International Journal of Veterinary Sciences and Animal Husbandry

ISSN: 2456-2912
VET 2024; 9(5): 567-572

www.veterinarypaper.com
Received: 09-08-2024
Accepted: 16-09-2024

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Antimicrobial properties of cashew nut (*Anacardium occidentale*) shell extract against pathogenic bacteria associated with bovine mastitis

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DOI: https://doi.org/10.22271/veterinary.2024.v9.i5i.1767

Abstract
The increasing bacterial resistance to available antimicrobials necessitates the exploration of new bioactive compounds, particularly from natural sources. Traditional animal healthcare often relies on medicinal plants, many of which possess potent antimicrobial properties. This study evaluated the acetone extract of *Anacardium occidentale* (cashew) nut shell for its phytochemical composition, antioxidant activity, and antimicrobial potential, both alone and in combination with antibiotics and other phytochemicals. The extract contained carbohydrates, saponins, triterpenes, phenols, flavonoids, proteins, and amino acids. The antioxidant activity of cashew nut shell extract was showing moderate activity. Antibacterial activity was assessed using microdilution assays, with minimum inhibitory concentrations (MICs) determined against *Escherichia coli* and *Staphylococcus aureus*. The extract demonstrated stronger activity against *S. aureus* (MIC = 0.078 mg/ml) than *E. coli* (MIC = 2.5 mg/ml). When combined with amoxicillin and enrofloxacin, the extract showed additive effects. Overall, cashew nut shell extract demonstrated significant antimicrobial activity, particularly against *S. aureus*, and shows promise when combined with existing antibiotics.

Keywords: *Anacardium occidentale*, cashew nut shell extract, antimicrobial property

Introduction

Traditional healthcare of animals includes the use of medicinal herbs to prevent and treat a range of diseases and problems encountered by livestock. Several secondary metabolites of plants (alkaloids, flavonoids, tannins, terpenes, quinones, essential oils, and resins) have shown potential antimicrobial activity [1], predominantly through mechanisms that lead to cell death [2]. Plant-derived antimicrobials are not only effective in killing microorganisms but also affect bacterial cellular events in the pathogenic process, offering relatively broad-spectrum antimicrobial activity. Consequently, bacteria, fungi, and viruses have a reduced ability to develop resistance to botanicals [3], making these agents valuable in eliminating resistant microbes [4].

Anacardium occidentale (cashew nut tree) is widely cultivated in India and many other tropical countries due to the high nutritional and commercial value of cashew nuts [5]. The extract from the pericarp of cashew nuts is a major byproduct of the cashew nut industry and has numerous medicinal applications [6]. It is primarily used for gastrointestinal and skin disorders caused by various fungal and bacterial species [7].

Mastitis is one of the most frequent infectious diseases in dairy cattle, accounting for the majority of antibiotics administered to dairy cows [8]. The source of infection for bovine mastitis often includes the infected cow for primary contagious pathogen *Staphylococcus aureus* (*S. aureus*) and the surrounding environment for the important environmental pathogen *Escherichia coli* (*E. coli*) [9]. The use of antimicrobials in food animals can affect both animal and human health by increasing the risk of antimicrobial residues and contributing to the selection or generation of antimicrobial-resistant pathogens [10]. The outcome of antimicrobial treatment for mild and moderate cases of mastitis can be difficult to predict when using selected antimicrobial agents, even with veterinary breakpoints being validated [11].

Germicidal pre-milking and post-milking teat dips have proven effective in reducing the incidence of new udder infections by killing bacteria on the teat surface [12]. However, teat dips/disinfectants may have drawbacks, such as affecting milking parlour efficiency, variability in the sensitivity of mastitis pathogen strains [13], potential irritation to teat skin, and imparting odour to milk.
In this context, the present *In vitro* study was carried out to screen cashew nut shell acetone extract (CNSE) for its antioxidant properties and antimicrobial activity against *E. coli* and *S. aureus*. The future aim is to use this extract as one of the ingredients in the formulation of herbal pre- and post-milking teat dips.

Materials and Methods

Plant Material and Chemicals

Cashew nuts were collected from a local farm; shells were manually removed, and powdered. Chemicals were procured from (Himedia and Merck, Mumbai, India).

Clinical Isolates Microbial Culture

E. coli and *S. aureus* were isolated from buffalo mastitis cases (College Veterinary Hospital), collected milk samples were processed, and cultured on EMB for *E. coli* and MSA for *S. aureus* [14]. Isolates were confirmed by PCR at (Department of Veterinary Microbiology, NTR College of Veterinary Science, Gannavaram).

Preparation of Extract

Fifty grams of powdered cashew nut material was soaked in acetone (1:10 ratio) and subjected to overnight stirring at room temperature. The mixture was filtered, and the filtrate was lyophilized until the weight remained constant to obtain the cashew nut shell extract (CNSE).

Phytochemical Analysis

CNSE was qualitatively screened for phenols, flavonoids, reducing sugars, saponins, triterpenes, proteins, carbohydrates, and alkaloids [15].

Antioxidant Activity

The antioxidant activity was evaluated by phosphomolybdenum assay [16]. Absorbance was measured at (695 nm) after incubating test compounds and ascorbic acid at 95°C. Antioxidant activity was expressed as ascorbic acid equivalents.

Antibacterial Properties

Minimum Inhibitory Concentration (MIC):

MIC was evaluated against *E. coli* and *S. aureus* (MTCC and clinical isolates) following [17] standards. Antibiotics (enrofloxacin, amoxicillin) and CNSE were serially diluted in a microtiter plate, and microbial growth was observed using iodonitrotetrazoline chloride (INT). The lowest concentration showing no growth was recorded as the MIC.

Biofilm Inhibition:

Biofilm inhibition was assessed following methods of Bazargani and Rohloff, (2016) [18] and O' Toole (2011) [19] with CNSE at MIC concentrations. Absorbance was measured at (OD 550 nm) after crystal violet staining, and inhibition was calculated as a percentage compared to the control. The mean absorbance of the samples was determined, the absorbance in blank well was subtracted from absorbance reading and percentage inhibition was then compared with the positive control.

$$\text{Percentage inhibition} = \frac{\text{OD negative control} - \text{OD experimental}}{\text{OD of negative control}} \times 100$$

Checkerboard Assay for Synergy Testing

The combined antimicrobial activity of CNSE with enrofloxacin (against *E. coli*) and with amoxicillin (against *S. aureus*) was evaluated using the checkerboard assay [20]. Fractional inhibitory concentration (FIC) indices were calculated to assess the type of interaction.
Stock solutions of CNSE, enrofloxacin, and amoxicillin (at double the MIC) were prepared, and serial two-fold dilutions were performed. A total of (50 µl) of Mueller-Hinton broth was distributed into each well of a microdilution plate. CNSE was serially diluted along the abscissa, while enrofloxacin or amoxicillin was diluted along the ordinate. To each well, (10 µl) of bacterial suspension (0.5 McFarland) was added.
The resulting checkerboard design had the highest concentration of both compounds at opposite corners, with each agent serially diluted across rows and columns. Controls (positive and negative) were included in columns (9 to 12). Plates were incubated at (37 °C) for 24 hours and then evaluated for bacterial growth. In order to assess the activities of combination, fractional inhibitory concentration (FIC) indices were calculated as $FIC_A + FIC_B$ where FIC_A and FIC_B are the fraction of minimum concentration that inhibited the bacterial growth for samples A in combination with sample B and for sample B in combination of sample A respectively.

$$FIC_A = \frac{MIC_A \text{ in combination with B}}{\text{MIC of A alone}}; \; FIC_B = \frac{MIC_B \text{ in combination with A}}{\text{MIC of B alone}}$$

FIC indices were calculated as follows to determine interaction type:
FIC index = $FIC_A + FIC_B$ and the results were interpreted as Synergistic: (FIC Index < 0.5), Additive: (FIC Index = 0.5–1.0), Indifferent: (FIC Index = 1.0–4.0), Antagonistic: (FIC Index > 4).

Results

The present study evaluated the acetone extract of cashew nut shell for phytochemical composition, *In vitro* antioxidant and antimicrobial activity against mastitis-causing bacteria *Staphylococcus aureus* and *Escherichia coli*, as well as the synergistic antimicrobial potential in combination with amoxicillin and enrofloxacin.

Phytochemical Screening of Acetone Extract of Cashew Nut Shell

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

Evaluation of Antioxidant Activity

The antioxidant activity of CNSE was assessed using the phosphomolybdenum assay, expressed as antioxidant capacity equivalent to µg/ml of ascorbic acid. A standard curve was plotted using ascorbic acid (10–60 µg/ml) at 695 nm. The estimated antioxidant activity of 0.1 mg/ml CNSE and enrofloxacin was 63.6 ± 0.64 and 33.605 ± 0.31 µg/ml of ascorbic acid, respectively (Table 1).

Antibacterial Activity against *Escherichia coli*

Using the microdilution assay, the MIC for enrofloxacin and CNSE against *Escherichia coli* was determined (Table 2). The concentrations range for estimation of MIC of enrofloxacin was from 10 to 0.0048 µg/ml, with inhibition at 0.03 µg/ml. For CNSE, the concentration range for estimation of MIC was from 10 to 0.0048 mg/ml, with inhibition at 2.5 mg/ml. The combination of enrofloxacin and CNSE resulted in an MIC of 0.0195 µg/ml to enrofloxacin and 1.25 mg/ml to CNSE.

Antibacterial Activity against *Staphylococcus aureus*

Similarly, MIC determination for amoxicillin and cashew nut shell extract against *Staphylococcus aureus* was conducted (Table 2). The concentration range for estimation of MIC for amoxicillin was from 10 to 0.0048 µg/ml, and showed inhibition at 0.078 µg/ml, and for CNSE the concentration range for estimation of MIC was from 10 to 0.0048 mg/ml, with inhibition showed at 2.5 mg/ml. In combination, the MIC for amoxicillin was 0.15 µg/ml, and for CNSE, it was 0.039 mg/ml.

Biofilm Inhibition Potential against *Escherichia coli*

The biofilm inhibition potential of enrofloxacin and CNSE was evaluated using the crystal violet assay (Table 2). Enrofloxacin exhibited the highest percentage of biofilm inhibition, both alone and in combination with CNSE. However, the combination showed a slightly lower inhibition percentage compared to enrofloxacin alone.

Biofilm Inhibition Potential against *Staphylococcus aureus*

The crystal violet assay was also used to assess biofilm inhibition against *S. aureus* (Table 2). CNSE alone and in combination with amoxicillin demonstrated a significantly higher percentage of biofilm inhibition compared to individual agents.

Additive antibacterial activity interaction against *Escherichia coli* by microdilution checkerboard method

The checkerboard assay was performed to evaluate the combined antibacterial effect of enrofloxacin and CNSE against *E. coli* (Table 2). The calculated FIC index was 1, indicating an additive antibacterial activity that is the combined effect of both agents was equivalent to the sum of their individual effects.

Additive Antibacterial Activity against *Staphylococcus aureus* by Microdilution Checkerboard Method

Similarly, amoxicillin and CNSE were assessed for their combined action against *S. aureus* (Table 2). The FIC index was 1, indicating additive antibacterial activity that is the combined effect of both agents was equivalent to the sum of their individual effects.

Discussion

Infectious diseases caused by bacteria and fungi continue to affect millions of humans and animals globally, remaining a significant cause of death and disability. The discovery of novel antibiotics has been essential in combating bacterial infections. However, the increasing development of antimicrobial resistance, largely attributed to the indiscriminate and improper use of antimicrobial agents, has emerged as a critical global concern [21]. Misuse of antibiotics over the decades has led to the development of multiple drug-resistant bacteria, a problem that is steadily intensifying [22]. This escalating resistance affects all major classes of antimicrobial drugs, presenting a serious global health threat [23, 24].

In recent years, the pace of new antimicrobial development has slowed considerably, while resistance rates have increased alarmingly [25]. Although efforts to develop new antibiotics are crucial, the rapid and widespread emergence of resistance to newly introduced agents highlights the need for alternative therapeutic strategies [26]. This calls for urgent exploration of novel antibacterial substances, especially from natural sources. Plant-derived antimicrobials offer an attractive alternative, with the added benefit of fewer side effects compared to synthetic drugs [27, 28]. Phytochemicals present an effective and economical solution to both treat infections and counter bacterial resistance [29]. Various plant species have demonstrated microbiostatic and microbiocidal properties, attributed to their secondary metabolites such as tannins, terpenes, and flavonoids [30, 31].

The indiscriminate use of antibiotics, especially in treating bovine mastitis caused by bacteria like *Staphylococcus aureus* and *Escherichia coli*, has exacerbated resistance [32, 34]. Consequently, medicinal plants with antioxidant, immunomodulatory, and antibacterial properties offer a promising alternative for treating udder infections in bovines [34], [35]. Although herbs may have limited antimicrobial efficacy on their own, their combination with synthetic antimicrobials often yields synergistic effects, enhancing the overall antibacterial action [36, 37, 38].

The cashew plant (*Anacardium occidentale*) is widely known for its medicinal properties, particularly the potent antibacterial activity of its nut shell oil, used traditionally for various bacterial ailments [39, 40]. Given its reported therapeutic properties, the present study was designed to evaluate the antibacterial and antioxidant activities of the acetone extract of *Anacardium occidentale* nut shell and its interactions with antibiotics like amoxicillin and enrofloxacin against mastitis-causing organisms.

Phytochemical screening of the acetone extract of cashew nut shell confirmed the presence of phenols, flavonoids, reducing sugars, saponins, triterpenes, proteins, carbohydrates and alkaloids. Similar studies on different parts of the cashew tree, such as leaves and bark, have reported comparable phytochemical profiles, which are linked to the plant's various pharmacological activities [41, 42].

The acetone extract of cashew nut shell demonstrated significant antioxidant activity, equivalent to 63.6±0.64 µg/ml of ascorbic acid, as assessed by the phosphomolybdenum assay. This aligns with previous studies that reported antioxidant activity in CNSE using different methods, such as DPPH radical scavenging assay [43, 44]. Similarly, enrofloxacin also exhibited potent antioxidant activity, as previously observed by Masadeh *et al*. (2012) [45].

The minimum inhibitory concentration (MIC) of CNSE against *Escherichia coli* was found to be 2.5 mg/ml, indicating the presence of substantial antibacterial activity. This is consistent with the findings of Hemashekhar *et al*. (2012) [40], who reported that anacardic acid-a major bioactive compound in cashew nut shell-possesses strong antibacterial properties. The antibacterial and antioxidant effects of the extract may be attributed to the presence of alkyl polyunsaturated phenols [46].

The MIC of cashew nut shell extract against *Staphylococcus aureus* was 0.078 mg/ml, demonstrating potent antibacterial activity. This could be due to the phenolic compounds in the extract, which affect the synthesis of peptidoglycan in bacterial cell walls, thereby inhibiting cell proliferation [47]. Similar results have been reported in other studies [43, 47].

The combination of cashew nut shell extract with enrofloxacin exhibited an additive effect against *Escherichia coli*, meaning that the sum of their individual effects equals their combined effect. This can be explained by the complementary modes of action of enrofloxacin, which disrupts bacterial DNA and protein synthesis, and cashew nut shell extract, which contains flavonoids and phenols that enhance antibacterial activity [40]. Similarly, the combination of cashew nut shell extract with amoxicillin showed an additive antibacterial effect against *Staphylococcus aureus*, indicating that the combination of synthetic and natural agents could be beneficial in treating resistant bacteria.

Biofilm inhibition was assessed using the crystal violet assay. The cashew nut shell extract did not exhibit biofilm inhibition against *Escherichia coli* at MIC concentrations. However, enrofloxacin alone showed the highest percentage of biofilm inhibition. Several studies have reported similar findings regarding the inhibition of biofilm formation by plant extracts and essential oils [18]. In contrast, the combination of cashew nut shell extract and amoxicillin demonstrated significant biofilm inhibition against *Staphylococcus aureus*, similar to studies on other plant extracts [48].

Table 1: Phytochemical screening and antioxidant potential of cashew nut shell acetone extract

Phytochemical screened	Reagents/Chemicals	Observation	Results
Phenol	Ferric chloride	Bluish black	+
Flavnoids	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's	Violet ring at the junction	+
Alkaloids	Dragendroff	Red precipitate	-
Antioxidant activity of 0.1 mg/ml of CNSAE is equivalent to	Phosphomolybdate reagent	----	63.6±0.64 µg/ml of ascorbic acid

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

Antimicrobial properties		***E. coli***	***S.aureus***
Minimum Inhibitory concentration (MIC)	Cashew Nut Shell Acetone Extract (CNSEA)	2.5 mg/ml	0.3125 mg/ml
	Enrofloxacin	0.03 µg/ml	
	Amoxicillin		0.078 µg/ml
MIC in combination with Enrofloxacin	CNSEA	1.25 mg/ml	
	Enrofloxicin	0.0195 µg/ml	
	FIC	1	
	Type of Interaction	Additive	
MIC in combination with Amoxicillin (units)	CNSEA		0.039 mg/ml
	Amoxicillin		0.15 µg/ml
	FIC		1
	Type of Interaction		Additive
Percentage inhibition of bio-film.		***E. coli***	***S.aureus***
Bio-film inhibition	Cashew Nut Shell Acetone Extract (CNSEA) Concentration (% inhibition)	No inhibition	0.07 mg/ml (74.2±0.21)
	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	CNSEA + Enrofloxacin Concentration (% inhibition)	5 mg/ml + 0.03 µg/ml (63.1±3.1)	
	CNSEA + Amoxicillin Concentration (% inhibition)		0.07 mg/ml + 0.3 µg/ml (74.2±1.09)

Values are mean±SEM; oneway ANOVA (SPSS).
Means with different alphabets as superscripts differ significantly ($p<0.05$).

Conclusion

The present study revealed that cashew nut shell extract possesses both antioxidant and antimicrobial properties, attributed to its rich phytochemical composition, including anacardic acid and other phenolic compounds. The extract demonstrated an additive antibacterial effect when combined with enrofloxacin and amoxicillin against *Escherichia coli* and *Staphylococcus aureus*, respectively. These findings suggest that cashew nut shell extract could serve as a valuable adjunct in antibiotic therapy, particularly in managing infections caused by resistant bacterial strains.

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How to Cite This Article
Kavya Y, Bharavi K, Srividya G, Theresa MTS, Bhadraiah A, Kumar KA, *et al*. Antimicrobial properties of cashew nut (Anacardium occidentale) shell extract against pathogenic bacteria associated with bovine mastitis. International Journal of Veterinary Sciences and Animal Husbandry. 2024;9(5):567-572.