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RESEARCH ARTICLE

ASSESSMENT OF ANTIBACTERIAL ACTIVITIES OF COTONEASTER FRUITS EXTRACT

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ABSTRACT

Methanol extract from cotoneaster fruit by using soxhlet apparatus was obtained. While aqueous extract was prepared by using magnetic heater. 1 g of solvent residue was dissolved in 10 ml of dimethyl sulphoxide, in same time, 1g of aqueous extract was dissolved in 10 ml of dimethyl sulphoxide, to obtain concentration of 100mg / ml, this depend for preparation of the 100, 50, 25 mg /ml for antimicrobial activity assay for decoctions, for aqueous extract 1 g of air-dried plant material was added to 10 ml. of distilled water, left for 24 hours, mean while for alcoholic extract 1 g of powdered fruit added to 10 ml methanol (70 %) left for 24 hours. This 100 mg /ml depend for preparation of the 100, 50, 25 mg /ml for antibacterial activity assay. Isolated pure bacteria used in this study were: *E. coli*, *Staphylococcus aureus*, *Bacillus subtlis*, *Pseudomonasspp* and *Proteus spp.* The results showed that agar well diffusion method was more valuable to determine the antibacterial effects of both aqueous and alcoholic extracts of cotoneaster sp. In comparison with agar disc method, the alcoholic extract was more effective and showed higher antibacterial effect against all bacteria sp. In comparison with aqueous extract also both aqueous and alcoholic extract were concentration dependent as they were more effective at concentration 50 mg /ml in comparison with 25 mg /ml . at 100 mg /ml were highly effective in comparison with 25 and 50 mg ml the plants used in this study could be potential source of new antimicrobial agents.

INTRODUCTION

The emergence of organisms resistant to nearly all classes of antimicrobial agents has become a serious public health concern in the past several years (Didem Dellorman Orhan 2012).The plants that exhibit great activity could be considered as a source of potential antimicrobial compounds. Many screening studies have been conducted to find new antimicrobial agents from natural or synthetic compounds for a variety of novel active compounds with different molecular targets that controls infections caused by microorganisms. Crude plant extracts that were used in traditional folk medicine for their antimicrobial properties are still widely used to treat infections. Therefore, it is worthwhile to study plants and plant products for activity against microorganisms (Kan *et al.*,2009). The discovery of antimicrobial agents from plants based on the evaluation of traditional plant extracts is very important research topic (Didem Dellorman Orhan2012). Burden *et al.* (Burden *et al.*, 1984) found that chemical structure Cotoneaster lacteal contain the material Cotonefuran (Phytoalexins), which is of bactericidal activity, (Kokubun *et al.*,1995) referred to isolation of phytoalexins as dibenzoefuran from C. acutifolius and classified them into alpha, beta, gamma, delta and epsilon, which has an inhibitory effects against plant fungi growth on plants, and effects against microorganism , which is the same material isolated by (Burden *et al.*,1984) from C. lacteal.

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MATERIALS AND METHODS

Specimen's collection and cultures preparation

The aim of this study was to evaluate the in vitro antibacterial activities of aqueous and alcoholic extracts of cotoneaster fruit collected from different sections of Baqubah, Diyala, Iraq.

Plant material

Preparation of extracts

Cotoneaster fruits were placed in shadeatlaboratory temperature till complete dryness, then grinded by electrical mixertill obtain a powder. Methanol extract from cotoneaster fruit by using soxhlet apparatus was used by filling 25 g of shade dried, powder of cotoneaster fruit in the thimble and extracted with 500 ml of methanol (70%) for 72 hours at 80 ^{o}C., then by using rotary evaporator to separate the dissolvent from extract. After complete solvent evaporation, the solvent extract was weighed and preserved at -20 ^{o}C. in airtight bottles until use (Hareborne1984; Donald *et al.*,1982).While aqueous extract was prepared by filling a 25 g of shade dried, powder of Cotoneaster fruit in a beaker contain 300ml distill water, using magnetic stirrer mixer at 6o^{o}c, and 800 speed for 72 hours.Then the extract was concentrated using rotary evaporator. After complete water evaporation, the extract weight and preserved at -20 ^{o}C.in airtight bottles until use (Hareborne1984; Donald *et al.*, 1982). Dilution 1 g of solvent resi-

due was dissolved in 10 ml of dimethyl sulphoxide, in same time,1g of aqueous extract was dissolved in 10 ml of dimethyl sulphoxide, to obtain concentration of 100mg / ml, this depend for preparation of the 100, 50, 25 mg /ml for antimicrobial activity assay (Karaman *et al.*, 2003; Okeke *et al.*, 2001; Srinivasa *et al.*,2001).For decoctions, for aqueous extract 1 g of air-dried plant material was added to 10 ml. of distilled water , left for 24 hours , mean while for alcoholic extract 1 g of powdered fruit added to 10ml methanol (70 %) left for 24 hours. This100mg /ml depend for preparation of the 100,50, 25 mg /ml for antibacterial activity assay.Isolated pure bacteria used in this study were: *E.coli, Staphylococcus aureus, Bacillus subtlis, and Pseudomonas and Proteus sp*. Obtained from Department of microbiology, College of Veterinary medicine, University of Baghdad, Baghdad, Iraq.

Antibacterial activities assay

Agar well diffusion method

The extract activities were carried by spreading o.1 ml of bacterial suspension prepared according (Bauer *et al.*, 1966) which contain 1x 10^8cell/ ml over the surface of Muller – Hinton agar plate, to obtain uniform growth, left the plate to dry for 5 minutes. Then well were prepared by usingPasteur pipette 5 mm diameter. These well filled by 50 microliter concentrated extract of either aqueous or alcoholic extract according to dilution used, leave the medium to settle for 1 hour in laboratory condition, then incubate for 24 h at 37 ^{o}C.and zone of inhibition if any around the well were measured in mm (millimeter). Each treatment consists of four repeat (Karaman*et al.*,2003; Srinivasa*et al.*,2001; Masika and Afolayan2002).

Disc diffusion method

Antibacterial activity of aqueous and methanol extracts were determined by disc diffusion method on Muller –Hinton agar (Steeland Torrie 1985). Sterile Whatman filter disc (5 mm diameter) were made using sterile cork borer (5 mm), these disc impregnated in the 50 microliter of aqueous or alcoholic extract. placed in Petri dishes according to concentration for 24 hours.Inoculums containing 10^8CFU / ml of bacteria were spread, with sterile swab moistened with the bacterial suspension.The disc also impregnated in 50 µL of solvent either distilled water or alcohol,served as a standard control.

The plates were incubated for 24 h at 37 ^{o}c and zone of inhibition if any around the disc were measured in mm (millimeter). Each treatment consists of four repeat (Karaman*et al.*,2003; Srinivasa *et al.*, 2001; Masika and Afolayan2002). Standard antibiotic disc; Rifampin 5, Doxycycline 30, Amoxicillin 25, Kanamycin 30 and Ampicillin – cloxacillin 30, forantibacterial activity tests were carried out against bacterial strains in used.

Statistical analysis

All values are expressed as the mean ± the standard error of the mean (SEM). The data were analyzed by using one way analysis of variance ANOVA, and then the test of the least significant difference LSD applied, in addition to Duncan test to find the significant differences between the means of inhibitory zones (Al-Badrani 2002).The significant level of test was P< 0.05.

RESULTS AND DISCUSSION

Comparison between susceptibility of bacteria sp. To different concentration of cotoneaster sp. extracts.Agar well diffusion method both aqueous and alcoholic Cotoneaster (orange fruits) extracts exhibited inhibitory activities against bacteria sp. with25; 50 and 100 mg / ml concentrations.In all bacteria sp., the inhibitory effects wereconcentration dependent in both aqueous and alcoholic extract. As the inhibitory zones were more at 50 mg /ml in comparison with 25 mg /ml, and the widest were at 100 mg /ml against all bacteria spp. Table -1- showed that aqueous extract in agar well diffusion method exhibit a significantly higher zones of inhibitions against all bacteria sp. in used at 50 mg /ml in comparison with 25 mg / mlexcept against Proteus sp. as there is no significant difference in zone of inhibitions. While at 100 mg /ml the aqueous extract of cotoneaster a significantly differences zones of inhibition against all bacteria sp. in used in comparison with concentration at 25 mg /ml, but showed a significantly different zones of inhibition against only Pseud., Bacillus and Staph. in comparison with 50 mg / ml.

Table -1- showed that the alcoholic extract of Cotoneaster sp. Exhibit an inhibitory effect against all bacteria sp. in used and was concentration dependent as it was significantly difference in comparison with inhibitory zones at 50 mg /ml and 25 mg /ml, and between the inhibitory effects at 100 mg /ml in comparison with 25 and 50 mg/ml.In agar well diffusion

Table 1. Agar well diffusion methods: Comparison between susceptibility of bacteria sp.to different concentration of cotoneaster sp. Extracts

Extracts	Conc.	Bacteria sp.				
		Pseudomonasspp	*Bacillus subtlis*	*E. coli*	*Proteus*	*Staphylococcus aureus*
Aqueous	25 mg/ml	9.00±	8.75±	9.25±	9.25 ±	10.0±
		0.58 –a-	0.48 –a-	1.03 -a-	0.85-a-	0.00-a-
	50mg/ml	11.00±	12.00±	12.00±	10.25 ±	12.75±
		0.41 -b-	0.82 -b-	0.71 -a-	0.85-a-	0.48-b-
	100mg/ml	13.25±	14.25±	14.25±	12.5±	16.75±
		0.48 -bc-	0.85–b*-	0.85–b*-	0.65-b*-	0.75-bc-
Alcoholic	25mg/ml	10.25±	10.25±	10.75±	09.25±	12.00±
		0.25 -a-	0.25 -a-	0.75–a	0.75 -a-	0.41 -a-
	50mg/ml	12.50±	13.75±	13.50±	11.25±	15.00±
		0.25-b-	0.63-b-	0.65-b-	0.75-a-	0.41-b-
	100mg/ml	14.75±	17.25±	15.75±	14.50±	21.25±
		0.25-bc-	1.11-bc-	0.75-b-	1.19-b-	0.48-bc-

Values : M± S.E.M. a, b,bc ;significantly differ at level of P <0.05

-b*- mean the significance was between concentration 100 and 25 mg /ml only.

method aqueous extract at 25 mg /ml the highest inhibition was against Staph. sp. and lowest inhibitory effect was against method aqueous extract at 25 mg /ml the highest inhibition as against Staph. sp. and lowest inhibitory effect was against Bacillus sp. While at 50 and 100 mg /ml the lowest inhibition was against Proteus, and highest was against *Staphylococcusaureus*. The alcoholic extract at 25, 50,and 100mg/ml exhibit the lowest inhibition against Proteus and highest against *Staphylococcus aureus*.

Agar disc method

In agar disc methodthe aqueous extract at 25 mg/ml did not exhibit any inhibitory zone except against Bacillus sp. While at 50 mg /ml the narrowest inhibitory zones were against Proteus, and widest were against*Pseudomonas spp*, meanwhile at 100 mg /ml the widest inhibitory zones were against Staph. Sp, and narrowest against Bacillus and E.coli. in alcoholic extract at 25, 50, and 100 mg/ml the Proteus showed the widest inhibitory zone and Staph. Sp.The narrowest inhibitory zone (Table -2).

Table -3- showed that alcoholic extract in agar well method showed a better effect against all bacteria in used in comparison with agar disc method at 25 mg /ml, while at 50 mg /ml there were a significantly higher inhibition against all bacteria sp. as acomparison with disc method except against Proteus as there was non-significant differences in inhibition, meanwhile at 100 mg /ml there were non-significant differences against Proteus, *Pseudomonas* and E. coli between agar well and agar disc method. Table -3- showed that aqueous extract in agar well method showed inhibitory zones of significance at 50 mg / ml in comparison with 25 mg / ml, and 100 mg / ml in comparison with both 25 and 50 mg / ml against all bacteria in used in comparison with agar disc method. In comparison between the disc method and agar well method, aqueous extract of the plant showed less inhibitory effects when compared with the same concentration of alcoholic extract against all bacterial spp. Beside of it failing of exhibiting any effect at 25 mg //ml concentration of plant except in Bacillus sp. (Table -3)

Table 2. Agar disc diffusion methods :Comparison between susceptibility of bacteria sp. to different concentration of cotoneaster sp. extracts

Extracts	Conc.	Bacteria sp.				
		Pseudomonasspp	*Bacillus subtlis*	*E. coli*	Proteus	*Staphylococcus aureus*
Aqueous	25mg/ml	0.0± 0.0 -a-	1.5± 0.87-a-	0.0± 0.0 -a-	0.0± 0.0-a-	0.0± 0.0-a-
	50mg/ml	5.5± 0.29 –b	5.0± 0.41-b-	4.75± 0.48-b-	4.5± 0.29 -b-	5.0± 0.41 -b-
	100mg/ml	10.0± 0.82 -bc-	09.5± 0.65-bc-	09.5± 0.65-bc-	9.75± 0.63–bc-	10.25± 0.25-bc
Alcoholic	25mg/ml	4.75± 0.85 –a-	5.0± 0.41-a-	5.75± 1.03 -a-	6.5± 0.5-a-	4.5± 0.29-a-
	50mg/ml	8.25± 0.85–b-	9.0± 0.41 -b-	9.25± 0.63–b-	11.0± 0.41-b-	14.75± 0.75-b-
	100mg/ml	13.5± 0.65-bc-	13.25± 1.11-bc-	14.0± 0.82-bc-	7.25± 0.48-bc-	12.75± 0.48-bc

Values : M± S.E.M. a, b, bc; significantly differ at level of P < 0.05

Table 3. Comparison between agar well and disc methods, aqueous and alcoholic extracts

Extracts	Conc	Method	Bacteria sp.				
			Pseudomonasspp	*Bacillus subtlis*	E. coli	Proteus	*Staphylococcus aureus*
Aqueous	25 mg/ml	Disc	0.0± 0.0	1.5± 0.87	0.0± 0.0	0.0± 0.0	0.0± 0.0
		Well	9.0± 0.58*	8.75± 0.47*	9.25± 1.03*	9.25± 0.85*	10.0± 0.0*
	50 mg/ml	Disc	5.5± 0.29	5.0± 0.41	4.75± 0.48	4.5± 0.29	5.0± 0.41
		Well	11.0± 0.41*	12.0± 0.82*	12.0± 0.71*	10.25± 0.85*	12.75± 0.48*
	100 mg/ml	Disc	10.0± 0.82	9.5± 0.65	9.5± 0.65	9.75± 0.63	10.25± 0.25
		Well	13.25± 0.48*	14.25± 0.85*	14.25± 0.85*	12.5± 0.65*	16.75± 0.75*
Alcoholic	25 mg/ml	Disc	4.75 ±0.85	5.0 ± 0.41	5.75± 1.03	6.5 ± 0.5	4.5 ± 0.29
		Well	10.25± 0.25*	10.25± 0.25*	10.75± 0.75*	9.25± 0.75*	12.0± 0.41*
	50 mg/ml	Disc	8.25± 0.86	9.0 ± 0.41	9.25± 0.63	11.0± 0.41	7.25± 0.48
		Well	12.25± 0.25*	13.75± 0.63*	13.5± 0.65*	11.25± 0.75	15.0± 0.41*
	100 mg/ml	Disc	13.5 ±0.65	13.25± 1.11	14.0± 0.82	14.75± 0.75	12.75± 0.48
		Well	14.75± 0.25	17.25± 1.11*	15.75± 0.75	14.5± 1.19	21.25± 0.48*

Values : M± S.E.M.* significantly differentin well method in comparison with disc method at the same concentration ; at level of P < 0.05

Table -4- showed that aqueous extract in disc method significantly inhibitory zones exhibited against only Bacillus sp. At 25 mg /ml, while against other bacteria sp. There was no significance difference. At 50 mg /ml there was no significance difference between bacteria sp. except between *Staphylococcus aureus* and Proteus. At 100 mg /ml there were no significance differences between all bacteria sp. While in well method there were no significant differences between bacteria sp. except between *Staphylococcus aureus.* and Bacillus at 25 mg /ml, at 50 mg / ml there were no significance difference between bacteria sp.Except between all bacteria sp. and Staph. While at 100 mg /ml there were only significantly difference between Staph .and each of *Pseudomonas**spp*** and Proteus.

Table 5- In comparison of bacterial sensitivity to alcoholic extract by disc method; showed that the inhibitory effect at 25 mg/ml, there were a significant differences between *Proteus sp.* With each of *Pseudomonas**spp*** *Staphylococcus aureus* and *Bacillus*; but not differ significantly with E. coli. At 50 mg /ml there were significant differences between *Staphylococcus aureus* and *E. coli*. and between *Proteus* with each of *E. coli*; Bacillus, Staph, and *Pseudomonas**spp*** At 100 mg /ml there were no significance differences in susceptibility of bacteria sp. in used except between *Proteus* and *Staphylococcus aureus.*

The alcoholic extract gave the highest and clear inhibitory effects against bacterial sp. in agar well diffusion method than in disc diffusion methods (Table -6).

Table 4. Comparison between bacterial sp. sensitivity; the disc and agar well diffusion method – aqueous extract

Extract	Bactsp. conc	Proteus	*Bacillus subtlis*	*Staphylococcus aureus*	E coli	*Pseudomonasspp*
Aqueous:	25 mg/ml	0.0±	1.5±	0.0±	0.0±	0.0±
Disc		0.0-a-	0.87-b-	0.11-a-	0.0-a-	0.0-a-
	50mg/ml	4.5±	5.0±	5.0±	4.75±	5.5±
		0.29-a-	0.41-a-	0.41-a-	0/48-a-	0.29-a*-
	100mg/ml	9.75±	9.5±	10.25±	9.5±	10.0±
		0.63-a-	0.65-a-	0.25-a-	0.65-a-	0.82-a-
Aqueous:	25 mg/ml	9.25±	8.75±	10.0±	9.25±	9.0±
Well		0.85-a-	0.47-a-	0.0-a**-	1.03-a	0.58-a-
	50mg /ml	10.25±	12.0±	12.75±	12.0±	11.0±
		0.85-a-	0.82-a-	0.48-b-	0.71- a-	0.41-a-
	100mg/ml	12.5±	14.25±	16.75	14.25±	13.25±
		0.65-a-	0.85-a-	0.75-a***-	0.85-a	0.48-a-

Values : M± S.E.M. a, b, bc ; significantly differ at level of P < 0.05
-a*- only significantly differ between Pseud. and Proteus
-a**- significance only between Staph and Bacillus
-a***significance between Staph.and each of Pseud. and Proteus

Table 5. Comparison between bacterial sp. Sensitivity; disc and agar well diffusion methods – alcoholic extract

Extract	Conc.	Bacterial spp .				
		Proteus	***Bacillus subtlis***	***Staphylococcus aureus***	**E coli**	***Pseudomonasspp***
Alcoholic:	25 mg/ml	6.5±	5.0±	4.5±	5.75±	4.75±
Disc		0.5-b*-	0.41-a-	0.29-a-	1.03-a-	0.85-a-
	50mg/ml	11.0±	9.0±	7.25±	9.25±	8.25±
		0.41-bc-	0.41-b-	0.48-a-	o.63-b-	0.86-a**
	100mg/ml	14.75±	13.25±	12.75±	14.0±	13.5±
		0.75-b-	1.11-a-	0.48-a-	0.82-a-	0.65-a-
Alcoholic:	25 mg/ml	9.25±	10.25±	12.0±	10.75±	10.25±
Well		0.75-a-	0.25-a-	0.41-b**-	0.75-a-	0.25-a-
	50mg /ml	11.25±	13.75±	15.0±	13.5±	12.25±
		0.75-a-	0.63-bc-	0.41-b-	0.65-bcd-	0.25-a-
	100mg/ml	14.5±	17.25±	21.25±	15.75±	14.75±
		1.19-a-	1.11-ab-	0.48-bc-	0.75-a-	0.25-a-

-b*-Proteus significantly differ in comparison with other only with E. coli non-significantly differ.
-a**- Pseud. non significantly differ with E coli and Bacillus.
-b**-staph significantly duffer with all except non-significant with *E.coli*.

Table 6. Agar well diffusion, Comparison between aqueous and alcoholic extract

Bact sp.	Extract	aqueous	Alcoholic	aqueous	alcoholic	aqueous	Alcoholic
	Conc.mg/ml	25	25	50	50	100	100
Pseudomonasspp		9.00±	10.25±	11.00±	12.5±	13.25±	14.75±
		0.58	0.25 *	0.41	0.25*	0.48	0.25*
Bacillus subtlis		8.75±	10.25±	12.00±	13.75±	14.25±	17.25±
		0.48	0.25 *	0.82	0.63	0.85	1.11
E.coli		9.25±	10.75±	12.00±	13.5±	14.25±	15.75±
		1.03	0.75	0.71	0.65	0.85	0.75
Proteus		9.25±	9.25±	10.25±	11.25±	12.5±	14.5±
		0.85	0.75	0.85	0.75	o.65	1.19*
Staphylococcus aureus		10.0±	12.0±	12.75±	15±	16.75±	21.25±
		0.00	0.41 *	0.48	0.41 *	0.75	0.48*

Values : M± S.E.M. a, b, bc : significantly differ at level of P < 0.05

Table 7. Agar disc method, comparison between aqueous and alcoholic extract

Bacterial sp.	aqueous	alcoholic	Aqueous	alcoholic	aqueous	alcoholic
	25mg/ml	25mg/ml	50mg/ml	50mg/ml	100mg/ml	100mg/ml
Pseudomonasspp	0.0±	4.75±	5.5±	8.25±	10±	13.5±
	0.0	0.85 *	0.29	0.85 *	0.82	0.65 *
Bacillus subtlis	1.5±	5.0±	5±	9.0±	9.5±	13.25±
	0.87	0.41*	0.41	0.41*	0.65	1.11 *
E.coli	0.0±	5.75±	4.75±	9.25±	9.5±	14.0±
	0.0	1.03*	0.48	0.63 *	0.65	0.82 *
Proteus	0.0±	6.5±	4.5±	11.0±	9.75±	14.75±
	0.0	0.5*	0.29	0.41 *	0.63	0.75 *
Staphylococcus aureus	0.0±	4.5±	5.0±	7.25±	10.25±	12.75±
	0.11	0.29*	0.41 -	0.48 *	0.25	0.48*

Values : M± S.E.M. a, b, bc ; significantly differ at level of $P < 0.05$

Table 8. Standard antibiotic discs

AntibioticBact. Spp.	E. coli	*Pseudomonasspp*	*Staphylococcus aureus*	Proteus	*Bacillus subtlis*
RA: RA5	8	10	26	9	17
DO : 30	11.5	16.5	32.5	16.5	27
AX: 25	-	9.0	32.5	20	20
K:30	10	9.5	29	16	13
APX:30	2	3.5	6	-	15

RA: Rifampin 5 mcg RA5 ; DO: Doxycycline 30mcg 30; AX Amoxicillin 25 mcg 25; K: Kanamycin 30mcg 30; APX: Ampicillin cloxacillin, 25 / 5 mcg 30.

The results revealed that by disc method, the aqueous extract at 25 mg /ml concentration did not exhibit inhibitory effect against the bacterial sp. in used except against Bacillus sp. (Table -7). While at 50 and 100 mg /ml concentration of aqueous extract of plant there were inhibitory effects against all bacterial species in used, with the highest effect was against *Pseudomonas* at 50 mg /ml and *Staphylococcus aureus*. At 100 mg /ml, meanwhile the lowest inhibitory effects were against Proteus at 50 mg /ml and against *E coli* and *Bacillus* at 100 mg /ml (Table -7).

Al-Badrani (2002) found that crude ethanol extract of cotoneaster fruit posses the antibacterial effects against staph. aureus and *Strept.pyogens*, but (Watt *et al.*,1962) referred that there was no effect of the plant cotoneaster as antibacterial. The antibacterial effects can attribute to phytoallexins of Cotonefuran type (Burden *et al.*, 1984; Kokubun *et al.*,1995). As noted above, the plants are rich in terpenoids and phenolic compounds known to posses' antimicrobial activity. In our study, no relationship between total phenol content and the antimicrobial activities of extracts was observed. The antimicrobial activity of plants may vary depending on the types of terpenoids and flavonoids. The results of the present study may suggest that all extracts presumably possess compounds with antimicrobial properties against some bacterial.In conclusion from results we can concluded thatagar well diffusion method was more valuable to determine the antibacterial effects of both aqueous and alcoholic extracts of cotoneaster sp. In comparison with agar disc method.The alcoholic extract was more effective and showed higher antibacterial effect against all bacteria sp. In comparison with aqueous extract .also both aqueous and alcoholic extract were concentration dependent as they were more effective atconcentration 50 mg /ml in comparison with 25 mg /ml. at 100 mg /ml were highly effective in comparison with 25 and 50 mg ml the plants used in this study could be potential source of new antimicrobial agents.

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