

PHYTOCHEMICAL ANALYSIS AND *IN VITRO* STUDY ON ANTIMICROBIAL, ANTI-INFLAMMATORY AND ANTIOXIDANT ACTIVITIES OF *TRIDAX PROCUMBENS* EXTRACTS AGAINST BOVINE MASTITIS PATHOGENS

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ABSTRACT

This study evaluated the antimicrobial, anti-inflammatory and antioxidant properties of *Tridax procumbens* leaf extracts. The ethanolic extract demonstrated significant antibacterial activity against common bovine mastitis pathogens, including *Staphylococcus* spp., *Bacillus* spp., *Klebsiella* spp., *E. coli* and *Pseudomonas* spp. Additionally, the extract exhibited potent antioxidant properties, exceeding those of ascorbic acid, along with a notable anti-inflammatory effect. Phytochemical analysis confirmed the presence of flavonoids, phenols, tannins and other bioactive compounds. These findings indicate that *Tridax procumbens* extracts have therapeutic potential for treating bovine mastitis, warranting further investigation.

Keywords: Anti-Inflammatory, Antimicrobial, Antioxidant properties, Bovine, Mastitis, *Tridax procumbens*, Phytochemical analysis

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Mastitis, an inflammatory condition affecting the mammary gland, poses a substantial threat to the global dairy industry. This condition is marked by changes in glandular tissue and milk composition, encompassing clinical, physical, chemical, and microbiological aspects (Gera and Guha, 2011). Clinical mastitis is an economic importance, especially to small and marginal farmers due to milk discard, treatment expenses, and other related costs (Halasa *et al.*, 2007). The growing issue of antibiotic resistance among mastitis-causing pathogens, coupled with diminished treatment efficacy, has become increasingly prevalent in veterinary care (Wang *et al.* 2006). Herders often rely on antibiotics to treat and prevent bovine mastitis, but the individual use of these medications has exacerbated antibiotic resistance of common mastitogens (Srinivasan *et al.*, 2007).

Mastitis treatment is anticipated to become increasingly challenging due to the rapid rise of antibiotic-resistant pathogens that can be transmitted through foodborne infections. Currently, the primary treatment approach involves administering antibiotics via intramammary infusion, intramuscular injection, or intravenous injection (Bhosale *et al.*, 2014). However, the growing issue of antibiotic resistance has reduced the

effectiveness of these treatments, and the presence of antibiotic residues in milk and dairy products poses a significant public health risk (Yang *et al.*, 2019). In search of alternative solutions, research has highlighted the potential of plant-based antibacterial agents, such as flavonoids, which exhibit therapeutic properties with minimal side effects (Lutterodt *et al.*, 1999). Notably, *Tridax procumbens*, a plant traditionally used in India for wound healing and other purposes, has demonstrated various pharmacological effects, including antimicrobial, anti-inflammatory and immunomodulatory activities (Taddel and Rosas-Romero, 2007). Given its potential, this study investigates the *in vitro* efficacy of *Tridax procumbens* against common pathogens causing bovine mastitis.

MATERIALS AND METHODS

Collection of Samples

A total of 264 milk samples were collected from cows exhibiting udder and milk abnormalities at the Veterinary Clinical Complex of Veterinary College and Research Institute, located in the Salem district of Tamil Nadu. The mastitis milk samples were aseptically collected in sterile containers from cows with mastitis and transported to the laboratory for further analysis, including

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bacterial culture and isolation. Based on ABST, categorized into resistant mastitis (n=176), non-resistant (n=52) and culturally negative (n=36).

Isolation and identification of mastitogens

Each milk sample was inoculated onto Muller Hinton Agar (M/s Hi-Media Laboratories Ltd., Mumbai, India) using the streak plate method and incubated at 37 °C. Growth was monitored at 24 and 48 hours. Colony morphology was examined and bacterial smears were prepared and characterized using Gram staining. Biochemical test kits (M/s Hi-Media Laboratories Ltd., Mumbai, India) were used to further characterize the isolates. Molecular characterization of the mastitis-causing pathogens was performed using polymerase chain reaction (PCR) with microorganism-specific primers. The following mastitogens can be detected using PCR with specific primers and conditions: *Staphylococcus aureus*, *E. coli*, *Bacillus cereus*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. For *S. aureus*, primers NUC1 F: ATGAAGTCAAATAAATCGCT and NUC2 R: TTTGGTGAAAATACTTCTC are used with PCR cycling of 94°C 2 min, 40 cycles of 94°C 2 min, 55°C 2 min, 72°C 3 min and a final 72°C 10 min, yielding a 458 bp product (Gandra *et al.*, 2011). For *E. coli*, primers *uspA* F: CCGATACGCTGCCAATCAGT and R: ACGCAGACCGTAGGCCAGAT are used with PCR cycling of 94°C 5 min, 30 cycles of 94°C 1 min, 55°C 1 min, 72°C 2 min and a final 72°C 5 min, yielding an 884 bp product (Singathia *et al.*, 2023).

For *Bacillus cereus*, primers F: AGAGTTTGATCCTGGCTCAG and R: AAGGAGGTGATCCAGCCGCA are used with PCR cycling of 95°C for 5 min, 30 cycles of 94°C 1 min, 52°C 1 min, 72°C for 90s and a final 72°C 10 min, yielding a 1500 bp product (Meng *et al.*, 2023). For *Klebsiella pneumoniae*, primers P f: ATTTGAAGAGGTTGCAAACGAT and Pr2: CCGAAGATGTTTCACTTCTGATT are used with PCR cycling of 94°C 10 min, 35 cycles of 94°C 30s, 57°C 20s, 72°C 20s and a final 72°C 10 min, yielding a 260 bp product (Osman *et al.*, 2020). For *Pseudomonas aeruginosa*, primers PA-SS-F: GGGGGATCTTCGGACCTCA and PA-SS-R: TCCTTAGAGTGCCACCCG are used with PCR cycling of 95°C 2 min, 25 cycles of 94°C 20 sec, 58°C 20 Sec, 72°C 40 Sec and a final 72°C 1min, yielding a 956 bp product (Spilker *et al.*, 2004).

Tridax procumbens plant collection

Fresh leaves of *Tridax procumbens* were collected from the vicinity of the Veterinary College and Research

Institute Campus in Salem, Tamil Nadu. The plant's taxonomic identity was confirmed by the Botany department at Rajah Serfoji Government College (Autonomous) in Thanjavur. The fresh plant material was washed with tap water, cut into small pieces, and shade-dried at room temperature for a week. The dried material was then powdered using a mechanical blender and stored in an airtight container.

Extract preparation

Aqueous and ethanolic extracts of *Tridax procumbens* were prepared using the cold maceration method at room temperature, with slight modifications to the protocols described by Monika *et al.* (2013) and Rana *et al.* (2022).

Aqueous extraction of *T. Procumbens* leaves

Ten grams of air-dried *T. Procumbens* powder was mixed with 100 mL of distilled water and kept at -20 °C for 7 days with intermittent shaking three times a day. It was filtered through eight layers of muslin cloth, then centrifuged at 5000 rpm for 10 min and filtered through Whatman No.1 filter paper. The supernatant was collected, dried and concentrated in a hot air oven at 55 °C for 12-24 hours. The extracts were collected and stored at -20 °C until further processing.

Ethanol extraction of *T. procumbens* leaves

Ten grams of air-dried *T. Procumbens* powder was mixed with 100 mL of 70% ethanol and kept at -20 °C for 7 days with intermittent shaking three times a day. It was then filtered through eight layers of muslin cloth, centrifuged at 5000 rpm for 10 minutes and then filtered through Whatman No. 1 filter paper. The supernatant was collected, dried, and concentrated in a hot air oven at 55 °C for 12 - 24 hours. The extracts were collected and stored at -20°C until further processing.

Phytochemical screening

Phytochemical analysis was conducted on the aqueous and ethanolic extracts using standard procedures to identify the phytoconstituents, following methods described by Sofowara (1993), Trease and Evans (1989), Harborne (1991) and Dharajiya *et al.* (2012).

Quantitative analysis of phytochemicals in *T. Procumbens* extracts

The total flavonoid content of the extracts was determined using a colorimetric method (Nabavi *et al.*, 2008), expressed as milligrams of catechin per gram of extract. Total phenolic content was measured using the Folin-Ciocalteu reagent method (Biglari *et al.*, 2008), with results expressed as milligrams of gallic acid equivalent

per gram of dry weight (mg GAE/g). The phenolic compound content was calculated using the formula $C = A/B$, where C represents the concentration (mg GAE/g dry weight), A is the equivalent gallic acid concentration from the calibration curve (mg) and B is the dry weight of the extract (g). Additionally, Thin Layer Chromatography (TLC) was used to quantify phenol and flavonoid compounds in the ethanolic extract.

Antibacterial activity of the extract of *T. Procumbens*

The antibacterial activity of aqueous and ethanolic extracts was evaluated against five mastitis-causing pathogens. For each bacterium (*Staphylococcus* spp., *Bacillus* spp., *Klebsiella* spp., *Escherichia coli* and *Pseudomonas* spp.), individually tested the antibacterial properties of the plant extracts (using the agar well diffusion method (Perez *et al.*, 1990; Nair and Chanda, 2005). Briefly, Mueller Hinton Agar plates were inoculated with 0.5 McFarland (108 CFU/mL) bacterial suspensions, and a well (0.85 cm) was created and filled with 100 μ L of extract. After overnight incubation at 37°C, antibacterial activity was assessed by measuring the zone of inhibition (mm). Controls included pure solvents and Gentamicin, and each experiment was repeated three times, with results presented as mean values $\pm$ standard deviation (SD) (Parekh and Chanda, 2007).

Antioxidant property of the *T. procumbens*

The antioxidant property of *T. procumbens* leaves was evaluated using a DPPH assay kit. To a methanolic solution of DPPH (90.25 mm), an equal volume of ethanolic leaf extract of *T. procumbens* was added and made up to 1.0 mL with methanolic DPPH. An equal amount of methanol was added to the control. After 20 min, the absorbance was recorded at 517 nm in a Systronics UV-visible Spectrophotometer. Ascorbic acid was used as a standard for comparison. The percentage inhibition of free radicals by DPPH was calculated using a specific equation.

$$\% \text{ Scavenging} = \frac{A \text{ Control OD} - A \text{ sample}}{A \text{ blank}} \times 100$$

Where A control is the absorbance of the control reaction (containing all reagents except the test compound), and A sample is the absorbance of the test compound.

Anti-inflammatory activity of *T. procumbens* extracts

The anti-inflammatory activity of *T. procumbens* was evaluated using the albumin denaturation inhibition technique, based on methods described by Mizushima and Kobayashi (1968) and Sakat *et al.* (2010) and followed with minor modifications. Briefly, the reaction mixture



Fig. 1. Results of qualitative analysis of Aqueous and Ethanolic *Tridax procumbens* leaves, showcasing positive and negative outcomes. 1. Shinoda test (Flavonoids), 2. FeCl₃ test (Phenol), 3. Lead acetate test (Tannin), 4. Foam test (Saponin), 5. Sodium chloride test (Coumarins), 6. KOH test (Quinine)



Fig. 2. Results of qualitative analysis of Aqueous and Ethanolic *Tridax procumbens* leaves, showcasing positive and negative outcomes. 1. Alkaloids, 2. Legal's test (Glycoside), 3. Sugar test, 4. Volatile oil test.

consisted of test extracts, and a 1% aqueous solution of bovine albumin fraction was prepared, and the pH of the reaction mixture was adjusted with 1N HCl. The sample extracts were incubated at 37°C for 20 min and then heated to 51 °C for 20 min, then the sample was cooled and the turbidity was measured at 660 nm with UV-Visible Spectrophotometer Model 371(M/s Elico India Ltd). The experiment was performed in triplicates.

RESULTS AND DISCUSSION

The phytochemical screening results for aqueous and ethanolic extracts of *Tridax procumbens* leaves are presented in Table 1. The analysis revealed a greater diversity of phytochemicals in the ethanolic extracts, including flavonoids, phenols, tannins, coumarins and quinones, compared to the aqueous extracts, which contained tannins, quinones, alkaloids and volatile oils (Figures 1 and 2). This study found that ethanolic extracts of *T. procumbens* contained a greater variety of

Table. 1. Qualitative phytochemical evaluation of Aqueous and Ethanolic extracts of *Tridax procumbens*

S.No.	Phytochemical	Aqueous extract	Ethanolic Extract
1.	Flavonoids	-	+
2.	Phenol	-	+
3.	Tannin	++	+
4.	Saponin	-	-
5.	Coumarin	-	+
6.	Quinone	+	+
7.	Alkaloids	+++	-
8.	Glycosides	-	-
9.	Sugar	-	-
10.	Volatile oil	++	-

Table. 2. Antibacterial activity of Aqueous and Ethanolic extracts of *Tridax Procumbens* (Diameter of Zone of inhibition in mm)

Mastitogens	Aqueous extracts (n=43)	Ethanolic extracts (n=43)	Positive control Gentamicin (n=43)
<i>Staphylococcus aureus</i> (n=8)	4.8±0.3	10.5±0.3	23.4±0.6
<i>Bacillus</i> spp. (n=8)	3.7±0.4	11.4±0.4	18.1±0.3
<i>E. coli</i> (n=8)	3.3±0.2	9.4±0.4	21.8±0.5
<i>Klebsiella</i> spp. (n=9)	NA	7.7±0.4	19±0.6
<i>Pseudomonas</i> spp. (n=9)	NA	6.8±0.3	20.2±0.6

NA – no activity

phytochemicals (flavonoids, phenols, tannin, coumarin and quinone) compared to aqueous extracts (tannin, quinone, alkaloids and volatile oil). These findings align with previous research, Syed *et al.* (2020) and Archana *et al.* (2022), reported the presence of glycosides, flavonoids, carbohydrates, polyphenols and tannins in ethanolic extracts, and glycosides, flavonoids, saponins, and polyphenols in water extracts. Similarly, Sawant and Godghate (2013) detected various phytochemicals, including saponin, steroids, diterpenes, alkaloids, flavonoids, phenols and tannins, in *T. procumbens* leave.

The extraction yield of *T. procumbens* leaves using different solvents showed that hydro-ethanolic solvent resulted in the highest yield (19.8%), followed by ethanolic solvent (12.8%) and aqueous extract (11.4%).

The aqueous extract of *T. procumbens* contained 1.037 mg/100g of phenols, with a total phenolic content of 68.65 mg GAE/g (Folin-Ciocalteu method), and 35 mg/g of flavonoids (aluminum chloride assay). The ethanolic extract contained 1.1 mg/100g of tannins. Further analysis using thin-layer chromatography (TLC) detected phenol ($R_f=0.5$) and flavonoid ($R_f=0.3$) in the ethanolic extract.

The antibacterial activity of *T. procumbens* extracts against common bovine mastitis pathogens showed that

the ethanolic extract exhibited stronger antibacterial effects than the aqueous extract (Table 2). The minimum inhibitory concentration (MIC) of the ethanolic extract revealed that Gram-positive bacteria were more susceptible to the extract than Gram-negative bacteria, requiring a lower amount of extract for inhibition. The antibacterial properties of *T. procumbens* are attributed to the presence of phytochemicals like flavonoids, alkaloids, saponins and tannins (Mundada and Shivhare, 2010). This study found that the ethanolic extract exhibited significant antibacterial activity against common mastitis pathogens, including *Staphylococcus* spp., *Bacillus* spp., *Klebsiella* spp., *E. coli* and *Pseudomonas* spp. In contrast, the aqueous extract showed minimal activity against some pathogens and no activity against others. These findings are consistent with previous research conducted by Aniel and Naidu (2010), who reported antibacterial activity in ethanolic extracts but not in aqueous extracts. Other studies have also demonstrated the antimicrobial effects of *T. procumbens* leaf extracts against both Gram-positive and Gram-negative bacteria (Udopa *et al.*, 2014; Chitra *et al.*, 2011). The present study confirms that the ethanolic extract has higher antibacterial activity than the aqueous extract, suggesting that *T. procumbens* leaves are a promising source of antimicrobial compounds (Bharathi *et al.*, 2012; Arulraj *et al.*, 2018).

The antioxidant activity of *T. procumbens* extracts was evaluated using the DPPH method. The ethanolic extract showed a higher percentage of inhibition than the standard antioxidant ascorbic acid, followed by the hydroethanolic extract and aqueous extract. This study found that the ethanolic extract of *T. procumbens* leaves exhibited higher antioxidant activity, with a greater percentage of inhibition than the standard antioxidant ascorbic acid, followed by hydroethanolic and aqueous extracts. These findings are consistent with Syed *et al.* (2020), who reported significant antioxidant activity in ethanolic extracts ($61.52 \pm 0.32\%$) and lower activity in the aqueous extracts ($25.57 \pm 0.38\%$). *T. procumbens* has been strongly proven for its medicinal and analgesic activities.

In animal studies, the protective effects are linked to the presence of flavonoids and sterols (Vinod *et al.*, 2013). *T. procumbens* is commonly used for pharmacological purposes because of its wide range of phytochemical properties. These include analgesic, anticoagulant, antileishmanial, antioxidant, anticancer, immunomodulatory, insecticidal, anthelmintic, cardiovascular, antiseptic, antimicrobial and insecticidal effects (Varsharani *et al.*, 2022). In this present study, compared to aqueous extracts, the ethanolic extracts provided better activity because of the compound's flavonoids and phenols, the presence of which was confirmed by qualitative and quantitative

screening of these compounds.

The anti-inflammatory activity of *T. procumbens* extracts was assessed using the albumin denaturation assay. The results showed that the ethanolic extract exhibited higher anti-inflammatory activity than the standard control, aspirin, followed by the hydroethanolic extract and aqueous extract.

Geographical variation significantly impacts the phytochemical composition of plants. Factors like climate, soil composition and altitude, which vary across different regions, influence the types and amounts of secondary metabolites (phytochemicals) a plant produces. Therefore, selected plants should be subjected to qualitative and quantitative fingerprinting and that should be matched with available published reports.

CONCLUSION

The ethanolic extract of *T. procumbens* leaves contains various bioactive compounds, indicating significant potential. This study demonstrates that hydroethanolic and ethanolic extracts exhibit potent antimicrobial, anti-inflammatory and antioxidant properties against common bovine mastitis pathogens. These findings suggest that *T. procumbens* extracts may serve as a valuable therapeutic option for treating bovine mastitis.

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