RESEARCH ARTICLE

Antimicrobial Potential of Polyherbal Formulation against Common Pathogens of Cow Mastitis

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Abstract

Mastitis is a global problem, which affects the health of animal, milk quality and economics of milk production. Multidrug-resistant *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Escherichia coli* are found to be increased in mastitis cases. There is a growing need for finding alternatives to antibiotics in the prevention and treatment of mastitis infections. Polyherbal formulations (PHF) are safe with wide therapeutic range and effective at low dose compared to single herbal formulation. Equal concentration of ethanolic extract of *Aerva lanata, Phyllanthus emblica, Cyperus rotundus* and *Boerhavia diffusa* formulation was used to assess the *in vitro* antibacterial activity against mastitis milk isolates *Staphylococcus aureus, Klebsiella pneumoniae and Escherichia coli* by well diffusion, agar diffusion and MIC by resazurin dye method. Zone of inhibition was greater in polyherbal formulation than herbal standard quercetin due to synergistic effects of multiple numbers of bioactive constituents. Polyherbal formulation had one fold low MIC value than gentamicin and one fold high MIC value than quercetin against *E. coli, S. aureus, K. pneumoniae.* Antimicrobial activity of the polyherbal formulation could be due to quercetin, a plant flavonoid quantified by using High Performance Thin Layer Chromatography (HPTLC).

Keywords: Antimicrobial, Cow, Mastitis, MIC, Polyherbal, Quercetin.

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Introduction

In India, economic loss due to mastitis is about Rs. 5750 lakhs per annum and the milk reduction by 21% (Bardhan, 2013). Due to invasion of both Gram positive and Gram negative bacteria in udder, a combination of antibiotics is administered through parenteral and intramammary route, but many times the results are not satisfactory due to bacterial resistance. Under these circumstances, there is a growing need for identifying alternatives to antibiotics in the prevention and treatment of mastitis. One such approach is the utilization of naturally occurring botanicals that have the potential to be used as an alternative or a complement to antibiotics. Traditional herbs which are rich in alkaloids, terpenoids, tannins, steroids, coumarins and flavonoids and do not normally cause resistance. Antimicrobial and hepatoprotective properties of *Aerva lanata* was reported by Goyal *et al.* (2011). *Phyllanthus emblica* is effective against *Escherichia coli* (Mondal *et al*., 2017). *Cyperus rotundus* oil possesses antimicrobial activity against *Staphylococcus aureus* (Zhang *et al.* 2017). Aqueous and ethanolic extracts of *Boerhavia diffusa* leaves is effective against *Staphylococcus aureus* and *Escherichia coli* (Adeku *et al*., 2022). The present study was undertaken to evaluate *in vitro* antibacterial activity of polyherbal formulation containing *Aerva lanata*, *Phyllanthus emblica, Cyperus rotundus* and *Boerhavia diffusa* against mastitis milk isolates *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Escherichia coli* from cows.

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Materials and Methods

Plant Materials and Test Microorganisms

Plant materials used in this study *consist* of whole plant of *Aerva lanata*, fruits of *Phyllanthus emblica*, roots of *Cyperus rotundus* and roots of *Boerhavia diffusa*, which were collected in and around Tirunelveli district, Tamil Nadu (India). Authentication of identified plants was done at Siddha

Central Research Institute, Chennai (form number-PCOG002-ACF). The extraction was done by Soxhlet apparatus using ethanol as solvent. The extract was concentrated using rotary evaporator and final yield was stored at 4°C. Crude extracts were taken in equal concentration for final polyherbal formulation.

Staphylococcus aureus, *Klebsiella pneumoniae* and *Escherichia coli* isolated from the cow mastitis milk samples were obtained from the Department of Veterinary Microbiology, VCRI, Tirunelveli. The bacterial cultures were maintained in nutrient broth at 37°C and maintained on nutrient agar slants at 4° C

Phytochemical Screening

Crude extracts were qualitatively screened for the phytochemicals responsible for antibacterial action such as saponins, tannins, phenols, alkaloids, phytotannin, flavonoids, terpenoids, volatile oil, carbohydrates, glycosides, and amino acids (Trease and Evans, 1989).

Quantification of Identified Marker Compound using HPTLC

Quantification of Quercetin was done using the mobile phase, toluene: ethyl acetate: formic acid in the ratio of 10.9:8.7:0.4 (v/v) (Merck). The aluminium plate precoated with silica gel G 60 F_{254} (Merck) which was 0.2 mm in thickness and 10 cm x 20 cm in size was used as stationary phase. Sample was prepared by diluting extract with methanol of HPLC grade to 100 mg/mL. The concentration of standard Quercetin (Sigma aldrich) was 10 mg/mL of methanol. Quantification of Quercetin was done using vision CATS software (Patel *et al*., 2017).

Agar Well and Disc Diffusion Methods

Antibacterial activities of polyherbal crude extracts were checked at different concentration against *S. aureus, K. pneumoniae* and *E. coli* using the agar well diffusion method at higher concentration (Chidambara Murthy *et al*., 2003). Eight wells were formed with a 6 mm diameter sterile cork borer, 2 mm from the edge of the plate. Three wells were aseptically filled with 50 µL of polyherbal extract at concentration of 600 µg/mL, 800 µg/mL, 1000 µg/mL and the remaining wells were filled with standard Quercetin at concentration of 600 µg/mL, 800 µg/mL, 1000 µg/mL. Positive controls used were Gentamicin 10 µg/mL and Amoxicillin- Clavulanic acid (50/10 µg/mL). All experiments were repeated three times and results recorded as mean values.

The paper disc diffusion test was used to screen the antibacterial activity of the polyherbal extracts at lower concentration (Mostafa *et al*., 2018). Each bacterial suspension (*S. aureus, K.pneumoniae* and *E. coli*) was diluted with a sterile physiological solution to 10^8 CFU/mL by comparing with 0.5 McFarland turbidity standards. 100 µL of the bacterial suspension were swabbed uniformly on the surface of MHA and the inoculum was allowed to dry for five minutes. Separate stock solution was prepared for polyherbal extract and quercetin by dissolving 10 mg in 10 mL of ethanol. The stock solution was then diluted with ethanol to concentrations of 300 µg/mL, 400 µg/mL and 500 µg/mL for polyherbal extract and quercetin. 20 µL of each dilution was impregnated in the sterilized blank discs, which was placed on the surface of the MHA. Positive controls used were the same as above. The inoculated plates with bacteria were incubated at 37 °C for 24 h. The zones of inhibitions were measured in mm diameters. All experiments were repeated three times and results recorded as mean values.

MIC by Resazurin Dye Assay

MIC values of polyherbal against *S. aureus, K. pneumoniae* and *E. coli* were determined using microdilution method modified with resazurin dye assay (Sarker *et al*., 2007). Individual bacterial inoculum was prepared by using standard plate cultures in sterile distilled water equivalent to 0.5 McFarland turbidity standards. The resazurin solution of 0.015% was prepared by using resazurin powder (HiMedia) in distilled water. Plates were wrapped with cling film to avoid the dehydration of bacteria. The plates were prepared in triplicate and incubated at 37°C for 24 h. Colour change from violet to pink or colorless was recorded as positive. The lowest concentration at which colour change occurred was taken as the MIC value.

Statistical Analysis

Results were expressed as Mean ± Standard Error (SEM). Data obtained were analyzed by one-way ANOVA followed by Duncan's test using SPSS version 22.0 software. Statistical significance was set at $p<0.05$.

Results and Discussion

The polyherbal formulation used in this investigation at different concentration inhibited the growth of mastitis causing pathogens such as *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Escherichia coli*. Preliminary phytochemical screening of polyherbal formulation indicated the presence of a number of bioactive constituents such as saponins, tannins, alkaloids, flavonoids, terpenoids, volatile oil, carbohydrates, glycosides. Among the phytochemical constituents tannins, saponins, flavonoids, alkaloids serve as defense mechanisms against predation by many microorganisms (Zongo *et al*., 2010). The antimicrobial activity in the present study may be due to the presence of these bioactive constituents. Significant zones of inhibitions were observed by well diffusion assay (Table 1) in the polyherbal formulation at different concentration (1000 µg/mL, 800 µg/mL, 600 µg/mL) and quercetin at different concentration (1000 µg/mL, 800 µg/mL, 600 µg/mL) against *Staphylococcus aureus, Klebsiella pneumoniae* and *Escherichia coli* compared to the standard gentamicin (50 µg/mL), amoxicillin- clavulanic acid (50/10 µg/mL). Similarly significant zones of inhibitions by disc diffusion assay were also observed in the polyherbal formulation at different lower concentration (500 µg/mL, 400 µg/mL, 300 µg/mL) and quercetin at different concentration (500 µg/mL, 400 µg/mL, 300 µg/mL) against *Staphylococcus aureus, Klebsiella pneumoniae* and *Escherichia coli* compared to the standard gentamicin (50 µg/mL), amoxicillin-clavulanic acid (50/10 µg/mL) (Table 2; Fig. 1)

Table 1: Antibacterial activity of polyherbal extract compared with standards by well diffusion method

	Zone of Inhibition (mm)							
Organism	**PHF 600 µg/mL**	**Quercetin 600 µg/mL**	**PHF 800 µg/mL**	**Quercetin 800 µg/mL**	**PHF 1000 µg/mL**	**Quercetin 1000 µg/mL**	**Gentamicin 50 µg/mL**	**Amox+Clav 50/10 µg/mL**
E. coli	18.3^c±0.42	14.8^d±0.60	20.0^b±0.57	16.0^d±0.36	24.5^a±0.42	17.5^c±0.42	14.3^d±0.49	14.5^d±0.52
S. aureus	29.1^b±0.47	13.8^d±0.47	30.0^b±0.57	16.5^d±0.42	32.5^a±0.42	17.0^d±0.36	25.5^c±0.42	25.2^c±0.38
K. pneumoniae	28.5^c±0.56	17.0^f±0.36	31.5^b±1.0	19.5^e±0.42	33.6^a±0.42	23.5^d±0.56	19.5^e±0.42	19.7^e±0.39

Values are mean ± SEM (n=3). Means bearing different superscripts within the row differ significantly (p<0.01).

Table 2: Antibacterial activity of polyherbal extract compared with standards by disc diffusion method

	Zone of Inhibition (mm)							
Organism	**PHF 300 µg/mL**	**Quercetin 300 µg/mL**	**PHF 400 µg/mL**	**Quercetin 400 µg/mL**	**PHF 500 µg/mL**	**Quercetin 500 µg/mL**	**Gentamicin 50 µg disc**	**Amox+Clav 50/10 µg disc**
E. coli	13.6^c±0.21	12.6^c±0.33	14.4^b ± 1.87	12.83^d±0.30	15.3^a±0.81	13.3^c ±0.33	15.5^a±0.42	15.2^a ±0.39
S. aureus	22.0^d±0.36	16.3^f±0.42	26.0^{ab}±0.4	17.1^f±0.30	26.5^a±0.42	18.0^e ±0.36	24.67^c± 0.42	24.23^c± 0.12
K. pneumonia	17.1^c±0.30	13.0^e±0.36	18.6^b ± 0.42	15.1^d±0.30	21.3^a±0.42	15.5^d ±0.41	21.0^a ± 0.57	20.8^a ± 0.41

Values are mean ± SEM (n=3). Means bearing different superscripts within the row differ significantly (p<0.05).

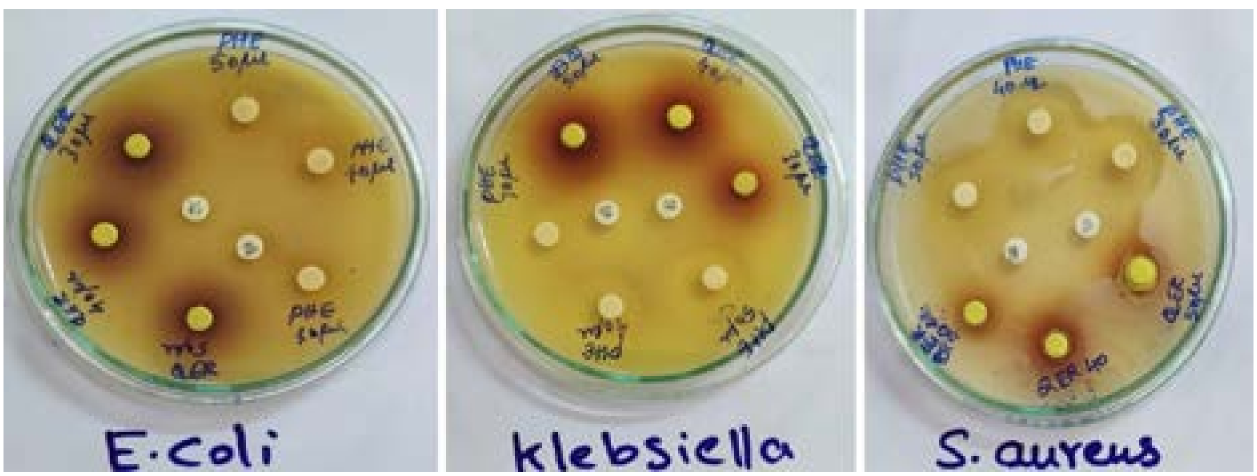

Fig. 1: Antibacterial activity of polyherbal formulation against E. coli, K. pneumonia and S. aureus (PHE-polyherbal extract, QER- quercetin, GEN-gentamicin, AMC- amoxicillin+clavulanate)

The polyherbal formulation prepared by using the ethanol as solvent inhibited both the Gram positive *(Staphylococcus aureus)*and Gram negative bacteria (*Escherichia coli, Klebsiella pneumonia*) by *in vitro* method indicating the broad spectrum antibacterial activity, as also reported by Masola *et al*. (2009). Inhibitory effect on both Gram positive and Gram negative bacteria has been reported while using ethanol, hexane and methanol as solvent for the extraction from plants (Joseph *et al*., 2011). The broad spectrum antibacterial activity could be due to the presence of polyphenolic compounds such as tannins and flavonoids. The antibacterial action of flavonoids is due to their ability to bind with extracellular and soluble protein and to complex with bacterial cell (Zongo *et al*., 2010). Gram-negative bacteria were more resistant to the plant-based organic extracts because the hydrophilic cell wall structure of Gram-negative bacterial cell is made up of a lipopolysaccharide that blocks the penetration of hydrophobic oil and prevents the accumulation of organic herbal extracts in target cell membrane (Djeussi *et al.*, 2013). Gram-positive bacteria should be more susceptible since they have only an outer peptidoglycans layer which is not an effective permeability barrier. But in contrary, the polyherbal formulations were effective against both Gram positive and Gram negative bacteria due to phytoconstituents present in them. In resazurin dye assay lowest MIC value was observed in gentamicin, when compared to polyherbal formulation and quercetin against *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Escherichia coli* (Table 3, Fig. 2). Polyherbal formulations had one fold low MIC value than gentamicin and one fold high MIC value than quercetin against *E.coli, S.aureus, K.pneumoniae*.

Table 3: MIC value of polyherbal extract, quercetin and gentamicin

Organism	Gentamicin µg/mL	PHE µg/mL	Quercetin µg/mL
E.coli	$41.6^{a} \pm 6.5$	$83.3^{a} \pm 13.1$	$166.6^{b} \pm 26.3$
S. aureus	$36.41^{a} \pm 5.1$	$72.91^{a} \pm 10.41$	$145.8^{b} \pm 20.8$
K. pneumonia	$31.25^{a} \pm 0.0$	$62.5^{a} \pm 0.0$	$145.8^{b} \pm 20.8$

Values are mean ± SEM (n=3). Means bearing different superscripts within the row differ significantly ($p<0.05$).

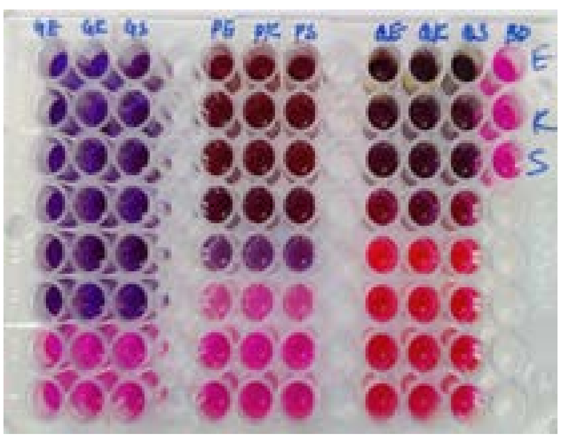

Fig. 2: Determination of MIC for gentamicin, polyherbal extract, quercetin against E. coli, S. aureus and K. pneumonia. Row 6 from well 1 to 3 confirms E. coli, K. pneumonia and S. aureus inhibited by gentamicin at 31.25 µg/mL. Row 5 from well 5 to 7 confirms E. coli, S. aureus and K. pneumonia inhibited by polyherbal extract at 62.5 µg/mL. Row 4 from well 9 to 11 confirms E. coli, K. pneumonia and S. aureus inhibited by quercetin at 125 µg/mL. Column 12 from well 1 to 3, negative control (E. coli, K. pneumonia, S. aureus with resazurin dye) showing change of colour from blue to pink due to growth of bacteria.

Further, antimicrobial property of polyherbal formulation could be due to quercetin, which was quantified by high performance thin layer chromatography. By plotting peak area against quercetin concentration, calibration plot was obtained. The co-efficient of variation for the calibrated polyherbal formulation samples was 4.99 % with correlation co-efficient of 99.47%, comparable with the standard quercetin. Using vision CATS software, the quercetin content of polyherbal formulation was enumerated to be 353 µg/100 mg of ethanolic extract (Fig. 3, 4). The antimicrobial activity due to quercetin was also reported by Baghel *et al.* (2017). Quercetin, a plant flavonoid, was shown to inhibit the growth of both Gram-positive and Gram-negative bacteria. The antibacterial action of quercetin could be due to cell membrane damage or alteration of membrane permeability, inhibition of nucleic acids and protein synthesis, mitochondrial dysfunction (Nguyen and Bhattacharya, 2022). Zone of inhibition was greater in polyherbal formulation than quercetin due to synergistic effects of multiple numbers of bioactive constituents

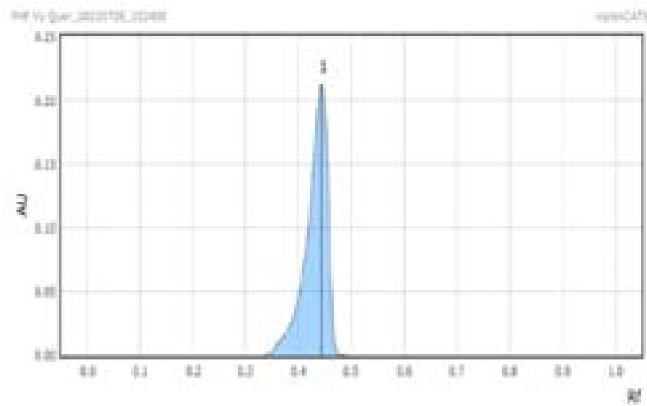

Fig. 3: HPTLC chromatogram PHF - Quercetin

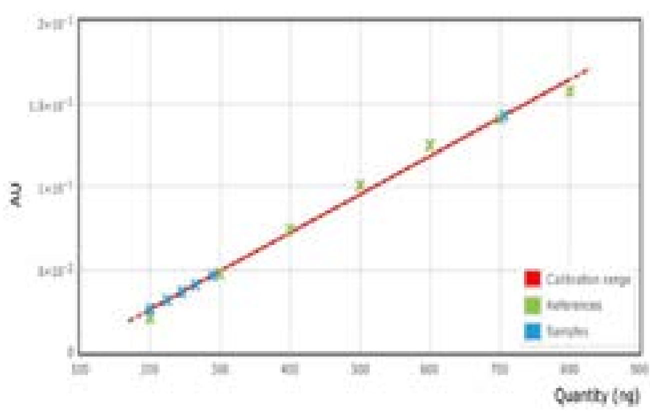

Fig. 4: Standard calibration curve - Quercetin quantification

Conclusion

The antibacterial effect of polyherbal formulation containing *Aerva lanata, Phyllanthus emblica, Cyperus rotundus* and *Boerhavia diffusa* is similar to standard gentamicin and amoxicillin-clavulanic acid which are commonly used for the treatment of mastitis at field condition. Hence, it can be concluded that after preclinical studies in animal model, this formulation can be used against mastitis, which will avoid the use of antibacterial and development of resistance.

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