Journal of Animal Research: v.5 n.4, p. 767-772. December 2015

DOI: 10.5958/2277-940X.2015.00127.8

Assessment of Antimicrobial Activity of Pomegranate Leaf Extract against Bacterial Isolates of Otitis Externa and Diarrhea of Dogs

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Received: 22 July, 2015 **Accepted:** 05 Nov., 2015

ABSTRACT

Bacterial pathogens were isolated from cases of otitis externa and diarrhoea in dogs at Durg and Raipur districts of Chhattisgarh. All isolates were further subjected for antibiogram profile using commonly used antibiotics. Total 105 bacterial isolates were obtained which included *Staphylococcus sp.* and *E. coli* as predominant pathogens followed by *Proteus, Klebsiella, Pseudomonas, Micrococcus* and *Salmonella*. In cases of otitis externa, ciprofloxacin was the most effective antibiotic (67.27%) followed by Gentamicin (61.81%), Cephalaxin (40%), Chloramphenicol (32.72%), Amoxycillin (21.81%), Ampicillin (20%), Doxycycline (16.36%), Penicillin (12.72%) and Erythromycin (3.63%). However, Chloromphenical (72%) was reported as the most effective drug against bacterial isolates of diarrhoea followed by Ciprofloxacin (70%), Gentamicin (52%), Cephalaxin (40%), Doxycyline (30%), Ampicillin (14%), Amoxycillin (6%) and Erythromycin (2%).Cold extract of pomegranate leaves was then assessed for antibacterial activity against all isolates by disc diffusion and tube dilution methods. The zones of inhibition of pomegranate leaves extract (PLE) against *Staphylococcus aureus, Pseudomonas aeruginosa, Proteus, Klebsiella, E. coli* and *Salmonella* were 18.8 ± 0.050, 5±0.70, 7.8±0.73, 16.2±0.86, 15.6±0.92 and 15.2±0.86 mm respectively. The zone of inhibition with *Staphylococcus* was significantly higher ($P<0.05$) than other bacteria. The minimum bactericidal concentration against *Staphylococcus aureus, Klebsiella, E. coli* and *Salmonella* were 33.33±8.34, 66.66±16.68, 66.66±16.68 and 33.33±8.34 mg/ml respectively. *Staphylococcus aureus* showed maximum sensitivity to pomegranate leaf extract amongst all the organisms studied, followed by *Klebsiella, E. coli* and *Salmonella*. Although, *Proteus* and *Pseudomonas* were refractory to PLE, but moderate sensitivity was noticed even against few multidrug resistant isolates.

Keywords: Pomegranate leaf extract; bacteria; otitis externa; diarrhoea; dog

Diarrhoea and otitis externa in dog are the common problem in present scenario to the dog lovers as well as pet practitioners (Kumar *et al*. 2014; Bhat *et al*. 2015). The most frequent microorganism associated with otitis externa in dogs are *Staphylococcus*, *Malassezia, Pseudomonas, Streptococcus* and *Proteus spp.* whereas *Escherichia coli*, *Proteus, Salmonella* and *Klebsiella* are common cause of diarrhoea in dogs (Petrov *et al*. 2013). Unfortunately, the resistance to antibiotics is increas ing, and these bacteria showed resistance to many

antibi otics. So, investigations must be conducted to discover the possibility of owning the plant capable to respond to these bacteria. Ayurveda explore the therapeutic utility of Indian medicinal herbs. The herbal remedies are economical and within the reach of common man. One of the promising options being aqueous extract of pomegranate (*Punica granatum*) leaves. *Punica granatum* a member of fam ily *Punicaceae* has a long history of antibacterial use dating back to biblical times (Charles, 2009). Different parts of plant including leaves, rinds of the fruit, root bark possesses antimicrobial activity and are effective against diarrhoea and dysentery (Jayaprakash *et al*. 2006). Therefore, present investigation was conducted to assess the antibacterial activity of leaves of pomegranate in light of its cleaning effect, anti inflammatory action as well as its potential to act against multiple drug resistant bacteria isolated from cases of otitis externa and diarrhoea in dogs.

MATERIAL AND METHODS

Clinical samples

The ear swabs and rectal swabs were collected from dogs suffering from otitis (n=23) and diarrhoea (n=30) at Durg and Raipur districts in Chhattisgarh. After 6 hours enrichment in peptone water, each sample was inoculated on Mannitol salt agar, EMB agar, MacConkey agar and Pseudomonas isolation agar for isolation of bacteria as per method described by Parker and Duerden (1990). On the basis of colony morphology, staining characteristics and biochemical tests viz. indole test, methyl red test, Voges-Proskauer test, citrate utilization, sugar fermentation, H_2S production on triple sugar iron and urease test; identity of bacteria was confirmed (Cruickshank *et al*. 1975).

Antibiotic sensitivity pattern of bacterial isolates

Commercially available antibiotic discs (Hi Media Laboratories Limited, Mumbai) were used to test sensitivity of bacteria against antibiotic. Antibiotic discs included Ciprofloxacin (Cf,10 µg), Gentamicin (G,30 µg), Cephalaxin (Cp,30 µg), Chloramphenicol (C,10 µg), Amoxycillin (Am,10 µg), Ampicillin (A,25 µg), Doxycycline (Do,10 µg), Penicillin (P,10 µg) and Erythromycin (E,10 µg). *In vitro* antibiotic sensitivity of isolates was studied by disc diffusion method (Bauer *et al*. 1966).

Preparation of pomegranate leaf extracts (PLE)

Mature dark green color pomegranate leaves were collected and shade dried (under fan) in the laboratory. Shade drying of 500 gm of pomegranate leaves yielded 130 gm of the powder. Then, 10% w/v suspension of cold extract was prepared in distilled water by dissolving the alcohol evaporated pomegranate leaf powder.

Assessment of antibacterial activity of cold extract of pomegranate leaves

The antibacterial activity of cold extract of pomegranate leaves was studied *in vitro* by disc diffusion (Bauer *et al*. 1966) and tube dilution methods (Ericsson and Sherris, 1971) against all isolates (antibiotic resistant as well as antibiotic sensitive bacterial isolates).

Disc diffusion method

Table 1. Prevalence of bacterial pathogens from Otitis externa and diarrhoea cases of dog

Name of bacterial isolate	Otitis externa		Diarrhoea	
	No. of isolates	Prevalence rate	No. of isolates	Prevalence rate
Escherichia coli	—	—	23	46%
Coagulase positive *Staphylococcocci*	7	12.72%	—	—
Coagulase negative *Staphylococcocci*	12	21.81%	—	—
Pseudomanas aeruginosa	14	25.45%	—	—
Proteus mirabilis	9	16.36%	7	14%
Proteus vulgaris	3	5.45%	4	8%
Klebsiella pneumoniae	5	9.09%	6	12%
Salmonella	—	—	4	8%
Klebsiella oxytoca	3	5.45%	3	6%
Micrococcus	2	3.63%	3	6%
Total	**55**		**50**	

Table 2: Antibiotic sensitivity pattern of Gram positive bacteria recovered from otitis externa

Name of bacteria	No. of isolates	Cf		G		Cp		P		A		Am	
		S	R	S	R	S	R	S	R	S	R	S	R
Coagulase +ve Staphylococcus spp.	7	5	2	6	1	5	2	2	5	4	3	1	6
Coagulase -ve Staphylococcus spp	12	12	0	10	2	7	5	4	8	5	7	9	3
Micrococcus	2	2	0	1	1	2	0	1	0	2	0	2	0

S = Sensitive, R = Resistant.

The blank discs (6.25 mm in diameter) were prepared from filter paper (0.3 mm thick) through punching. The discs, in a group of five were weighed in electronic monopan balance and the average weight of each disc was calculated. The discs were sterilized by autoclaving at 121^{O}C (15 lbs pressure) for 15 minute. The suspension of cold extract was allowed to soak on the discs drop by drop and the discs were dried. The process was repeated thrice. The impregnated discs after complete drying were again weighed in-group of five to know the amount of extract soaked on each disc and was calculated by subtracting the weight of extract socked disc to the blank disc. The collective weight of five filter paper blank discs, after several repetition, was identical *i.e.* 14.7 ± 0.27 mg. The combined weight of five discs impregnated with extract was 64.7 ± 0.27 mg, so the amount of cold extract impregnated on each disc was calculated as 10 ± 0.27 mg. 6 hrs fresh culture of each micro-organism was inoculated on the surface of nutrient agar plates. Subsequently, filter paper discs (6.25 mm diameters) with extract were impregnated on surface of each inoculated plates at 37^{o}C for 24 hrs. The sensitivity of bacterial isolate against pomegranate leaf extract was observed by assessing the zone of inhibition for particular isolate.

Tube dilution technique

In this technique the smallest amount of extract required to inhibit the growth of fixed dilution of organism *in-vitro* was determined. This amount was referred as minimum inhibitory concentration (MIC). Fresh bacterial culture of each isolate was taken in eight sterile tubes; 10 ml in first tube and 5 ml in remaining tubes. The first tube was added with 200 mg of leaf extract and the extract was diluted by making two fold serial dilutions up to seventh tube. Eighth tube was considered as control for the leaf extract. All the tubes were incubated at 37^{O}C for 24 hrs. Maximum dilution of the leaf extract showing absence of microbial growth determines MIC. Further, a loop full of the mixture was streaked on to nutrient agar plates and incubated so as confirm viability of bacteria. No growth on solid media was adjudged to be the bactericidal effect and interpreted as minimum bactericidal concentration (MBC). It is the lowest concentration of an antibacterial agent required to kill a particular bacterium.

Statistical analysis

Mean differences were analyzed by applying GLM (General Linear Model) for factorial experiments using SPSS computer software package (Version 16.0.0.247 ©2007).

RESULTS AND DISCUSSION

A total of 105 isolates (55 isolates from otitis and 50 from diarrhoea) were obtained in present investigation (Table 1). Predominant bacteria associated with otitis externa were *Staphylococcus* spp. followed by *Pseudomonas, Proteus, Klebsiella* and *Micrococcus* sp. In agreement with present observation, Oliveira *et al.* (2005) also reported *Staphylococcus* as predominant pathogen in otitis cases of dogs. With slight differences in prevalence rate, *Pseudomonas* (Fernandez *et al.* 2006), *Klebsiella* (Sarerler and Krkan, 2004) and *Proteus* (Ahmed, 2000) were frequently encountered in cases of otitis externa in dogs.

During present investigation, *Escherichia coli* occupied prime position in cases of diarrhoea followed by *Proteus*, *Klebsiella*, *Salmonella* and *Micrococcus* sp. Likewise, James *et al.* (2001) reported higher incidence of *E. coli* in diarrhoeic dogs. Prevalence of other isolates encountered in diarrhoea *viz. Proteus*, *Klebsiella* and *Salmonella* collaborate with the findings of Edward and Hall (2004).

Overall sensitivity of bacterial isolates recovered from otitis externa (Table 2 and 3) revealed that ciprofloxacin was the most effective antibiotic (67.27%) followed by Gentamicin (61.81%), Cephalaxin (40%),

Table 3: Antibiotic sensitivity pattern of Gram negative bacteria recovered from otitis externa

Organism	No. of isolates	Cf		G		E		Do		C		Cp	
		S	R	S	R	S	R	S	R	S	R	S	R
Pseudomonas aeruginosa	14	3	11	4	10	0	14	0	14	0	14	2	12
Proteus mirabilis	9	7	2	4	5	1	8	6	3	8	1	4	5
Proteus vulgaris	3	3	0	2	1	1	2	3	0	3	0	2	1
Klebsiella pneumoniae	5	3	2	4	1	0	5	0	5	5	0	0	5
Klebsiella oxytoca	3	2	1	3	0	0	3	0	3	2	1	0	3

S= Sensitive; R= Resistant.

Table 4: Antibiotic sensitivity pattern of Gram negative bacteria recovered from diarrhea

Organism	No. of isolates	Cf		G		E		Do		C		Cp		A		Am	
		S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R
Escherichia Coli	23	16	7	13	10	0	23	7	16	23	0	10	13	4	19	0	23
Proteus sp	11	9	2	6	5	0	11	5	6	7	4	3	8	0	11	0	11
Klebsiella sp	9	7	2	5	4	1	8	0	9	0	9	4	5	0	9	0	9
Salmonella sp	4	1	3	2	2	0	4	2	2	4	0	3	1	0	4	0	4
Micrococcus	3	2	1	0	3	0	3	1	2	2	1	0	3	3	0	3	0

S= Sensitive R= Resistant

Table 5 : Sensitivity of multiple drug resistant bacteria to herbal extract

Organism	No. of isolates	No. of multiple drug resistant isolates	Number of isolates sensitive to PLE		
			S	M	R
Pseudomonas aeruginosa	14	14	00	02	12
Proteus spp	23	04	00	06	17
Klebsiella spp	17	05	13	04	00
Escherichia Coli	23	07	18	05	00
Salmonella spp	04	01	04	00	00

S = Sensitive; M = Moderate; R = Resistant.

Chloramphenicol (32.72%), Amoxycillin (21.81%), Ampicillin (20%), Doxycycline (16.36%) and least sensitive to Penicillin (12.72%) as well as Erythromycin (3.63%). Likewise, Sarerler and Krkan (2004) reported ciprofloxacin as most effective drug in otitis cases. *Pseudomonas aeruginosa* showed maximum resistance to all antibiotics used in the experiment and was moderately sensitive to Gentamicin (28.57%), Ciprofloxacin (21.4%) and Cephalexin (14.28%); which is in congruence with the observation of Martin *et al*. (2000). Against bacterial isolates of diarrhoea, Chloromphenical (72%) was reported as the most effective drug (Table 4) followed by Ciprofloxacin (70%), Gentamicin (52%), Cephalaxin (40%), Doxycyline (30%), Ampicillin (14%), Amoxycillin (6%) and Erythromycin (2%).

The zones of inhibition of PLE against *Staphylococcus aureus, Pseudomonas aeruginosa*, *Proteus*, *Klebsiella, E. coli* and *Salmonella* were 18.8 ± 0.050, 5 ± 0.70, 7.8 ± 0.73, 16.2 ± 0.86, 15.6 ± 0.92 and 15.2 ± 0.86 mm, respectively. The zone of inhibition with *Staphylococcus aureus* was significantly higher (P<0.5) than *Pseudomonas aeruginosa*, *Proteus*, *Kelbsiella*, *E. coli* and *Salmonella.* The MBC against *Staphylococcus aureus, Klebsiella., E. Coli* and *Salmonella* were 33.33 ± 8.34, 66.66 ± 16.68, 66.66 ± 16.68 and 33.33 ± 8.34 mg/ml, respectively.

Antibacterial potency of PLE against bacterial isolate during present study is well supported by Hayouni *et al*. (2011) who reported significant antibacterial activity of extract against wound bacteria, including strains of *Pseudomonas aeruginosa*, *S. aureus*, *E. coli*, *Klebsiella pneumoniae*, *Salmonella* Anatum, *S*. Typhimurium, and *Streptococcus pneumonia*. Zone of inhibition of PLE against *S. aureus* in present report is in congruence with observation of Chaitra *et al*. (2012) who found that extracts exhibited maximum inhibition against *S. aureus* (16 ± 0.20 mm). Likewise, in a study Tayel *et al*. (2012) observed that a pomegranate peel extract at 250 µg/ml was most effective at inhibiting antibiotic resistant strains of *Salmonella* Typhimurium and *Staphylococcus aureus*. There is evidence from a number of *in vitro* experiments that pomegranate extracts moderately to strongly inhibit cultured MRSA strains also (Su *et al*. 2012). More or less similar findings were reported by Voravuthikunchai *et al*. (2004) where aqueous extract of pomegranate were found sensitive to *E. coli* and produced 7 to 17 mm zone of inhibition, however MBC reported was less i.e. 0.39 mg/ml.

Sensitivity of bacterial isolates against antibiotics and PLE was compared (Table 5) and it is observed that all the isolates (sensitive as well multidrug resistant) of *Klebsiella*, *Salmonella* and *E. coli* were found sensitive to PLE. In present study, *Proteus* and *Pseudomonas* were refractory to pomegranate leaf extract, although moderate sensitivity was observed with few multidrug resistant isolates. On contrary, Santhanamari *et al.* (2011) reported higher antimicrobial potency of *Punica granatum* which inhibited 75% of resistant isolates of *Pseudomonas aeruginosa.* Similarly, Gislene *et al.* (2000) found more than 7 mm zone of inhibition of *Pseudomonas* against pomegranate leaf extract. It is opined that moderate sensitivity of *Pseudomonas* and *Proteus* during present investigation might be due to some factors like soluble pyocinin pigment and swarming factor, respectively, which might be involved in inhibiting action of leaf extract against *Proteus* and *Pseudomonas*. On the other hand, isolates of *Staphylococcus*, *Salmonella*, *E. coli* and *Klebsiella* hardly produce any apparently visible factor during their growth on suitable laboratory medium. Growth of all isolates of these bacteria was totally prevented in the presence of leaf extract impregnated disc. Pomegranate leaf extract have cleaning effect besides antimicrobial potential (Gislene *et al*. 2000). Cleaning removes the nidus for reinfection, bacterial debris, allow antimicrobial element to penetrate entire canal and also prevent purulent debris from inactivating some antimicrobial elements ultimately results in efficient overall antimicrobial activity. The pomegranate leaf extract also possess the anti inflammatory potential which helps in preventing the stenosis of ear canal due to increased inflammatory reaction and accumulation of debris (Jayaprakash *et al*. 2006).

Bacteria associated with otitis and diarrhoea are often resistant to multiple antibacterial agents and hence choice of herbal extract of pomegranate leaves for *in vitro* sensitivity test may prove to be effective against antibiotic resistant bacteria, too. From the results of antibacterial activity of the extracts against the resistant isolates it has been observed that *Staphylococcus aureus, E. coli, Salmonella* and *Klebsiella* were more susceptible to the employed plant extracts than *Pseudomonas aeruginosa* and *Proteus*. Different solvents may be considered for preparation of pomegranate leaf extracts for its wider applications. The *in-vitro* laboratory experiment need validation *in vivo* by appropriately designed experimental trial on laboratory animals.

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