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Evaluation of the antimicrobial efficacy of aerial root extract of *Ficus benghalensis* against mastitis-causing bacterial pathogens in bovine

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Abstract
This study aimed to evaluate the phytochemical constituents, antioxidant activity, biofilm inhibition potential, and antibacterial efficacy of the methanol extract of *Ficus benghalensis* aerial roots (FBARE), along with its synergistic antibacterial effects when combined with the synthetic antibiotics enrofloxacin and amoxicillin. The study targeted clinical isolates of *Escherichia coli* and *Staphylococcus aureus*, known pathogens responsible for bovine mastitis. Phytochemical analysis revealed the presence of alkaloids, carbohydrates, reducing sugars, saponins, triterpenes, phenols, flavonoids, proteins, and amino acids in the extract. The antioxidant activity of FBARE was found to be equivalent to 54.6±0.142 µg/mL of ascorbic acid per 0.1 mg/mL of extract. The antibacterial activity, demonstrated by a minimum inhibitory concentration (MIC) of 2.5 mg/mL, was effective against clinical isolates of both *E. coli* and *S. aureus*. FBARE exhibited an additive effect when combined with enrofloxacin and amoxicillin, enhancing their antibacterial efficacy. Additionally, FBARE significantly inhibited biofilm formation, with biofilm inhibition rates of 70.7±0.76% for *E. coli* and 70.14±0.57% for *S. aureus*. Overall, the additive antibacterial and significant increase in the percentage of anti-biofilm properties of FBARE may offer a promising strategy for combating clinical strains of mastitis-causing bacteria, potentially reducing the risk of resistance development.

Keywords: *Ficus benghalensis*, aerial roots, bovine mastitis, phytochemical, antimicrobial properties

Introduction
In recent years, antimicrobial resistance (AMR) has become a significant global health concern, emerging as a central issue in both human and veterinary medicine. The widespread availability and inappropriate use of antimicrobials have contributed to the accelerated development of AMR, posing a major threat to public health. As noted by Barbosa and Levy (2000) [1], antimicrobial resistance is an increasingly pressing issue, drawing the attention of policymakers who are now prioritizing long-term action plans to address this growing challenge.

Conventional antimicrobial agents are associated with the risk of developing acquired drug resistance. This poses a dual threat to both animal and human health due to the selection and spread of resistant strains (Marshall and Levy, 2011; Saini *et al*., 2012) [2, 3]. The evolution of antibiotic resistance in mastitis-causing pathogens, such as *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*), exemplifies this problem, with no long-lasting remedies available for effective control. The limited number of antimicrobial drug classes and the frequent occurrence of cross-resistance between and within these classes further complicate the situation. As a result, there is an urgent need for alternative strategies or more effective antimicrobial agents (Bayramov and Neff, 2017) [4].

One promising approach to combat the rise of bacterial resistance is the use of natural plant-based products. Many plants possess diverse pharmacological properties, and *Ficus benghalensis*, commonly known as the Indian Banyan Tree, is one such plant with remarkable medicinal properties. Ancient medicine systems such as Ayurveda extensively used this tree to treat a wide range of ailments (Papitha *et al*., 2017) [5].

Previous studies have reported the presence of several bioactive compounds in *Ficus benghalensis* (Verma *et al*., 2015) [6], including lupenyl acetate, α-amyrenyl acetate, γ-

sitosterol, palmitic acid, and lupeol. These compounds have demonstrated various pharmacological activities, such as antibacterial, antioxidant, anti-inflammatory, anti-diabetic, and anticancer properties. Among these, *Ficus benghalensis* extracts exhibited high antioxidant activity when compared to standard gallic acid through FRAP assay. Additionally, the synergistic effects of plant extracts in combination with antibiotics have been shown to enhance antimicrobial activity, with several studies documenting additive effects in such combinations (Adwan and Mhanna, 2008; Niculae *et al*., 2015; Mun *et al*., 2013) [7, 8, 9].

In light of these considerations, the present study aims to evaluate the methanol extract of the aerial prop roots of *Ficus benghalensis* for its antimicrobial activity against mastitis-causing organisms. The specific objectives include assessing the antibacterial activity of *Ficus benghalensis* against *Staphylococcus aureus* and *Escherichia coli*, evaluating the *in vitro* antibacterial efficacy, and determining the antibiofilm activity in combination with the synthetic antibiotics enrofloxacin and amoxicillin.

Materials and Methods

All the chemicals are of analytical grade and were procured from (Himedia and Merck) Mumbai, India.

Preparation of Extract

Aerial roots of *F. benghalensis* were collected from the Guntur region, Andhra Pradesh, authenticated by a botanist, shade-dried, and powdered. Twenty grams of the powder was soaked in methanol (1:10 ratio) and stirred overnight at room temperature. The filtrate was lyophilized to a constant weight. The extract (FBARE) was mixed with DMSO to prepare a 500 mg/mL stock solution, which was diluted with 1% DMSO to obtain a 10 mg/mL working solution.

Test Bacteria and Culture Media

Freeze-dried cultures of *E. coli* and *S. aureus* were revived using tryptic soya broth, streaked onto eosin methylene blue (EMB) and mannitol salt agar (MSA) respectively, and incubated. Clinical isolates from milk samples were processed via centrifugation and cultured in brain heart infusion broth (BHI) at 37 °C (Cruickshank *et al*., 1975) [10]. Specific media (EMB for *E. coli* and MSA for *S. aureus*) were used to study the isolates, and PCR confirmed their identity.

Qualitative Phytochemical Analysis

FBARE was tested for alkaloids, carbohydrates, reducing sugars, saponins, triterpenes, phenols, flavonoids, proteins, and amino acids using standard methods (Swargiary *et al*., 2016) [11].

Total Antioxidant Activity

The total antioxidant capacity of the sample was evaluated by phospho-molybdenum method (Prieto *et al*., 1999) [12]. Serial six concentrations of standards of ascorbic acid ranging from 10-60 µg/ml were made in distilled water. An aliquot of 0.2 ml of the sample solution was mixed with 2 ml of the phosphomolybdate reagent (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). For the blank, 0.2 ml of distilled water was mixed with 2 ml of the reagent. The test tubes were incubated in a water bath at 95°C for 90 min. After incubation, the test tubes were cooled and the absorbance was measured at 695 nm for the extinction coefficient for vitamin C reduced phosphomolybdenum complex from six serial concentrations of ascorbic acid versus absorbance by using the line of the best-fit method. Antioxidant activity of FBARE expressed as equivalent to Ascorbic acid that formed the green phosphomolybdenum complex.

Antibacterial Activity Assay

Preparation of Cation-Adjusted Muller-Hinton Broth (CAMHB)

CAMHB was prepared, autoclaved, and cooled. Magnesium (0.2 ml of $MgCl_2$ solution) and calcium ions (0.4 ml of $CaCl_2$ solution) were added to the chilled broth to achieve final concentrations of 10 mg/L and 20 mg/L, respectively, with a pH of 7.2-7.4 (CLSI, 2012) [13].

Estimation of MIC by Broth Dilution Method

The minimum inhibitory concentration (MIC) was evaluated in a 96-well microplate (CLSI, 2006) [14]. Serial dilutions of antibiotics and FBARE were prepared in CAMHB. Bacterial cultures (0.5 McFarland units) were added to each well, and positive and negative controls were maintained. Plates were incubated at 37 °C for 18 hours, and microbial growth was determined using iodonitrotetrazoline chloride (INT). MIC was defined as the lowest concentration showing no color change, indicating bacterial inhibition.

Biofilm Inhibition Assay

FBARE, amoxicillin, and enrofloxacin (at MIC concentrations) were tested for biofilm inhibition (Bazargani and Rohloff, 2016) [15]. Cultures were incubated with test compounds in a microtiter plate, washed, stained with crystal violet, and de-stained with ethanol. Absorbance at 590 nm was measured, and percentage inhibition was calculated against controls.

Antibacterial Activity in Combination with Antibiotics

The microdilution checkerboard method (Schelz *et al*., 2006; Orhan *et al*., 2005) [16, 17] was used to evaluate interactions between FBARE and antibiotics. Serial dilutions of FBARE, enrofloxacin, and amoxicillin were prepared, and bacterial cultures were added. Fractional inhibitory concentration (FIC) indices were calculated to determine synergistic, additive, indifferent, or antagonistic effects. (fig.1)

Statistical Analysis

Statistical analysis was performed using SPSS 19.0. One-way ANOVA with Duncan's post-hoc test was used to compare biofilm inhibition, represented as Mean ± SEM. A value of $P < 0.05$ was considered significant.

Results

The present study evaluated the FBARE for the qualitative phytochemicals analysis, *in-vitro* antioxidant activity, *in-vitro* antimicrobial activity, and antibiofilm activity against mastitis-causing bacteria *Staphylococcus aureus* and *Escherichia coli*, clinical isolates cultures and also evaluated *in-vitro* antimicrobial activity interaction with synthetic antibiotics enrofloxacin and amoxycillin respectively.

Phytochemical analysis: The phytochemical analysis revealed the presence of various secondary metabolites including alkaloids, reducing sugars, saponins, triterpene, phenols, flavonoids, and proteins (Table 1).

Antioxidant activity

The antioxidant activity of FBARE was expressed as

antioxidant activity equivalent to µg/ml of ascorbic acid. A standard curve was plotted with OD values at 695 nm obtained against six serial concentrations of ascorbic acid that reduced phosphomolybdenum complex and by using the line of the best-fit method extinction coefficient for ascorbic acid was calculated. (fig.2) Antioxidant activity of FBARE expressed as equivalent of Ascorbic acid that formed the green phosphomolybdenum complex.

The estimated antioxidant activity of 0.1 mg/ml of FBARE and enrofloxacin was 54.6±70.146 µg/ml and 33.60±0.31 µg/ml of ascorbic acid respectively (table 1).

Antibacterial activity of FBARE against *Escherichia coli*

E. coli growth inhibition was observed at a concentration of 2.5 mg/ml for FBARE and in combination with enrofloxacin, the FBARE inhibited the growth at a concentration of 1.25 mg/ml (fig.3) (table 2).

Antibacterial activity of FBARE against *Staphylococcus aureus*

S. aureus growth inhibition was observed at a concentration of 2.5 mg/ml for FBARE and in combination with amoxycillin inhibited the growth at 0.625 mg/ml concentration. (fig.4) (table 2).

Biofilm Inhibition Potential Against *Escherichia coli*

The biofilm inhibition potential of enrofloxacin and FBARE against *E. coli* was evaluated using the crystal violet assay (Table 2). FBARE inhibited the 70.78±0.76% of biofilm formation at 2.5 mg/ml concentration; whereas in combination with enrofloxacin 83.5±1.2% biofilm formation was inhibited at 2.5 mg/ml of FBARE and 0.03 µg/ml concentration of enrofloxacin. Enrofloxacin alone inhibited 80.8±0.64% of biofilm formation at 0.03 µg/ml concentration (Fig 5).

Biofilm inhibition potential against *Staphylococcus aureus*

The biofilm inhibition potential of amoxycillin and FBARE was evaluated against *S. aureus* using the crystal violet assay (Table 2). FBARE inhibited the 70.14±0.57% of biofilm formation at 2.5 mg/ml concentration; whereas in combination with amoxycillin 73.2±1.02% biofilm formation was inhibited at 2.5 mg/ml of FBARE and 0.3 µg/ml concentration of amoxycillin. Amoxycillin alone inhibited 59.8±1.73% of biofilm formation at 0.03 µg/ml concentration (Fig 6).

Antibacterial activity interaction of FBARE with synthetic antibiotics

Antibacterial activity interaction of FBARE with antibiotics enrofloxacin and amoxicillin against *E. coli* and *Staphylococcus aureus* respectively by micro-dilution checker board method was evaluated for combined action and type of interaction by estimating fractional inhibitory concentration index.

FBARE in combination with Enrofloxacin

The FIC index of FBARE in combination with enrofloxacin was 1 which indicates the additive antibacterial activity of Enrofloxacin in combination with FBARE that is, the combined action is equal to the sum of the individual effects (fig 7) (Table 2).

FBARE in combination with amoxycillin

The FIC index of FBARE in combination with amoxycillin was 1 which indicates the additive antibacterial activity of Amoxycillin in combination with FBARE that is, the combined action is equal to the sum of the individual effects (fig 8) (Table 2).

Discussion

The current study explored the antimicrobial and antioxidant properties of *Ficus benghalensis* aerial root extract (FBARE) and evaluated its antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* clinical isolates. Additionally, we examined the interaction between FBARE and synthetic antibiotics such as enrofloxacin and amoxycillin, as well as the biofilm inhibition potential of FBARE.

Phytochemical screening of FBARE revealed secondary metabolites including alkaloids, reducing sugars, saponins, triterpenes, phenols, flavonoids, and proteins, aligning with findings by Tuse *et al*. (2011) [18]. These bioactive compounds are known to contribute significantly to the plant's medicinal properties. The antioxidant activity of FBARE, measured at 54.67 µg/ml ascorbic acid equivalent, supports previous studies by Verma *et al*. (2015) [6], which reported the presence of bioactive compounds such as quinic acid, palmitic acid, and ergosterol acetate responsible for antioxidant effects. Similarly, Rajkumar *et al*. (2011) [19] found methanolic extracts of *Ficus benghalensis* to exhibit high antioxidant and antiradical activities.

The antibacterial efficacy of FBARE was demonstrated against the clinical isolates of *E. coli* and *S. aureus*. The MIC of FBARE was 2.5 mg/mL, consistent with the results from Verma *et al*. (2015) [6], who found *Ficus benghalensis* to be effective against gram-positive and gram-negative bacteria. The antimicrobial activity can be attributed to phenols and flavonoids, which disrupt bacterial cytoplasmic membranes and interact with proteins and microbial enzymes (Maria *et al*., 2018) [20]. These phytochemicals may enhance the permeability of bacterial membranes, potentiating the antibacterial effects.

The biofilm inhibition potential of FBARE was evaluated using crystal violet staining, with significant inhibition observed against *E. coli* and *S. aureus*. In combination with enrofloxacin, FBARE exhibited significantly enhanced biofilm inhibition, particularly against *E. coli*, demonstrating 83.5±1.2% inhibition at MIC values. FBARE alone inhibited biofilm formation by 70.78±0.76%, while enrofloxacin alone showed 80.8±0.64% inhibition. The results align with studies by Awolola *et al*. (2017) [21], who reported potent anti-adhesion potential in methanol extracts of *Ficus* species.

Against *S. aureus*, FBARE exhibited 70.14±0.57% biofilm inhibition, which was further enhanced when combined with amoxicillin to 73.2±1.02%. Flavonoids in FBARE may act on the enzyme sortase, crucial for anchoring protein virulence factors in Gram-positive bacteria, thereby suppressing biofilm formation (Maria *et al*., 2018) [20]. These findings suggest that FBARE could serve as a complementary agent in combination therapy to combat biofilm-related infections in veterinary and clinical settings.

Our study is the first to evaluate the interaction of FBARE with synthetic antibiotics using the microdilution checkerboard method. The combination of FBARE with enrofloxacin against *E. coli* demonstrated an additive effect (FIC index of 1), indicating that the combined antibacterial action is equal to the sum of individual effects. Enrofloxacin inhibits bacterial DNA synthesis, while flavonoids and phenols in FBARE disrupt bacterial membranes, together providing a complementary mechanism of action (Verma *et*

al., 2015) [6].

Similarly, FBARE exhibited additive antibacterial activity when combined with amoxicillin against *S. aureus*. Amoxicillin targets bacterial cell wall synthesis, while FBARE's phytochemicals further enhance antibacterial effects by acting on bacterial proteins and membranes, aligning with previous reports of *Ficus benghalensis*' efficacy (Verma *et al*., 2015) [6].

Table 1: Phytochemical analysis of *Ficus benghalensis* aerial root extract

Phytochemical screened	Reagents/Chemicals	Observation	Results
Phenols	Ferric chloride	Bluish black	+
Flavonoids	Lead acetate	Yellow colour precipitate	+
Reducing sugar	Benedicts	Orange-red precipitate	+
Saponins	Distilled water	Foam formation	+
Triterpenes	Salkowski	Golden Yellow	+
Proteins	Xanthoproteic	Yellow colour	+
Carbohydrates	Molisch	No Violet ring was observed at the junction	-
Alkaloids	Dragendroff	Red precipitate	+
The antioxidant activity of 0.1 mg/ml of FBARE is equivalent to	Phosphomolybdate reagent	----	54.67±0.146 µg/ml of ascorbic acid

Table 2: Antimicrobial properties of FBARE against clinical isolates of *E. coli* and *S. aureus*

Antimicrobial properties		***E. coli***	***S.aureus***
Minimum Inhibitory concentration (MIC)	*Ficus benghalensis* aerial root extract Extract (FBARE)	2.5 mg/ml	2.5 mg/ml
	Enrofloxacin	0.03 µg/ml	
	Amoxicillin		0.3125 µg/ml
MIC in combination with Enrofloxacin	FBARE	1.25 mg/ml	
	Enrofloxacin	0.0195 µg/ml	
	FIC	1	
	Type of Interaction	Additive	
MIC in combination with Amoxicillin (units)	FBARE		1.25 mg/ml
	Amoxicillin		0.625 µg/ml
	FIC		1
	Type of Interaction		Additive
Percentage inhibition of bio-film.		***E. coli***	***S.aureus***
Bio-film inhibition	*Ficus benghalensis* aerial root extract Extract (FBARE) Concentration (% inhibition)	2.5 mg/ml (70.78±0.76%)	2.5 mg/ml (70.14±0.57%)
	Enrofloxacin Concentration (% inhibition)	0.03 µg/ml (80.8±0.64%)	
	Amoxicillin Concentration (% inhibition)		0.3 µg/ml (59.8±1.73%)
Bio-film inhibition in combination	FBARE + Enrofloxacin Concentration (% inhibition)	2.5 mg/ml + 0.03 µg/ml (83.5±1.2%)	
	FBARE + Amoxicillin Concentration (% inhibition)		2.5 mg/ml + 0.3 µg/ml (73.2±1.02%)

Values are mean ± SEM; oneway ANOVA (SPSS).

Means with different alphabets as superscripts differ significantly ($p<0.05$).

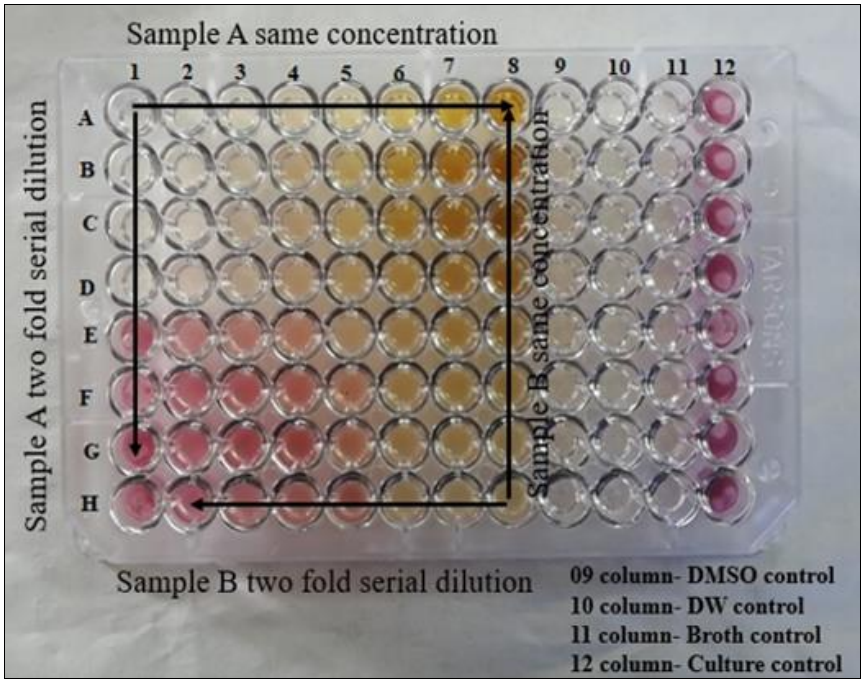


Fig 1: Microtiter plate showing combinations of two test samples adding dilutions and directions in various wells

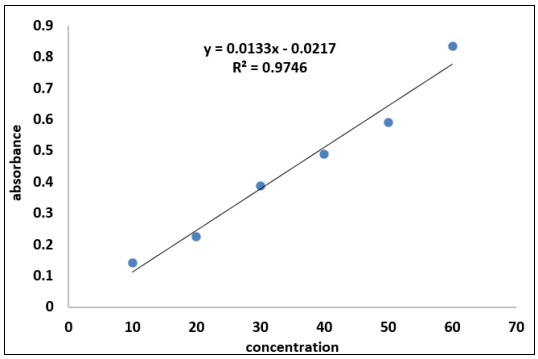


Fig 2: Antioxidant activity of test compounds by Phosphomolybdenum assay

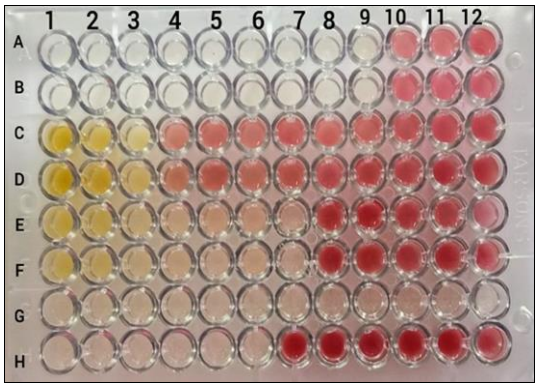


Fig 3: Microtiter plate showing inhibition of *Escherichia coli* growth on a serial dilution of *Ficus benghalensis* aerial root extract (FBARE) and Enrofloxacin
ROW A and B: 100 µl of Broth+ 100 µl of Enrofloxacin serial dilution from 10 µg/mL to 0.0048 µg/mL + Culture
ROW C and D: 100 µl of Broth+ 100 µl of FBARE Serial dilution from 10 mg/mL to 0.0048 mg/mL+ Culture
ROW E and F: 100 µl of Broth+ combination of 50 µl of Enrofloxacin from 10 µg/mL to 0.0048 µg/mL and 50 µl of FBARE from 10 mg/mL to 0.0048 mg/mL + Culture
ROW G: 1 to 6 – Broth+ 0.1 N NaOH Control; 7 to 12 – Broth+ 1% DMSO Control
ROW H: 1 to 6 – Broth Control; 7 to 12 – Broth+ Culture

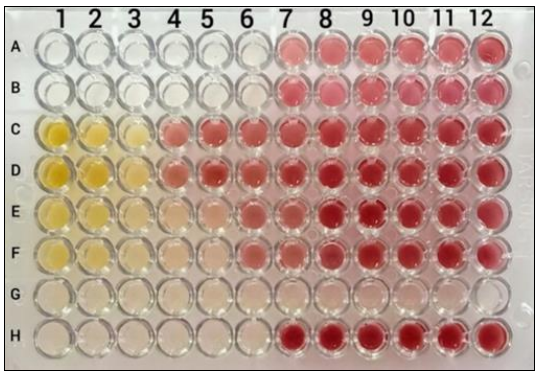
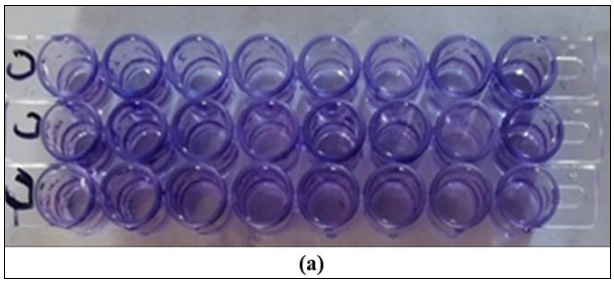


Fig 4: Microtiter plate showing inhibition of *Staphylococcus aureus* growth on a serial dilution of *Ficus benghalensis* aerial root extract and Amoxycillin
ROW A and B: 100 µl of Broth+100 µlof Amoxycillin serial dilution from 10 µg/mL to 0.0048 µg/mL + Culture
ROW C and D: 100 µl of Broth+ 100 µl of FBARE serial dilution from 10 mg/mL to 0.0048 mg/mL+ Culture
ROW E and F: 100 µl of Broth+ combination of 50 µl Amoxycillin from 10 µg/mL to 0.0048 µg/mL and 50 µl FBARE from 10 mg/mL to 0.0048 mg/mL + Culture
ROW G: 1 to 6 – Broth+ Distilled water Control; 7 to 12 – Broth+ 1% DMSO Control
ROW H: 1 to 6 – Broth Control; 7 to 12 – Broth+ Culture



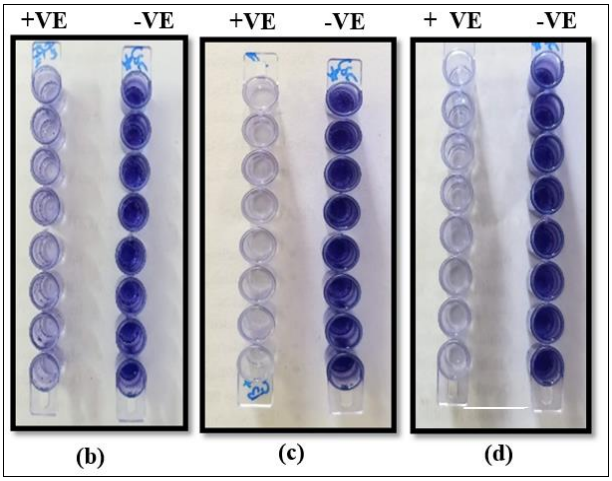
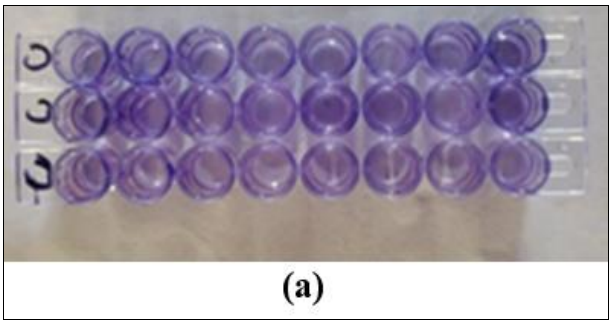


Fig 5: (a) biofilm formation by *E. coli*
(b**)** Inhibition of biofilm by *Ficus* (+ve) produced by *E. coli* (-ve)
(c) Inhibition of biofilm by enrofloxacin(+ve) produced by *E. coli* (-ve)
(d) Inhibition of biofilm by *Ficus* + enrofloxacin (+ve) produced by *E. coli* (-ve)



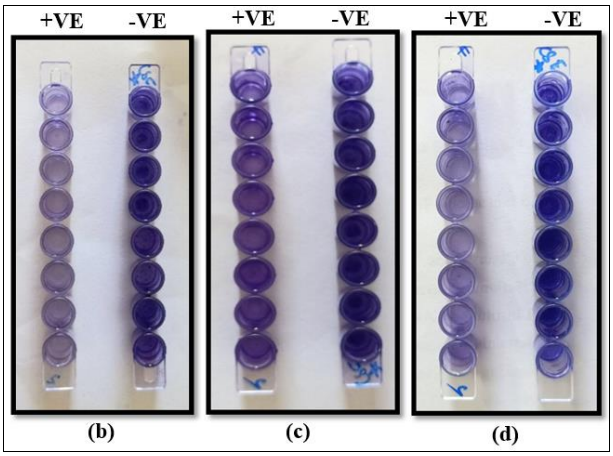


Fig 6: (a) Biofilm formation by *Staphylococcus aureus*
(b) Inhibition of biofilm by *Ficus* (+ve) produced by *S.aureus*(-ve)
(c) Inhibition of biofilm by *amoxicillin(+ve)* produced by *S.aureus*(-ve)
(d) Inhibition of biofilm by *Ficus* + *amoxicillin(+ve)* produced by *S. aureus* (-ve)

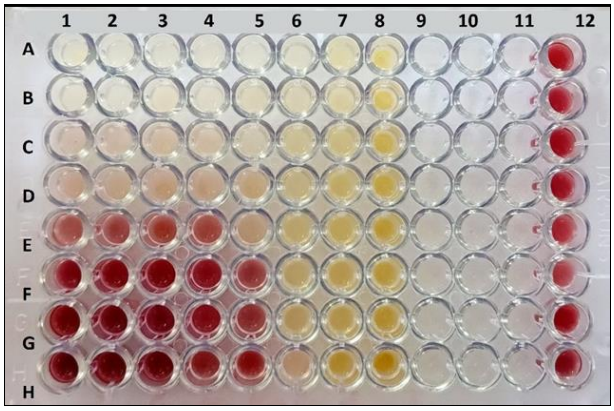


Fig 7: Microtiter plate showing microdilution checkerboard for Enrofloxacin and FBARE for combined antibacterial activity
A wells of columns 1-8 contain 50µl of serially diluted Enrofloxacin starting from 0.3125 µg/mL (A well) to 0.0048 µg/mL (G well).
8-2 wells of rows H to A contain 50 µl of serially diluted *Ficus benghalensis* aerial root extract (FBARE) starting from 20 mg/mL (8 well) to 0.3125 mg/mL (2 well)

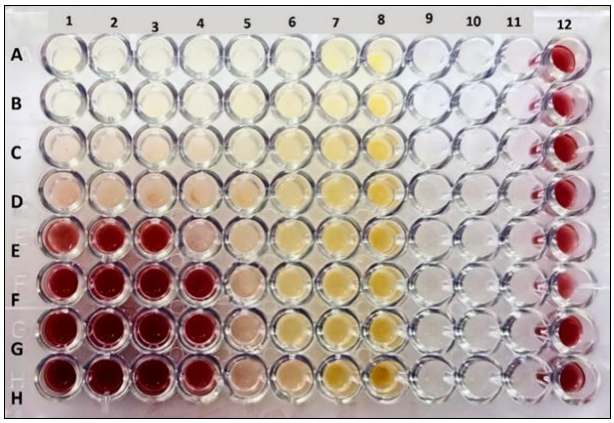


Fig 8: Microtiter plate showing microdilution checkerboard for Amoxycillin and *Ficus benghalensis* aerial root extract for combined antibacterial activity
A-G wells of columns 1-8 contain 50 µl of serially diluted Amoxycillin starting from 5 µg/mL (A well) to 0.078 µg/mL (G well).
8-2 wells of rows H to A contain 50 µl of serially diluted FBARE starting from 20 mg/mL (8 well) to 0.3125 mg/mL (2 well)

Conclusion

The aerial roots of *Ficus benghalensis* treasure various phytochemicals which are excellent sources to exhibit various important pharmacological activities. These active metabolites in the Methanol extract of tender prop roots exhibited significant antibacterial activity against both gram-positive and gram-negative bacteria causing bovine mastitis also exhibit antibiofilm activity. In combination with conventional antibiotics, enrofloxacin, and amoxycillin, inhibited *in-vitro* bacterial growth at reduced concentrations with additive antibacterial activity and also a significant increase in the percentage of biofilm formation by the above organisms. Further studies are required to separate various phytochemicals and establish the exact antimicrobial constituent.

Conflict of Interest

Not available.

Financial Support

Not available.

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