International Journal Of

Recent Scientific Research

ISSN: 0976-3031

Volume: 7(5) May -2016

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THE OFFICIAL PUBLICATION OF
INTERNATIONAL JOURNAL OF RECENT SCIENTIFIC RESEARCH (IJRSR)
http://www.recentscientific.com/ recentscientific@gmail.com

ISSN: 0976-3031

Available Online at http://www.recentscientific.com

International Journal of Recent Scientific Research
Vol. 7, Issue, 5, pp. 11440-11444, May, 2016

International Journal of Recent Scientific Research

Research Article

ISOLATION, SCREENING OF MASTITIS CAUSING BACTERIA AND ANTIBACTERIAL EFFECT OF POLY HERBAL FORMULATION

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ARTICLE INFO

Article History:

Received 11th February, 2016
Received in revised form 14th March, 2016
Accepted 18th April, 2016
Published online 28th May, 2016

Key Words:

Mastitis, Cow, Blood, *P. guajava* leaves, *T. foeum-graecum* seeds, Antibacterial

ABSTRACT

Bovine mastitis in dairy cattle is the most devastating inflammatory reaction of udder tissue caused by invasion of bacteria. Mastitis affects the entire dairy industries badly throughout the world. Overuse of antibiotics and that followed by generation of antibiotic-resistant bacteria encourages us to search for an alternative way of treatment. Here we report screening and characterization of mastitis causing bacteria form cow blood and their remedy by using herbal extracts of Guava and Fenugreek. Miobiological and biochemical examination revealed the abundance of Gram+ve (*S. aureus*) and Gram–ve (*E. coli*, *P. aeruginosa* and *Salmonella* spp.) bacteria in mastitis infected cow blood. Antibacterial activity against these isolates was checked by using disk diffusion method at different concentrations of *P. guajava* leaves and *T. foeum-graecum* seeds herbal exbact. The present study revealed that methanolic extract of both herb displays a potent antibacterial activity (at 1000µg concentration) while aqueous extract of *P. guajava* leaves doesn't show any bactericidal property. Antibacterial efficacy of methanolic extract of both herbs and aqueous extract of *T. foeum-graecum* seeds were in this order *E. coli* > *Salmonella* spp. > *P. aeruginosa* > *S. aureus* and *E. coli* > *S. aureus* > *P. aeruginosa* > *Salmonella* spp. respectively. Thus the study revealed that Guava leaves and Fenugreek seeds extracts can be a potential source to treat or blocking of bacterial pathogens which causes mastitis in cow.

INTRODUCTION

Mastitis is a major source of economic loss to the dairy industry and milk quality. Prevalence of mastitis are major factors in determining farm probability. Dairy herd managers ranked mastitis as the most important disease on their farms. The worldwide losses due to the disease are approximately $35 billion annually. Despite the fact that a sustained extension and educational effort has been ongoing since the early 1970s, mastitis remains the single health factor Which is most influential in affecting milk production, with both clinical and subclinical mastitis (Keefe, 1997). The etiology of bovine mastitis can be classified into contagious pathogens such as *Staphylococcus aureus, Streptococcus agalactiae,* environmental pathogens *viz; S .dysgalactiae, S. uberis, Corynebacterium bovis* and Coagulase negative *Staphylococcus*, *Pseudomonas aeruginosa, Staphylococcus epidermidis, Brucella melitensis, Escherichia coli, Klebsiella pneumonia, Klebsiella oxytoca, Enterobacter aerogenes, Pasteurella species, Proteus species, Prototheca zopfii, Prototheca wickerhamii, Mycoplasma species* (Watts, 1988). The economic impacts of bovine mastitis and intramammary infection have lead to the development of various therapeutic strategies to control them. Many drugs belonging to various therapeutic classes have been assessed in recat year (Koivula *et al.,* 2005). During the clinical mastitis cases the cow is first milked out and then introduced with intramammary infusion of antibiotics. Treating sub clinical mastitis with antimicrobials during lactation is not practiced because of high cost of treatment and poor efficacy. Herbal therapy is gaining much attention nowadays due to increased drug resistance and significance in terms of treating mastitis (Barkema *et al.,* 2006; Deb *et al.,* 2013).

Guava *(Psidium guajava*) also known as 'Amrood' in Hindi, is a very common plant in Asian countries, but is increasingly available in the western world also particularly as more of its health benefits were revealed. The young leaves of the *P. guajava* contain a number of beneficial constituents including antioxidants and flavonoids which are used in traditional medicine in tropical countries. Its leaves play an important role in treatment of diarrhea, dysentery, constipation, cough, cold, skin care, high blood pressure, weight loss and scurvy

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(Gutiérrez *et al.,* 2008). There are so many other health benefits also of *P. guajava* it is hard to know where to begin. Similarly Fenugreek (*Trigonella foenum-graecum)* also known as 'Methi' in Hindi is an important ingredient in Indian households. Fenugreek is a rich reservoir of medicinal properties that imparts many health benefits (Premanath *et al.,* 2011). Keeping so many tremendous positive benefit of *P. guajava* leaves and *T. foenum-graecum* seeds on human health encourage us to test their antibacterial potential against mastitis causing bacterial isolates from cow blood. These bacterial pathogens were isolated and screened from mastitis infected cow blood.

MATERIALS AND METHODS

Sample collection

Blood samples from mastitis infected cow were collected in sterile containers of local dairy located near to Patanjali University, Haridwar (UKH). Samples were sealed, labeled and kept at reduced temperature in laboratory.

Isolation of bacteria

Collected blood samples were serially diluted up to 10^{-4}. For it, 1 ml of blood sample was mixed in 10 ml of sterilized distilled water. Then, 1 ml of mixture was transferred to the tube containing 9 ml. of sterilized distilled water. Process was continued up to 4^{th} tube to get 10^{-4} concentration. Each concentration of blood samples were placed on separate plates and incubated at 37°C for 24 hours. Plates were observed after 24 hours. Isolated bacterial colonies were observed for colony characteristics and CFU count (Sanders, 2012),

Isolation of pure culture

Colonies obtained in Nutrient agar media plate were picked and purified by streaking on selective media *viz;* Blood agar, Brilliant green agar, Cetrimide agar and MacConkey agar. All media plates were incubated at 37°C for 24 hours. Plates were observed after 24 hours. Colonies characteristics and CFU count were observed and recorded.

Morphological and biochemical studies of pure culture

After culture and pure bacterial isolation, the isolates were identified by morphology of colony, Gram staining and by standard biochemical tests (Cappuccino and Sherman, 2008).

Plant material and extraction

Fresh leaves were detached from *P. guajava* trees (8-12 feet) grown wild in nearby areas of Patanjali Food and Herbal Park, Haridwar (UKH). Meanwhile *T. foenum-graecum* seeds were collected from local market of Haridwar. Separately a known amount of leaves and seeds (100 g each) were washed thoroughly and oven dried at 45 ºC. Leaves and seeds were powdered by using mortar and pestle. Thus prepared powder of leaves and seeds (10 gram each) were separately extracted 3 times with methanol and water (25-30 ml) for 24 h. Each time it was filtered through 0.45-µm filter paper and collected in a beaker. The extract thus obtained was dried over reflection water bath. Dried extracts were weighed and stored at 4ºC (Gupta and Ganjewala, 2013; Ganjewala *et al.,* 2013)

Antibacterial assay

Antibacterial activities of methanolic and aqueous extracts of leaves and seeds were assessed against Gram+ve (*Staphylococcus aureus)* and Gram–ve (*Escherichia coli, Pseudomonas aeruginosa, Salmonella* spp.) bacteria by agar well diffusion method. The culture plates were prepared by first sterilizing the nutrient agar (36gm in 1000 ml) in an autoclave at 121ºC at 15lbs for 15 minutes and then by pouring 20 ml media into sterilized Petri plates. 1 ml inoculum suspension was spread uniformly over the agar medium using sterile glass rod. Wells were made by using sterile cork borer (6 mm) in each plate. 100µl of each plant extracts (at concentration of 200, 400, 600, 800 and 1000 µg/ml) was added aseptically into the well. Simultaneously, a control without addition of plant extracts was also run. Plates were incubated at 37ºC for 24 hrs. After incubation, growth and the zone of inhibition (mm) produced due to the plant extracts were observed and compared with the controls. Each test was done in triplicates. The antibacterial activity was expressed as the mean of diameter of the inhibition (Ganjewala *et al.,* 2014).

RESULTS

Blood samples were collected from infected cow and bacteria were isolated by using serial dilution up to 10^{-4} concentration. After 24 hours of incubation colony characteristics *viz*; size, appearance, form, margin and elevation revealed different kind of colony forms. However, CFU count was recorded as $9.5x10^3$*cfu*/mL on nutrient agar media (Table 1).

These bacterial isolates were inoculated on selective media *viz;* Blood agar, Brilliant green agar, Cetrimide agar and MacConkey agar for selective pure culture isolations. Selective media, Blood agar, Brilliant green agar, Cetrimide agar and MacConkey agar shows CFU count $1.5x10^3$, $5.5x10^2$, $6.5x10^2$ and $7.5x10^3$*cfu/* mL respectively. Colonies on these selective media also shows typical colony characteristics (Table 2).

Microscopic examination of these bacterial isolates from each selective media shows typical bacterial cell appearance in reference to Motility test, Gram's, Endospoer, and Capsule staining (Table 3). Further number of biochemical examination confirms the presence of typical bacterial isolate from selective media. Like *S. aureus* (SA) form Blood agar, *Salmonella* spp. (SS) from Brilliant green agar, *P. aeruginosa* (PA) Cetrimide agar and *E. coli* (EC) from MacConkey agar were identified (Table 4).

Table 1 Colony characteristics of bacterial isolates from mastitis infected cow blood

Media	Colony characteristic						
	***Cfu/* mL**	**Size**	**Appearance**	**Pigmentation**	**Form**	**Margin**	**Elevation**
Nutrient agar media	$9.5x10^3$ *cfu/* mL	Pinpoint, moderate or large colonies	Shiny and smooth in appearance	Non pigmented to green pigmented cells	Circular and irregular colonies	Entire, irregular and lobate margin	Flat, raised and umbonate

Table 2 Colony characteristics on selective media of bacterial isolates

Colony characteristics	Selective media			
	Blood agar	Brilliant green agar	Cetrimide agar	MacConkey Agar
cfu/ mL	$1.5x10^3$	$5.5x10^2$	$6.5x10^2$	$7.5x10^3$
Size	Larger size	Moderate size	Large size	Pinpoint to small size
Pigmentation	Golden pigments	Non-pigmented	Blue-green pigments	Pink pigments
Appearance	Golden appearance	Red to pink white colonies	Light green blue colonies	Pink colonies
Form	Round with sharp border	Round with a sharp border	Irregular peripheral edges	Circular peripheral
Margin	Entire margin	Entire margin	Serrate margin	Entire margin
Elevation	Convex	Raised	Umbonate	Umbonate

Table 3 Microscopic examination of selective bacterial isolates

Selective media	Gram's reaction	Motility	Endospore staining	Capsule staining
Blood agar	+ve cocci	Non motile	Non-sporing	Non capsulated
Brilliant green agar	-ve rod	Motile	Non-sporing	Capsulated
Cetrimide agar	-ve rod	Motile	Non-sporing	Non capsulated
MacConkey agar	-ve rod	Motile	Non-sporing	Non capsulated

Table 4 Biochemical profile of selective bacterial isolates

Biochemical test	Blood agar	Brilliant green agar	Cetrimide agar	MacConkey Agar
Indole production	-	-	-	+
Methyl red	+	+	-	+
Vogus proskaure	±	-	-	-
Citrate utilization	-	+	+	-
Catalase	+	+	+	+
Urease	-	-	-	-
Oxidase	-	-	+	-
Nitrate reduction	+	+	+	+
Gelatin liquefaction	+	-	+	-
H_2S production	-	+	-	-
Starch hydrolysis	-	-	-	-
Lactose	A	-	-	AG
Dextrose	A	AG	-	AG
Sucrose	A	AG±	-	A±
Identification isolated	*S. aureus*	*Salmonella spp.*	*P. aeruginosa*	*E. coli*

AG=Acid and gas; ± = Variable reaction

For the test of antibacterial agent against these bacterial pathogens, *P. guajava* leaves and *T. foeum-graecum* seeds were selected. Both plant parts were extracted with different polarity of solvent water and methanol. The yield of *P. guajava* leaves and *T. foeum-graecum* seeds aqueous extracts were 50 and 15% while yield in methanolic extract were 25 and 10% respectively (Table 5).

Table 5 Percentage yield of *P. guajava* leaves and *T. foeum-graecum* seeds of the screened solvent extracts

S. No.	Plant	Solvent	
		Water	Methanol
1.	*P. guajava*	50%	25%
2.	*T. foeum-graecum*	15%	10%

The antibacterial profiles of the aqueous extracts of *viz., P. guajava* leaves and *T. foeum-graecum* seeds are presented in Table 6. Results revealed that only *T. foeum-graecum* seeds extracts showed fairly well bactericidal activity against all pathogenic bacteria isolates. Among the pathogens, *E. coli* was more sensitive with zone of inhibition 21 mm at 1000µg/ml concentration. Other pathogens also show a degree of sensitivity towards *T. foeum-graecum* seeds extracts. Methanolic extract of both plant parts carries potent antibacterial activity. Extract of *P. guajava* leaves and *T. foeum-graecum* seeds (1000µg/ml) did best antibacterial activity against with zone of inhibition 18 mm and 20 mm respectively. However, both extracts were significantly effective against all other pathogens *S. aureus*, *Salmonella* spp. and *P. aeruginosa* (Table 7). In comparison to the standard antibiotics used, antibacterial potential of the extracts of the samples were found moderately effective.

Table 6 Antibacterial activities of aqueous extract of *Psidium guajava* leaves and *T. foeum-graecum* seeds

Concen. (µg/ml)	Zone of inhibition (mm)							
	Psidium guajava leaves				*T. foeum-graecum* seeds			
	EC	PA	SS	SA	EC	PA	SS	SA
200	Nil				16	13.5	12.5	13
400					18	14	13	13.5
600					19.5	14	15	14
800					20	15.5	15.5	15
1000					**21**	17	17	19

Inhibition zones are the mean including borer (5 mm) diameter ± standard deviation

Table 7 Antibacterial activities of methanolic extracts of *Psidium guajava* leaves and *T. foeum-graecum* seeds

Concen. (µg/ml)	Zone of inhibition (mm)							
	Psidium guajava leaves				*T. foeum-graecum* seeds			
	EC	PA	SS	SA	EC	PA	SS	SA
200	13	12	14	-	14	14	15	12
400	11	13	14	-	15	15	16	12
600	13	13.5	15	11	16	15.5	17	13
800	14	14	15	12	20	16	17.5	13
1000	**18**	14	15	12.5	**20**	16	18	14

Inhibition zones are the mean including borer (5 mm) diameter ± standard deviation
Here **EC**-*Escherichia coli,* **PA**-*Pseudomonas aeruginosa*, **SS**- *Salmonella* spp. and **SA**- *Staphylococcus aureus*

DISCUSSION

The present study revealed that prominent CFU count on nutrient agar media plates favors that prevalence of pathogenic bacteria in infected cow blood. Further, selective media isolates primary broth culture were characterized by colony characteristics, microscopic and biochemical examinations. It reveals the prevalence of *E. coli*, *S. aureus*, *P. aeruginosa* and *Salmonella* spp. 78%, 15%, 6% and 5% (as per *cfu*/mL) respectively in causing bovine mastitis. These results are consistent with the previously published reports on bovine mastitis causing agents (Farooq *et al.,* 2008; Deb *et al.,* 2013; Bebor *et al.,* 2014). However, most of the reports favor the presence of *Staphylococcus* spp. in mastitis infected blood (Petersson-Wolfe *et al.,* 2010; Deb *et al.,* 2013; Hosseinzadeh and Saei, 2014)

The present study also found that the aqueous extract of *T. foenum-graecum* seeds and methanol extract of both plant parts (*P. guajava* leaves and *T. foenum-graceum* seeds) possessed in vitro antibacterial activity against mastitis-causing bacteria isolates from blood samples. These findings were in agreement with the previously published reports that the guava leaf extract and fenugreek seeds had a potent antibacterial activity against various bacteria including *E. coli*; *S. aureus* (Premanath *et al.,* 2011; Biswas *et al.,* 2013) and *P. aeruginosa* (Garode and

Waghode, 2014; ElNour *et al.,* 2015). *Salmonella* spp. was also shown great sensitivity towards guava leaves in earlier findings (Biswas *et al.,* 2013). The in vitro antibacterial potential against mastitis causing bacterial Isplates by both plant extracts could be due to presence of some active phyto chemicals such as phenolic compounds, flavonoids, essential oils, saponins, glycosides terpenoids, triterpene, tannins, carotenoid, and a number of other fixed substances in guvaya leaves as well as in fenugreek seeds (Nascimento *et al.,* 2000; Biswas *et al.,* 2013). However, aqueous extract of guava leaves did not displayed any antibacterial activity against bacterial isolates. It could be due to the type of solvent, phyto chemical constituents in the guava leaves which may differ attributable to the geographical locations, biochemical variations within the species. However, the present study, active ingredients of the guava leaf and fenugreek seed extract were not analyzed because of available well-established identifications of phytochemical. In future, molecular method to identify mastitis causing gene in these bacterial isolates could be a significant tool to trace the role and treatment of the same Would the serious threat to cattles.

Acknowledgments

The corresponding author of the manuscript would like to thank Head, Patanjali Ayurveda (P) Ltd, Padartha, Haridwar, Uttarakhand, India, for providing necessary support and facilities.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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How to cite this article:

Acharya Balkrishna *et al*. 2016, Isolation, Screening of Mastitis Causing Bacteria and Antibacterial Effect of Poly Herbal Formulation. *Int J Recent Sci Res.* 7(5), pp. 11440-11444.

TSSN 0976-3031