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Divyasri T

Department of Veterinary
Pharmacology and Toxicology,
NTR College of Veterinary Science
Sri Venkateswara Veterinary
University, Gannavaram,
Andhra Pradesh, India

Bharavi K

Department of Veterinary
Pharmacology and Toxicology,
College of Veterinary Science, Sri
Venkateswara Veterinary University,
Tirupati, Andhra Pradesh, India

Srividya G

Department of Veterinary
Pharmacology and Toxicology, NTR
College of Veterinary Science, Sri
Venkateswara Veterinary University,
Gannavaram, Andhra Pradesh, India

Srinivasa Rao G

Department of Veterinary
Pharmacology and Toxicology, NTR
College of Veterinary Science
Sri Venkateswara Veterinary
University, Gannavaram, Andhra
Pradesh, India

Aswani Kumar K

Department of Veterinary
Biochemistry, NTR College of
Veterinary Science, Sri Venkateswara
Veterinary University, Gannavaram,
Andhra Pradesh, India

Corresponding Author:

Bharavi K

Department of Veterinary
Pharmacology and Toxicology,
College of Veterinary Science, Sri
Venkateswara Veterinary University,
Tirupati, Andhra Pradesh, India

Antibacterial activity of acetone extract of *Boswellia ovalifoliolata* (Indian Frankincense) resin and its synergistic effects with selected phytochemicals against mastitis-causing pathogens

Divyasri T, Bharavi K, Srividya G, Srinivasa Rao G and Aswani Kumar K

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Abstract

Mastitis significantly impacts the dairy industry and public health, with increasing bacterial resistance to antibiotics necessitating alternative antimicrobial agents. This study evaluates the antibacterial and biofilm inhibition potential of acetone extract of *Boswellia ovalifoliolata* resin (BORE), its synergistic effects with antibiotics (enrofloxacin, amoxicillin) and phytochemicals (eugenol, thymol, carvacrol) against *Escherichia coli* and *Staphylococcus aureus*.

Phytochemical analysis revealed the presence of carbohydrates, saponins, triterpenes, phenols, and flavonoids. Antioxidant activity of 0.1 mg/mL BORE was equivalent to 63.1 ± 0.89 $\mu\text{g/mL}$ of ascorbic acid. MIC values of BORE against *E. coli* and *S. aureus* were 2.5 mg/mL and 0.3125 mg/mL, respectively. Combined antibacterial action with antibiotics and phytochemicals showed additive effects. BORE also significantly reduced biofilm formation, with enhanced inhibition in combination with other agents.

In conclusion, BORE demonstrated potent antimicrobial, antioxidant, and biofilm inhibition properties, highlighting its potential as a natural adjunct in managing bovine mastitis.

Keywords: *Boswellia ovalifoliolata* resin, antimicrobial activity, mastitis, phytochemicals

Introduction

The overuse and suboptimal application of antimicrobials in animal husbandry have been significant drivers of Antimicrobial Resistance (AMR), particularly in the dairy industry [1]. While antibiotics have substantially improved livestock health and productivity, they have also contributed to the emergence of resistant bacterial strains, complicating mastitis treatment [2, 3]. The indiscriminate use of antibiotics has led to high treatment costs, drug residues in milk, and the rapid spread of resistance [4].

Despite the growing prevalence of AMR, the pace of new antibiotic development has slowed, leading to an urgent need for alternative strategies [5]. One promising approach involves combining antibiotics with other bioactive compounds to restore antibacterial activity and combat resistance [6, 7]. Phytochemicals, derived from medicinal plants, have shown potent antimicrobial activity and the ability to enhance antibiotic efficacy while reducing adverse effects [8, 9]. Medicinal plants have historically been used to treat livestock diseases, with many demonstrating activity against bacterial efflux pumps, quorum sensing, and biofilm formation [10, 11]. Secondary metabolites such as tannins, terpenes, quinones, alkaloids, phenols, and flavonoids are key contributors to these antimicrobial effects [12]. Some plant-derived compounds act in synergy with antibiotics or sensitize pathogens to previously ineffective antibiotics [13, 14]. *Boswellia ovalifoliolata* resin, traditionally used to treat ailments such as ulcers, rheumatic pain, and dysentery, possesses significant antibacterial activity and immunostimulant properties [15]. Given its potential, this study evaluates the acetone extract of *Boswellia ovalifoliolata* resin for its antibacterial activity against mastitis-causing pathogens (*Staphylococcus aureus* and *Escherichia coli*) and its *in vitro* combined antibacterial effects with selected phytochemicals.

Materials and Methods

This study aimed to evaluate the antibacterial activity and biofilm inhibition potential of acetone extract of *Boswellia ovalifoliolata* resin (BORE), its synergistic effects with antibiotics (enrofloxacin and amoxicillin), and phytochemicals (eugenol, thymol, and carvacrol) against mastitis-causing pathogens (*Escherichia coli* and *Staphylococcus aureus*).

All the drugs and chemicals used in the experiment are analytical grade and were obtained from standard and authorized manufacturers.

The resin was collected from Tirupati, Andhra Pradesh, and authenticated by a botanist (Dr. K. Madhava Chetty, Sri Venkateswara University). Twenty grams of powdered resin was extracted with acetone in a 1:10 ratio. The mixture was stirred overnight, filtered, and lyophilized until constant weight was achieved. Stock solutions of BORE (500 mg/mL), antibiotics, and phytochemicals were prepared. BORE was diluted to a working concentration of 10 mg/mL, and antibiotics and phytochemicals were diluted to appropriate concentrations for antibacterial testing.

Phytochemical analysis of BORE was conducted using standard methods [16]. Tests included Dragendorff's test for alkaloids, Molisch's and Benedict's tests for carbohydrates and reducing sugars, foam test for saponins, Salkowski's test for triterpenes, ferric chloride test for phenols, lead acetate test for flavonoids, and Xanthoproteic and Ninhydrin tests for proteins and amino acids.

Antioxidant activity was assessed using the phosphomolybdenum assay [17]. Reaction mixtures included varying concentrations of BORE (0.1–0.6 mg/mL) and standard ascorbic acid (10–60 µg/mL). Samples were incubated with phosphomolybdate reagent at 95°C for 90 minutes, and absorbance was measured at 695 nm. Antioxidant potential was expressed as ascorbic acid equivalents using the extinction coefficient calculated for vitamin C.

Antibacterial activity was evaluated against clinical isolates of *E. coli* and *S. aureus*. Cultures were revived on selective media (Eosin Methylene Blue agar for *E. coli* and Mannitol Salt Agar for *S. aureus*). Isolated colonies were confirmed via PCR (NTR College of Veterinary Science). MIC was determined using the broth microdilution method [18]. Serial dilutions of BORE (10 to 0.0048 mg/mL), antibiotics (5 to 0.0024 µg/mL), and phytochemicals (25 to 0.012 mg/mL) were prepared in cation-adjusted Mueller-Hinton broth in 1st well to 12th well of micro titer plate. Each well received 10 µL of bacterial suspension (equivalent to 0.5 McFarland turbidity), and plates were incubated at 37°C for 18 hours. Growth was assessed by adding iodonitrotetrazolium chloride (INT) as an indicator. MIC was defined as the lowest concentration showing no visible growth.

Antibacterial activity in combinations was assessed using the checkerboard method [19, 20] (Fig 1). Serial two-fold dilutions of BORE and antibiotics/phytochemicals were combined in a microtiter plate. The first agent (*Boswellia ovalifoliolata* resin extract) of the combination in serially diluted concentrations was added along the abscissa (A to G wells of 1 to 8 columns added serially diluted concentration starting from the highest concentration in A well to lowest concentration in G well), while the second agent (enrofloxacin/amoxicillin or phytochemicals) in serially diluted concentration was added along the ordinate (concentration starting from the lowest in 2nd well to highest in 8th well of entire rows H to A). The resulting checkerboard contains a combination of two

compounds with the highest concentration of two compounds at opposite corners (A1 and H8). 10 µL of prepared bacterial culture suspension with turbidity equivalent of 0.5 McFarland units was added to all the wells of microtiter trays. Bacterial growth was evaluated as described above. Fractional inhibitory concentration (FIC) indices were calculated to classify interactions as synergistic (<0.5), additive (0.5–1.0), indifferent (1.0–4.0), or antagonistic (>4.0).

Biofilm inhibition was evaluated using a crystal violet assay [21]. Bacterial cultures were incubated with BORE, antibiotics, and phytochemicals at MIC concentrations. After incubation, wells were stained with crystal violet, destained with ethanol, and absorbance at 590 nm was recorded. Percent biofilm inhibition was calculated relative to untreated controls. Statistical analyses were performed using SPSS (IBM SPSS 19.0). One-way ANOVA followed by Duncan's post-hoc test compared biofilm inhibition percentages, expressed as Mean±SEM. A P-value <0.05 was considered statistically significant.

Results

The present study evaluated the acetone extract of *Boswellia ovalifoliolata* resin (BORE) for its phytochemical content, antioxidant activity, and antibacterial properties against mastitis-causing organisms (*Escherichia coli* and *Staphylococcus aureus*), including its synergy with antibiotics and phytochemicals.

- **Phytochemical Screening:** Qualitative analysis revealed that BORE contains alkaloids, carbohydrates, reducing sugars, saponins, triterpenes, phenols, flavonoids, and proteins, as confirmed by standard phytochemical tests (Table 1).
- **Antioxidant Activity:** The antioxidant potential of BORE (0.1 mg/mL) and other phytochemicals were evaluated using the phosphomolybdenum assay and expressed as ascorbic acid equivalents. BORE exhibited an antioxidant activity equivalent to 63.1±0.89 µg/mL of ascorbic acid, compared to 74.8±0.56, 53.8±0.32, and 106±1.21 µg/mL of ascorbic acid for eugenol, thymol, and carvacrol, respectively (Table 2).

Antibacterial Activity

- **Against *Escherichia coli*:** The MIC of BORE was 2.5 mg/mL, while eugenol, thymol, and carvacrol exhibited MICs of 1.562 mg/mL each. Enrofloxacin, tested as a control, showed an MIC of 0.078 µg/mL (Table 3) (Figure 2).
- **Against *Staphylococcus aureus*:** The MIC of BORE was 0.312 mg/mL, and carvacrol showed the lowest MIC (0.390 mg/mL) among the phytochemicals. Amoxicillin, the control antibiotic, displayed an MIC of 0.312 µg/mL (Tables 3) (Figure 3).
- **Antibacterial activity in combination:** Combined antibacterial activity was assessed using the checkerboard method. FIC indices indicated additive effects for BORE combined with enrofloxacin, amoxicillin, and phytochemicals (eugenol, thymol, and carvacrol) against both *E. coli* and *S. aureus*. The type of interaction is detailed in Tables 4 and 5.

Biofilm Inhibition

BORE, antibiotics, and phytochemicals were evaluated for biofilm inhibition using the crystal violet assay. For *E. coli*, the highest inhibition (77.8±0.61%) was observed with enrofloxacin, while combinations of enrofloxacin with BORE

achieved $71.8 \pm 0.28\%$ inhibition. BORE alone showed no significant inhibition (Table 6).

For *S. aureus*, BORE and carvacrol exhibited significant biofilm inhibition ($79.4 \pm 0.77\%$ and $75.4 \pm 1.68\%$, respectively). The combination of BORE with carvacrol produced the highest inhibition ($80.5 \pm 0.71\%$), while amoxicillin alone achieved $59.8 \pm 1.73\%$ (Tables 6).

Discussion

The indiscriminate use of antibiotics has led to widespread antimicrobial resistance (AMR), creating a global health crisis [4]. In dairy farming, the overuse of antibiotics as prophylactics and growth promoters has significantly contributed to the evolution of resistant bacterial strains, particularly those causing mastitis [2, 3]. The challenge is exacerbated by the slow development of new antimicrobial agents and the rapid emergence of resistance to newly introduced antibiotics [5, 6].

Phytochemicals offer a promising alternative due to their antimicrobial, antioxidant, and immunomodulatory properties [22, 23]. These natural compounds can also synergize with conventional antibiotics, enhancing their efficacy against resistant pathogens [24]. This study focused on the acetone extract of *Boswellia ovalifoliolata* resin, which contains bioactive compounds such as triterpenes, phenols, and flavonoids [16], evaluating its antibacterial and biofilm inhibition properties against *Escherichia coli* and

Staphylococcus aureus. The extract exhibited antioxidant activity equivalent to $63.1 \mu\text{g/mL}$ of ascorbic acid, consistent with reports attributing this activity to phenolic content [25]. It demonstrated minimum inhibitory concentrations (MICs) of 2.5 mg/mL against *E. coli* and 0.625 mg/mL against *S. aureus*, confirming its antibacterial potential [15, 26]. Phytochemicals such as eugenol, thymol, and carvacrol also displayed notable activity, with MICs of 1.562 mg/mL , 1.562 mg/mL , and 0.781 mg/mL , respectively, in line with previous findings [27, 28].

The combination of *Boswellia ovalifoliolata* extract with antibiotics and phytochemicals showed additive antibacterial effects. For instance, enrofloxacin combined with the resin extract enhanced antibacterial activity against *E. coli*, attributed to the disruption of bacterial membranes and DNA synthesis inhibition [29, 26]. Similarly, combinations with amoxicillin, eugenol, thymol, and carvacrol displayed synergistic or additive effects against *S. aureus* [30, 31].

While the extract lacked biofilm inhibition against *E. coli*, it demonstrated significant inhibition against *S. aureus*, both alone and in combination with carvacrol, reflecting its potential to disrupt biofilm formation by interfering with bacterial cell walls and membranes [32, 33]. These findings highlight the therapeutic potential of *Boswellia ovalifoliolata* resin as an adjunct to antibiotics in managing resistant bacterial infections and mastitis in livestock.

Table 1: Phytochemical analysis of *Boswellia ovalifoliolata* resin extract

Constituents	Results
Alkaloids (Dragendroff's test)	+
Carbohydrates (Molisch test)	+
Reducing sugars (Benedict's test)	+
Saponins (Foam test)	+
Triterpenes (Salkowski test)	+
Phenols (Ferric chloride test)	+
Flavonoids (Lead acetate test)	+
Proteins (Xanthoproteic test)	+
Amino acids (Ninhydrin test)	-

Table 2: Antioxidant activity by Phosphomolybdenum assay

Test compounds	Concentration	Antioxidant activity equivalent to ascorbic acid ($\mu\text{g/mL}$)
<i>Boswellia ovalifoliolata</i> resin extract	0.1 mg/mL	63.1 ± 0.89
Enrofloxacin	0.1 mg/mL	33.605 ± 0.31
Eugenol	0.1 mg/mL	74.80 ± 0.56
Thymol	0.1 mg/mL	53.80 ± 0.32
Carvacrol	10 mg/mL	106 ± 1.21

Table 3: MIC values of *Boswellia ovalifoliolata* resin extract along with various phytochemicals and enrofloxacin/amoxicillin against clinical isolate cultures of *Escherichia coli* and *Staphylococcus aureus*

Test compound	Minimum Inhibitory Concentration of clinical isolates	
	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>
Enrofloxacin ($\mu\text{g/mL}$)	0.078	--
Amoxicillin ($\mu\text{g/mL}$)	--	0.312
<i>Boswellia ovalifoliolata</i> resin extract (mg/mL)	2.5	0.312
Eugenol (mg/mL)	1.562	3.125
Thymol (mg/mL)	1.562	1.562
Carvacrol (mg/mL)	1.562	0.390

Table 4: Fractional inhibitory concentration and type of interaction between the enrofloxacin, *Boswellia ovalifoliolata* resin extract, and various phytochemicals against *Escherichia coli*

Test compound	MIC alone	MIC combination	FIC	Type of interaction
Enrofloxacin (µg/mL)	0.078	0.078	1	Additive
<i>Boswellia ovalifoliolata</i> resin extract (mg/mL)	2.5	2.5		
<i>Boswellia ovalifoliolata</i> resin extract (mg/mL)	2.5	1.25	1	Additive
Eugenol (mg/mL)	1.562	1.562	1	Additive
<i>Boswellia ovalifoliolata</i> resin extract (mg/mL)	2.5	2.5		
Thymol (mg/mL)	3.125	1.562	1	Additive
<i>Boswellia ovalifoliolata</i> resin extract (mg/mL)	2.5	2.5		
Carvacrol (mg/mL)	1.562	1.562	1	Additive

Table 5: Fractional inhibitory concentration and the type of interaction between amoxicillin, *Boswellia ovalifoliolata* resin extract, and various phytochemicals against *Staphylococcus aureus*

Test compound	MIC alone	MIC combination	FIC	Type of interaction
Amoxicillin (µg/mL)	0.078	0.039	0.6	Additive
<i>Boswellia ovalifoliolata</i> resin extract (mg/mL)	0.3125	0.3125		
<i>Boswellia ovalifoliolata</i> resin extract (mg/mL)	0.3125	0.3125	1	Additive
Eugenol (mg/mL)	3.125	3.125	0.	Additive
<i>Boswellia ovalifoliolata</i> resin extract (mg/mL)	0.3125	0.156		
Thymol (mg/mL)	1.562	1.562	0.7	Additive
<i>Boswellia ovalifoliolata</i> resin extract (mg/mL)	0.3125	0.156		
Carvacrol (mg/mL)	0.390	0.390	0.7	Additive

Table 6: Percentage inhibition of biofilm against *Escherichia coli*.

Test compound	Concentration	Percent biofilm inhibition
Enrofloxacin	0.078 µg/mL	77.8±0.61 ^A
<i>Boswellia ovalifoliolata</i> resin extract	2.5 mg/mL	No inhibition
<i>Boswellia ovalifoliolata</i> resin extract+ Enrofloxacin	2.5 mg/mL+0.078 µg/mL	71.8±0.28 ^{AB}
Eugenol	1.562 mg/mL	60.4±0.28 ^B
Thymol	1.562 mg/mL	63.8±0.32 ^{CD}
Carvacrol	1.562 mg/mL	58.8±0.53 ^{AB}
<i>Boswellia ovalifoliolata</i> resin extract+ Eugenol	2.5 mg/mL+1.562 mg/mL	61.8±0.60 ^{BC}
<i>Boswellia ovalifoliolata</i> resin extract+ Thymol	2.5 mg/mL+1.562mg/mL	65.8±0.89 ^D
<i>Boswellia ovalifoliolata</i> resin extract+ Carvacrol	5 mg/mL+1.562 mg/mL	56.6±0.69 ^A

Values are Mean ± SEM; one-way ANOVA (SPSS).

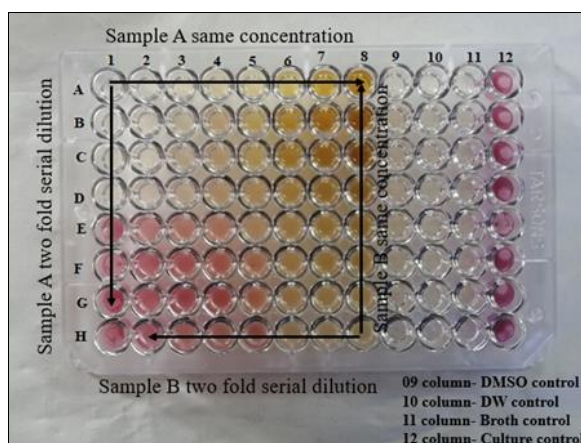
Means with different alphabets as superscripts differ significantly ($p < 0.00$).

Table 7: Percentage inhibition of biofilm against *Staphylococcus aureus*

Test compound	Concentration	Percent biofilm inhibition
Amoxicillin	0.312 µg/mL	59.8±1.73 ^E
<i>Boswellia ovalifoliolata</i> resin extract	0.312 mg/mL	79.4±0.77 ^C
<i>Boswellia ovalifoliolata</i> resin extract+ Amoxicillin	0.312 mg/mL +0.312 µg/mL	61.5±0.72 ^E
Eugenol	3.125 mg/mL	60.7±0.98 ^A
Thymol	1.562 mg/mL	70.3±0.53 ^B
Carvacrol	0.390 mg/mL	75.4±1.68 ^C
<i>Boswellia ovalifoliolata</i> resin extract+ Eugenol	0.312 mg/mL +3.125 mg/mL	56.4±1.67 ^A
<i>Boswellia ovalifoliolata</i> resin extract+ Thymol	0.312 mg/mL +1.562 mg/mL	68.7±0.20 ^B
<i>Boswellia ovalifoliolata</i> resin extract+ Carvacrol	0.312 mg/mL +0.390 mg/mL	80.5±0.71 ^C

Values are mean ± SEM; one-way ANOVA (SPSS).

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

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

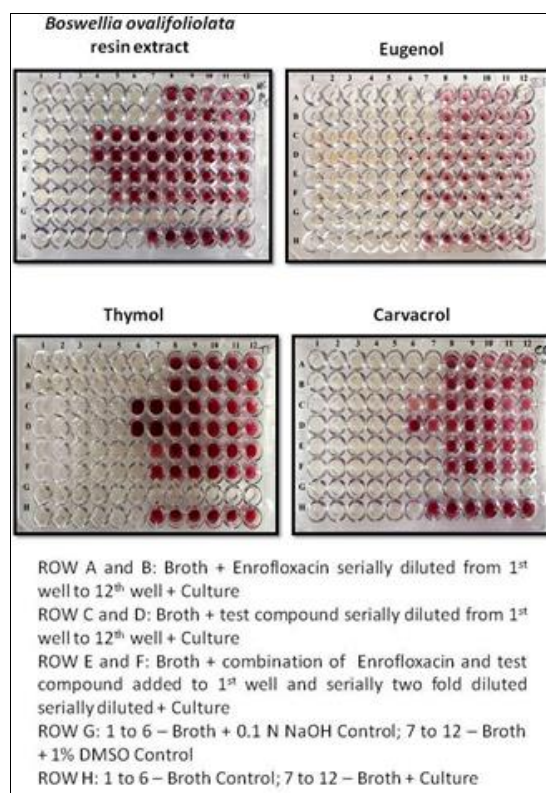


Fig 2: Microtiter plate showing inhibition of *Escherichia coli* growth on a serial dilution of various phytochemicals and Enrofloxacin

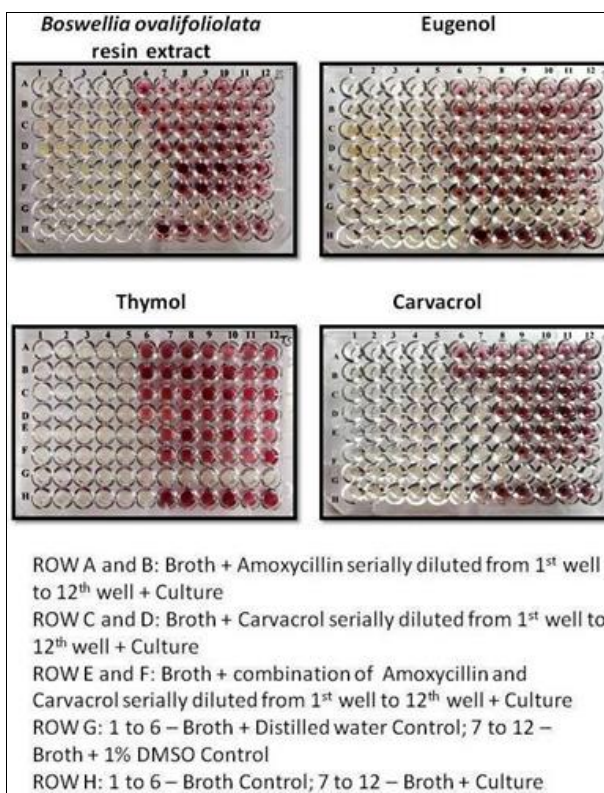


Fig 3: Microtiter plate showing inhibition of *Staphylococcus aureus* growth on a serial dilution of various phytochemicals and Amoxycillin

Conclusion

In conclusion, *Boswellia ovalifoliolata* resin extract, rich in bioactive phytochemicals, exhibits potent antioxidant, antimicrobial, and antibiofilm properties. Its additive effects with antibiotics and phytochemicals suggest its potential antimicrobial phytochemical to use in combination with classical antibiotics and other phytochemicals against resistant mastitis-causing bacteria, offering an effective alternative strategy to conventional antimicrobial therapies.

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Conflict of interest

There is no conflict of interest with anyone

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