction of various ingredients to prepare appropriate drugs. For instance a traditional medicine made from *Tamarindus indica* proved to be very effective against number of diseases such as gastrointestinal disorders, fever, jaundice and dysentery (Munimbazi & Bullerman, 1998). The antimicrobial and antitoxin properties of a number of medicinal plants have also been documented (Aziz, 2008; Bakht *et al*., 2013). About 84% people of the country rely on traditional medicines whenever they are required to take medicines for any kind of ailment (Khan *et al*., 2010). The reality is that both traditional and eastern medicinal system is mainly based on the properties of the active ingredient present in medicinal plants of the region.

Bacteria have an established antimicrobial resistance to the exposed ecosystem. The logic behind the availability of bacteria in diverse habitat is either the existing genes are modified by point mutation or horizontal gene transfer becomes involved in acquisition of resistant genes (Gayathri & Kannabiran, 2009). Synergism in toxicology is usually refers to the comparison between the health effects caused by chemicals in a combination or in individual application. It is found that combined chemicals have greater effects on health (Ali *et al.*, 2007; Khan *et al*., 2013) rather than their individual effects. Synergism between antimicrobial agents and bioactive plant is a new and recently reported concept. *In vitro* antimicrobial studies of *Rhus coriaria*, *Psidum guajava* and *Lawsonia inermis* were carried out against different bacterial species (Nakano & Zuber, 1998; Pankey & Ashcraft, 2010; Gunes *et al.*, 2013). The main objectives of the present study were to find out some new herbal sources against the locally isolated bacteria and to search new bioactive molecules as sources of bactericide. In addition, exploration of folk medicinal plants of the country was also aimed.

Materials and Methods

Materials: All the chemicals were of analytical grade and purchased from BDH/Merck. The experiments were performed in a laminar air flow cabinet (Stream Line Lab Products) and glassware was sterilized in a hot air oven (Memmert, Germany) at 105°C for 24 h. Nutrient (NB) agar medium (nutrient broth, 8 g/l; agar, 20 g/l; pH 7.2) was used for plating while for the microbiological experiments, sterilization was carried out at 121°C, 15 lbs/in^2 pressure, for 15 min.

Bacterial strains: Six bacterial strains were selected for the study which included *Staphylococcus aureus, Bacillus subtillus, Pseudomonas aeruginosa, B. pumillus, Salmonella typhimurium* and *Escherichia coli*. The indigenous microbial strains were obtained from Drug Testing Lab (DTL), Birdwood Road, Lahore.

Selection of plants: A range of different plants were collected from various localities and habitats of Pakistan on the basis of their medicinal properties (Table 1). Fig. 1 shows the original plant specimens used for the determination of antimicrobial and synergistic activity against indigenous bacteria. These plants were identified in our Lab at *LCWU, Lahore* and their voucher specimens were deposited in Prem Madan Herbarium, Lahore (Mughal *et al.*, 2009). The whole plant of *Ranunculus muricatus* (butter cup) was collected from the adjacent *Wapda Town, Lahore* (Voucher specimen # 0155). The leaves of *Ricinus communis* (castor oil) were collected from *LCWU, Lahore* (Voucher specimen # 0168). The

whole plant of *Heliotropium curassavicum* (podina booti) was collected from *Wapda Town, Lahore* (Voucher specimen # 0172). The leaves of *Nerium oleander* (kaner) were collected from *Allama Iqbal Campus, University of the Punjab, Lahore* (Voucher specimen # 0176). The fruits of *Citrullus colocynthis* (tumma) were purchased from a market of *Mughal Pura, Lahore* (Voucher Specimen # 0204). The leaves of *Withania somnifera* (ashwaganda) were collected from *LCWU, Lahore* (Voucher specimen # 0439).

Drying and grinding: The parts of different plants were kept on a newspaper under shade for drying at room temperature for one week or more as appropriate. Then these plant parts were disrupted in a grinder and converted into fine powder. Later, these were kept in separate bottles at 4ºC in a cold-cabinet and labeled accordingly.

Preparation of plant extracts: Methanolic extractions as reported by Qureshi *et al.,* (2007) were carried out in the present study. Methanolic pet ether and dichloromethane extracts of all plants were also tested. In Table 2 is depicted a comparative analysis of composition of all the selected plants. For this, soxhelet apparatus was used for extraction using methanol as a solvent. Plant materials were filled in thimble and placed in the apparatus. The temperature was kept at 60ºC for all the plant materials. The solvent was evaporated by using rotary evaporator. Plant extracts were transferred in 250 ml capacity Erlenmeyer flasks and fixed in the evaporator. The extracts were then made free of methanol and the crude extracts were stored at 4^{o}C in different reagent bottles.

Ranunculus muricatus ***Ricinus communis*** ***Nerium oleander***

Withania somnifera ***Heliotropium curassavicum*** ***Citrullus colocynthis***

Fig. 1. Original snapshots of plants used for the determination of antimicrobial and synergistic activity against indigenous bacteria.

Table 1. Taxonomic information and medicinal importance of the selected plants.

Plant specimens (family)	Vernacular name	Common name	Habit	Medicinal properties	Bibliography
Ranunculus muricatus (Ranunculaceae)	Chambul	Butter cup	Herb	Decoction of the plant is useful in various diseases of cattle's and also effective in cough and asthma	Tippu *et al*., (2006); Khan *et al*., (2013)
Ricinus communis (Euphorbiaceae)	Harnaoli, Arund	Castor oil	Shrub	Seed is used as contraceptive; seed is purgative; seeds are taken as contraceptive; oil extracted from seeds is used to easy delivery and to lessen labour pain	Ushimaru *et al*., (2007)
Nerium oleander (Apocynaceae)	dogbane	Oleander	Shrub	The roots have been used externally in traditional medicine for treating cancer, ulcers and leprosy	Balunus & Kinghom (2005)
Withania somnifera (Solanaceae)	Asgandh	Winter cherry	Herb	Plant is diuretic, astringent, diaphoretic, aphrodisiac and tonic in nature; extract of the plant is used to treat rheumatism; poultice of leaves on burns has soothing effect; root powder has infertility effect; crushed leaves along with berries in water, given at the time of labour make the birth easy	Nascimento *et al*., (2000); Rasool & Varalakshmi (2006); Shah *et al*., (2013)
Heliotropium curassavicum (Boraginaceae)	Akrri	Salt heliotrope	Herb	Plant is used as anti-septic to kin ailments	Qureshi *et al*., (2007); Mughal (2008)
Citrullus colocynthis (Cucurbitaceae)	Gharroonmba	Colocynth	Herb	Powdered leaves mixed with water are laxative while root and seed have purgative nature; one seed taken early in the morning for three weeks is good to cure diabetes	Shah *et al*., (2013)

Table 2. Comparative analysis of crude methanolic extracts of selected plants*.

Plant specimens	Weight of plant material (g)	Weight of crude extract (g)	Crude extract (%)
R. muricatus	17.69	136.1	769.36
R. communis	16.89	141.5	837.77
C. colocynthis	28.99	90.1	310.79
W. somnifera	22.7	161.7	712.33
H. curassavicum	33.95	125.4	369.366
N. oleander	26.9	131.2	487.73

*Extracts were prepared using methanol as a solvent at 60°C.

Antibacterial activity: Agar well diffusion method (AWDM) was used to determine the antibacterial activity of plants extracts after *Ushimaru et al.,* (2007) using all the identified microbial strains viz., *E. coli*, *P. aeruginosa*, *B. subtilis*, *B. pumillus, S. typhimurium* and *S. aureus*.

Bioassay screening: In many drug discovery programs, plant extracts have served as an important source for molecular diversity (Nascimento *et al.,* 2000). Ten ml of fresh culture of individual organisms in nutrient broth containing approx. 10^6 colony forming units (CFU) was prepared. Nutrient agar (500 ml) was prepared, sterilized and cooled to about 45-48°C. Bacterial culture (0.1 ml) was inoculated and mixed well in the medium. Nutrient agar (10-15 ml) containing bacterial culture was poured in sterile Petri dishes and allowed to solidify at an ambient temperature of 25ºC. Four wells were made in each plate with steel borer of 7 mm. Different concentrations of the plant extracts were added in the wells and incubated at 37°C for 20-24 h. Methanol (100%) was used as a negative control. Zones of inhibition for *P. aeruginosa, S. aureus, E. coli, B. pumilus, B. subtilis* and *S. typhimurium* growths were observed to be 16, 10, 30, 17, 22 and 15 mm, respectively. Reference antibiotics i.e., Amoxicillin, Levofloxcin, Ampicillin, Venomycin, Tetracycline, Ciprofloxcin and Penicillin were supplemented in other wells as positive control. The mean diameter of the resulting inhibition zones was measured and recorded as antimicrobial activity. Minimal inhibitory concentration (MIC) value of each plant extract was calculated.

Minimum inhibitory concentration (MIC): Plant extracts was used to evaluate the MIC which showed antimicrobial activity following the method described by Mughal *et al.,* (2010). Shortly, various concentrations ranging from 0.1-500 mg/l were prepared by using sterile dH_2O as a diluent and AWDM was used to find out MIC. The entire experiment was run in triplicate and the means were measured.

Synergistic bacterial activity: Methanolic extracts from experimental medicinal plants showed better antimicrobial activity, so these plants were included in the study of synergistic effects. The purpose was to use the crude plant extracts as drugs and increase their inhibitory effects against different microbes, some of which have acquired resistance many of the available antibiotics as reported by Kathleen *et al*., (2005). The plant's selection was done on the basis of ethno medicinal importance and their availability for synergistic activity (Levine & Anderson, 1932). In short, synergistic antimicrobial activity of different plant extracts was determined by taking 25 µg ml^{-1} of each extract following AWDM. The mean diameter of the resulting inhibition zones was measured in millimeters and recorded as antimicrobial activity. The MIC was also evaluated. All the experiment was run in triplicate and the means were recorded using one-way analysis of variance (one way ANOVA) after Kathleen *et al.,* (2005).

Results and Discussion

The antibacterial activity exhibited by extracts of different ethno medicinal plants using AWDM under the influence of six varied species of bacteria such as, *S. aureus*, *E. coli*, *B. subtilis*, *B. pumillus, S. typhimurium and P. aeroginosa.* The inhibition zones were estimated and the MIC was evaluated (Table 3). Methanolic pet ether and dichloromethane extracts of all plants (*Ranunculus muricatus, Ricinus communis, Heliotropium curassavicum, Nerium oleander, Citrulus colocynthis* and *Withania somnifera*) with concentration (250, 100, 50, 10, 5.0-, and 1.0 µg/ml) were used against each of the six bacterial strains. For the extraction of antimicrobial substances methanol was found to be best solvent on the basis of organism inhibition frequency and obtainable diameter of inhibition zones as reported by Mahesh & Satish (2008). The definite antibacterial activity and antifungal activity was showed by methanolic extracts of leaves of *A. nilotica, S. cordifolia, T. cordifolia, W. somnifera and Z. mauritiana* when compared to extracts of roots and bark. The effectiveness of an extract depends on the solvent used as reported by Rasool & Varalakshmi (2006). The maximum activity was shown by extract of *R. muricatus* in methanol against *S. typhimurium* and *P. aeroginosa* except *S. aureus, B. subtilis, B. pumillus* and *E. coli*. While the extracts of *R. communis* showed very poor activity against *B. pumillus* except *S. typhimurium P. aerogenosa, B. subtilis, E. coli* and *S. aureus* (Table 4). However, Ali *et al.,* (2007) studied that Yemeni traditional healers used 20 different plants for the treatment of infectious diseases after screening the antibacterial activity of their ethanolic extract for Gram positive or Gram negative bacteria. Antibacterial activity varied greatly among 14 ethanolic extracts. In first separation for the partioning of extracts in ethanol ethyl acetate and water was used. The test system proved that *Lawsonia inermis* ethyl acetate extract is the best one against all types of bacterial strains as reported

earlier by Aburjai *et al*., (2001) and Ferrara (2005). The comparison of synergistic screening of methanolic extracts of plants among themselves and with six other plants collected from Northern Punjab including *R. muricatus* (Ranunculaceae), *R. communis* (Euphorbiaceae), *H. curassavicum* (Boraginaceae), *N. oleander* (Apocynaceae), *C. colocynthis* (Cucurbitaceae) and *W. somnifera* (Solanaceae)- were done against six bacterial strains namely *S. aureus, E. coli, P. aeroginosa*, *B. subtilis, B. pumillus* and *S. typhimurium*. *R. muricatus* with *R. communis* extracts showed the highest antimicrobial activity against *B. pumilus* and *B. subtilis* (MIC 1µg/ml), however these plants showed poor activity against *S. aureus* and *E. coli* (Table 5). On the other hand, *R. muricatus* with *H. curassavicum* gave moderate activity against *B. pumilus* and *B. subtilis* (MIC 1 µg/ml) but no activity against *E. coli* and *S. aureus.* The results showed that *R. muricatus* with *N. oleander* exhibited good activity against *B. pumilus* and *B. subtilis* (MIC 1 µg/ml) but it showed poor activity against *S. aureus* and *E. coli.* Similarly, *R. muricatus* with *C. colocynthis* revealed the best activity against *B. pumilus* and *B. subtilis* (MIC 1 µg/ml) but it showed poor activity against *S. aureus* and *E. coli.* While extracts of *R. muricatus* with *W. somnifera* extracts gave the highest activity against *B. pumilus* and *B. subtilis* (5 and 10 µg/ml MIC, respectively) but it did not show any activity against *S. aureus* and *E. coli* (Table 6). The statistical analysis of zones of inhibition of crude methanolic extracts of medicinal plants (1 µg/ml) revealed the best value with *N. oleander* (Table 7). Previously, Ali *et al.,* (2007) and Mughal *et al*., (2009) also reported similar findings with the utilization of various other microbial cultures and plant extracts. In addition, Mughal *et al*., (2010) also investigated crude ethanolic extracts of ten medicinal plants including *Melilotus alba, Ranunnculus sceleratus, Senecio chrysanthemoides, Euphorbia splendens, Foeniculum vulgare, Brassica compesteris, Chenopodium album, Eichhornia crassipes, Capparis spinosa* and *Hibiscus rosa-sinesis* (widely used in Chinese system of medicine) which were tested to determine antimicrobial activity against 5 species of microorganism including *B. cereus*, *E. coli*, *S. aureus*, *C. albicans* and *P. aeruginosa*. Few plants (5/10) were found to exhibit antibacterial activity for more than one microorganism species and *Chelidonium majus*, *Sanguisorba officinalis*, and *Tussilago farfara* were found best to exhibit antimicrobial activity. The antimicrobial activity of plant extracts was synergistic with antibiotics tested as reported by Pankey & Ashcraft (2010) and Munazir *et al.,* (2012). It was further suggested that the antibacterial activity of *H. indicus*, *F. bengalensis* and *P. marspium* is greatly significant against pathogenic bacteria in aqueous extracts.

Table 4. MIC value of crude methanolic extracts of medicinal plants.

Bacterial strains	MIC (µg/ml)					
	H. curassavicum	***N. oleander***	***R. muricatus***	***R. communis***	***W. somnifera***	***C. colosythis***
B. pumilus	1	1	5	5	1	1
B. subtilis	1	1	5	1	1	1
S. aureus	1	1	1	1	1	1
E. coli	5	1	1	1	*nzi*	*nzi*
P. aeruginosa	1	5	5	5	*nzi*	*nzi*
S. typhimurium	1	1	5	1	*nzi*	*nzi*

'*nzi*' means no zones of inhibitions observed

Table 5. Zones of inhibition of crude methanolic extracts of *Ranunculus muricatus* with other selected plants.

Plant specimens	Bacterial strains	Zones of inhibition (mm)					
		1 µg/ml	**5 µg/ml**	**10 µg/ml**	**50 µg/ml**	**100 µg/ml**	**250 µg/ml**
R. communis							
	B. pumilus	4	6	11	18	*nzi*	*nzi*
	B. subtilis	4	8	16	16	10	*nzi*
H. curassavicum							
	B. pumilus	10	15	20	14	*nzi*	*nzi*
	B. subtilis	10	24	24	28	25	21
N. oleander							
	B. pumilus	8	10	19	20	25	20
	B. subtilis	3	11	17	16	*nzi*	*nzi*
C. colocynthis							
	B. pumilus	10	10	18	*nzi*	*nzi*	*nzi*
	B. subtilis	4	10	16	12	10	*nzi*
W. somnifera							
	B. pumilus	*nzi*	6	21	25	*nzi*	*nzi*
	B. subtilis	*nzi*	*nzi*	14	10	*nzi*	*nzi*

S. aureus and *E. coli* produced no zones of inhibition (*nzi)* with the plant combinations of *R. muricantus*

Table 3. Zones of inhibition of crude methanolic extracts of selected plants.

Plant specimens	Bacterial strains	Zones of inhibition (mm)					
		1 µg/ml	5 µg/ml	10 µg/ml	50 µg/ml	100 µg/ml	250 µg/ml
R. muricatus	*B. pumilus*	8	11	15	20	13	17
	B. subtilis	6	9	11	15	*nzi*	*nzi*
	S. aureus	*nzi*	2	8	17	*nzi*	*nzi*
	E. coli	*nzi*	11	17	25	13	18
	P. aeruginosa	11	16	18	21	*nzi*	*nzi*
	S. typhimurium	10	15	20	25	*nzi*	*nzi*
R. communis	*B. pumilus*	*nzi*	6	11	18	31	38
	B. subtilis	9	15	20	25	18	23
	S. aureus	3	7	13	18	20	26
	E. coli	2	8	16	24	19	27
	P. aeruginosa	*nzi*	14	17	20	29	36
	S. typhimurium	9	16	20	24	27	30
H. curassavicum	*B. pumilus*	10	15	20	25	16	21
	B. subtilis	8	15	20	25	24	28
	S. aureus	*nzi*	*nzi*	3	15	15	17
	E. coli	*nzi*	4	13	20	17	26
	P. aeruginosa	6	9	11	16	27	32
	S. typhimurium	7	9	11	15	18	25
N. oleander	*B. pumilus*	11	15	19	24	23	28
	B. subtilis	20	25	31	35	23	38
	S. aureus	2	9	16	25	45	52
	E. coli	13	18	21	29	18	25
	P. aeruginosa	*nzi*	9	14	18	22	28
	S. typhimurium	6	9	15	20	30	34
W. somnifera	*B. pumilus*	9	16	21	30	25	30
	B. subtilis	13	18	23	30	45	50
	S. aureus	11	16	20	25	25	29
C. colocynthis	*B. pumilus*	10	19	26	30	15	22
	B. subtilis	8	11	17	20	15	22
	S. aureus	4	9	11	15	15	20

E. coli, P. aeruginosa and S. typhimurium produced no zones of inhibition (*nzi*) with the crude methanolic extracts of *W. somnifera* and *C. colocynthis*

Table 6. MIC value of crude methanolic extracts of *Ranunculus muricatus* with other medicinal plants.

Bacterial strains	MIC (µg/ml)				
	R. muricatus* + *R. communis	***R. muricatus* + *H. curassavicum***	***R. muricatus* + *N. oleander***	***R. muricatus* + *C. colosythis***	***R. muricatus* + *W. somnifera***
B. pumilus	1	1	1	1	5
B. subtilis	1	1	1	4	10

S. aureus and *E. coli* did not exhibited MIC value of crude methanolic extracts of *R. muricatus* with other medicinal plants

Table 7. Statistical analysis of zones of inhibition of crude methanolic extracts of medicinal plants (1 µg/ml).

Bacterial strains	Zones of inhibition (mm)					
	R. muricatus	***R. communis***	***H. curassavicum***	***N. oleander***	***C. colosythis***	***W. somnifera***
B. pumilus	8 ± 0.5	*nzi*	10 ± 0.5	11 ± 0.5	10 ± 0.5	9 ± 0.5
B. subtilis	6 ± 0.5	9 ± 0.5	8 ± 0.5	20 ± 0.5	8 ± 0.5	13 ± 0.5
S. aureus	*nzi*	3 ± 0.5	*nzi*	2 ± 0.5	4 ± 0.5	11 ± 0.5
E. coli	*nzi*	2 ± 0.5	*nzi*	13 ± 0.5	*nzi*	*nzi*
P. aeruginosa	11 ± 0.5	*nzi*	6 ± 0.5	*nzi*	*nzi*	*nzi*
S. typhimurium	10 ± 0.5	9 ± 0.5	7 ± 0.5	6 ± 0.5	*nzi*	*nzi*

Good activity >15, Moderate activity >12, Poor activity < 12
± shows the values of standard deviation (sd) among the three parallel replicates
'*nzi*' means no zones of inhibitions observed

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