RESEARCH ARTICLE Indian Journal of Animal Research, Volume 59 Issue 1: 93-100 (January 2025)

In vitro Antibacterial Efficacy of 7 Plant Extracts on *Staphylococcus aureus* Isolated From Equine Skin Lesions

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10.18805/IJAR.B-5316

ABSTRACT

Background: *Staphylococcus aureus* is a prevalent opportunistic pathogen which is increasingly associated with various equine dermatological afflictions. The burgeoning issue of antibacterial resistance against this bacterium necessitates novel therapeutic approaches. This study, executed from May to November (2022) at the National Research Centre on Equine, Equine Production Centre, Bikaner, aimed to isolate and identify *S. aureus* from equine dermal lesions and to assess the *in vitro* efficacy of both organic (methanolic, aqueousand ethanolic) and inorganic (chloroform and petroleum ether) phytoextracts from *Calotropis gigentean*, *Capparis decidua*, *Leptadenia pyrotechnica*, *Aerva javanica*, *Azadirachta indica*, *Aloe vera* and *Eucalyptus camaldulensis*.

Method: The study utilised agar well diffusion and broth dilution techniques to assess the antimicrobial efficacy of these extracts against *S. aureus*.

Result: Microscopic analysis of gram-stained smears from cultures, alongside a suite of biochemical assays and polymerase chain reaction (PCR), corroborated the presence of *S. aureus*. The antimicrobial screening disclosed that both organic and inorganic extracts of *E. camaldulensis* manifested the most pronounced antibacterial activity, exhibiting zones of inhibition ranging from 15 mm to 21 mmand minimum inhibitory concentrations between 1.56 to 3.13 mg/mL. Furthermore, extracts from *A. indica* (chloroform, methanolicand ethanolic) and *A. vera* (methanolic and ethanolic) also demonstrated antibacterial effectiveness against this pathogen, with inhibition zones extending from 15 mm to 17.33 mm (MIC: 3.13 to 25 mg/mL) and 9 mm to 12 mm (MIC: 12.5 to 25 mg/mL), respectively. Moreover, the outcomes of this investigation substantiate the antibacterial capabilities of *E. camaldulensis*, *A. indica* and *A. vera* against dermatological pathogens, advocating their inclusion in topical antibacterial formulations as a strategic countermeasure to the escalating challenge of drug resistance.

Key words: Antibacterial activity, Extracts, Minimum inhibitory concentration, PCR.

INTRODUCTION

Staphylococcus aureus is a facultative anaerobic, Gram-positiveand catalase-positive coccus which typically forms clustered aggregations of single cells (Saleh *et al*., 2018). It is a primary etiological agent in a spectrum of skin and soft tissue infections. These range from superficial cutaneous manifestations such as impetigo and infected abrasions, to more intricate dermatological conditions like cellulitis, subcutaneous abscesses, folliculitis/furunculosis and infected chronic ulcers and wounds (Krishnan and Wong, 2015). Additionally, the pathogen is known to impede the wound healing process through multiple mechanisms. These include the sustained secretion of inflammatory mediators, metabolic by-productsand toxins, along with the continuous activation of neutrophils. The latter results in the release of cytolytic enzymes and free oxygen radicals, further exacerbating the healing process (Kumar *et al*., 2019). Beyond localized infections, *S. aureus* is also implicated in severe, potentially life-threatening conditions such as bacteraemia, endocarditis, pneumoniaand toxic shock syndrome (Habib *et al*., 2015).

Herbal plants offer several notable advantages: they are readily available at a relatively lower cost, typically exhibit fewer side effects and are better tolerated by patients. This enhanced acceptance is bolstered by their extensive historical usage (Tabassum and Hamdani, 2014; Basak *et al*., 2020; Ahuja *et al*., 2021). Plants produces many secondary metabolites to fight against various biotic and abiotic stress, plants growing in the desert areas are specially prone to the various stress conditions (Masmoudi *et al*., 2019; Zhedi *et al*., 2021). Major horse breeding tract for Marwari, kathiawari and sindhi breeds is also situated in the subtropical desert and semi desert climatic conditions of the north western India. So we hypothesized that if antibacterial activity of plants of this area is found that can provide a cheap and alternative treatment for the wounds in horses and other livestock species.

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How to cite this article: Kumari, M., Sankhala, L.N., Kumar, L., Legha, R.A. and Dedar, R.K. (2025). *In vitro* Antibacterial Efficacy of 7 Plant Extracts on *Staphylococcus aureus* Isolated From Equine Skin Lesions. Indian Journal of Animal Research. 59(1): 93-100. doi: 10.18805/IJAR.B-5316.

Submitted: 29-01-2024 **Accepted:** 05-06-2024 **Online:** 25-07-2024

The objective of this work is 1) to isolate and identify *S. aureus* bacteria isolated from horse skin diseases; 2) to consider antibiotic susceptibility tests against this pathogen; and 3) to explore the antimicrobial effects of seven locally available plants such as *C. deciduas, C. gigantean, L. pyrotechnica, A. javanica, A. indica, A. vera*and *E. camaldulensis* against *S. aureus* from horse skin diseases.

MATERIALS AND METHODS

Collection of sample and transportation

The samples (skin scraping and pus sample) from skin lesions of diseased horses from different geographical locations in Rajasthan were collected under aseptic conditions using sterile cotton buds and placed in a test tube containing phosphate buffered saline and nutrient broth, thereafter, transported to the lab of NRCE, Bikaner and maintained at 40°C in an ice box. Ethical approval for this study was obtained from the institutional animal ethical committee of CVAS, Bikaner (Rajasthan), vide order no. CVAS/IAEC/2022-23/24.

Identification and isolation of bacterial pathogen

The swab specimens were systematically streaked onto culture plates containing nutrient agar, blood agarand mannitol salt agar using a sterile inoculation loop. Subsequently, the plates were placed in an incubator maintained at a temperature of 37°C for a duration ranging from 24 to 48 hours. After the incubation period, the cultures were thoroughly examined for any significant signs of growth. Identification of the cultured microorganisms was then carried out based on a triad of criteria: their distinctive morphological characteristics as observed on the media, the results of gram staining reactions and the specific patterns yielded in a series of biochemical assays. These assays included tests for carbohydrate fermentation, catalase activityand indole production. The carbohydrate fermentation test was performed by the HiCarbohydrateTM kit (KB009A) and the catalase test and indole test were performed according to Mannan *et al*. (2009). The isolated bacterial pathogen was further confirmed by amplification of *S. aureus*-specific 16s rRNA gene, which gives an amplicon of size 1250 bp.

Extraction of bacterial genomic DNA

The DNA of the bacterial pathogen was extracted utilizing the commercially available DNA-Sure Blood Mini Kit (catalog number NP-61107) provided by Genetix Biotech Asia Pvt. Ltd., based in New Delhi.

PCR amplification of 16S rRNA genes

The 16S rRNA genes were amplified using *Staphylococcus aureus*-specific primers that targeted the 16S rRNA sequence. The forward strand primer sequence used was 5′-AGAGTTTGATCCTGGCTCAG-3′ and the reverse strand primer sequence was 5′-GGTTACCTTGTTACGACTT-3′. This procedure was executed to facilitate the identification of *S. aureus*, adhering to the methodology delineated by Saleh *et al*. (2018).

Preparation of *S. aureus* bacterial subculture

From a pure culture, 3 to 5 selected colonies of *Staphylococcus aureus* were carefully transferred to a tube containing 10 ml of Muller Hinton Broth (manufactured by Himedia). Subsequently, the liquid was delicately stirred to guarantee even distribution of the bacterial colonies. Following this, the tube was placed in an incubator at of 37°C temperature. The incubation process was extended until the bacterial suspension reached the same level of cloudiness as a 0.5 McFarland Standard. The turbidity level indicates a bacterial concentration of roughly 1.5×10^8 CFUs per millilitre (cfu/ml).

Antibiotic susceptibility assay

Antibiotic sensitivity testing of the *S. aureus* was determined using the discs diffusion method (Kahsay *et al*., 2014) and corresponding to the Clinical and Laboratory Standards Institute (CLSI) recommendations. The sensitive to six antibiotics such as Amoxicillin+clavunic acid (30 μg), Ciprofloxacin (5 μg), Co-trimaxazole (25 μg), Ceftriaxone (30 10^{-1} μg), Penicillin (10 μg) and Cefixime (10 μg) was estimated in the present study.

Preparation of plant extracts using various solvents

Leaves of various plant species, namely *C. decidua* (Kair), *C. gigentean* (Milkweed), *L. pyrotechnica* (Khimp), *A. javanica* (Kapok bush), *A. indica* (Neem), *A. vera* (Gwarpatha) and *E. camaldulensis* (Safeda) were meticulously collected. The leaves were washed individually under running tap water to eliminate soil particles and other foreign debris. Post washing, these leaves were air-dried at ambient room temperature, ensuring they were kept in shaded conditions to prevent direct sunlight exposure. Subsequently, the dried leaves were ground into a fine powder. For the extraction process, a consistent quantity of powdered plant material, precisely 20 grams, was immersed in 400 ml of various solvents, distilled water, chloroform, petroleum ether, ethanol and methanol separately for each plant typeand left to soak for a period of 72 hours. During this soaking phase, each mixture was stirred at 24-hour intervals using a sterile sonicator machine to ensure uniform extraction. The leaves were washed individually under running tap water to eliminate soil particles and other foreign debris. The filtrates thus obtained were then concentrated under vacuum conditions using a rotary evaporator to remove the solvents. This process resulted in the formation of concentrated plant extracts. Finally, stock solutions of each plant extract were prepared using 10% Dimethyl Sulfoxide to dissolve both polar and non-polar phytoconstituents at a concentration of 10 mg/ml. An exception was made for the water extract, which was dissolved in sterile distilled water instead of DMSO.

Screening of antibacterial activity of plant extract against isolated pathogen

Sterile Petri dishes were filled with around 20 ml of either nutritional agar or Muller-Hinton Agar (MHA) to allow it to set. After the sterile cotton swab was used to seed the MHA plates with the 0.5 McFarland standard bacterial culture, the plates were left to dry. Six wells were created in each plate using sterile micropipette tips with a diameter of 6.0 mm. Then, 200 microliters of plant extract were applied to each well. To allow the extracts to diffuse into the agar, the plates were left at room temperature for 1-2 hours before being incubated at 37°C for 24 hours. The inhibition zones surrounding the wells were measured in millimetres after incubation.

Estimation of MIC of plant extracts by agar micro-dilution method

The efficacy of plant extracts against *Staphylococcus aureus* was assessed by determining the MIC using the broth micro-dilution method. This procedure was performed in a 96-well microtitre plate, following minimal modifications based on the standard CLSI method (Kahsay *et al*., 2014). 100 microliters of Mueller-Hinton agar broth was distributed into all wells of the plate, excluding column 1. Following the process of labelling the plate and cover, a volume of 200 µl of plant extract with a concentration of 100 mg/mL was introduced into column 1. Subsequently, a volume of 100 µl was moved from column 1 to column 2 to achieve a twofold dilution. This process was then repeated successively until the 10th column. Identical guidelines were used to the series, resulting in the removal of 100 µl from column 10. Columns 1-11 were filled with 100 µl of the 0.5 McFarland standard bacterial culture using a multipipettor. Column 12 was designated as a sterility control. The plates were incubated at a temperature of 37°C for a period of 18-24 hours. Following the incubation period, a volume of 40 µl of Resazurin dye at a concentration of 0.001% was introduced into each well. The plates were subsequently placed in an incubator for an extra duration of 2 hours. The transition from the colour blue to pink signified the presence of viable microorganisms.

RESULTS AND DISCUSSION

Isolation and characterization of *Staphylococcus aureus* from skin lesion of horses

Bacterial culture

Yellow coloured, smooth, concave colony appeared on nutrient agar plate containing 10% sodium chloride after aerobic incubation of culture from nutrient broth at 37°C for 24 hours. Thereafter, a colony from nutrient agar transferred to Mannitol salt agar and yellow coloured colonies appeared on MSA agar which changed colour of the agar from pink to yellow due to fermentation.

Morphological features

Gram staining revealed presence of gram positive cocci in form of bunches of grapes like or cluster.

Biochemical test results

The organism fermented lactose, maltose, fructose, dextrose, galactose, trehalose, sucrose, mannose and mannitol, but was unable to ferment xylose, melibiose, raffinoseand l-arabinose. The organism showed positive results for catalase but negative result for indole test. Likewise, Kumar *et al*. (2019) reported that 14 isolates of *Staphylococcus* isolated out of 15 (isolated from canines) were positive for Mannitol, Lactose, Trehalose, Maltose, VP test, AP test, ONPG, Urease and Arginine but negative for Sucrose, Arabinose and Rafinose. In same line, Muktha *et al*. (2015) reported that *Staphylococcus aureus* isolated from respiratory track of horse were fermented five basic sugar (Dextrose, Sucrose, Lactose, Maltose, Mannitol) and positive for catalase and coagulase test.

Bacterial DNA isolation and PCR test

The bacterial DNA that was obtained was subjected to PCR amplification, specifically targeting the 16S rRNA genes. After performing the polymerase chain reaction (PCR), the resulting products were examined using agarose gel electrophoresis with a concentration of 1.5%. Subsequently, the samples were stained with ethidium bromide. Characteristic bands at 1250 base pairs were seen during this technique, as depicted in Fig 1.

Antibiotic sensitivity pattern

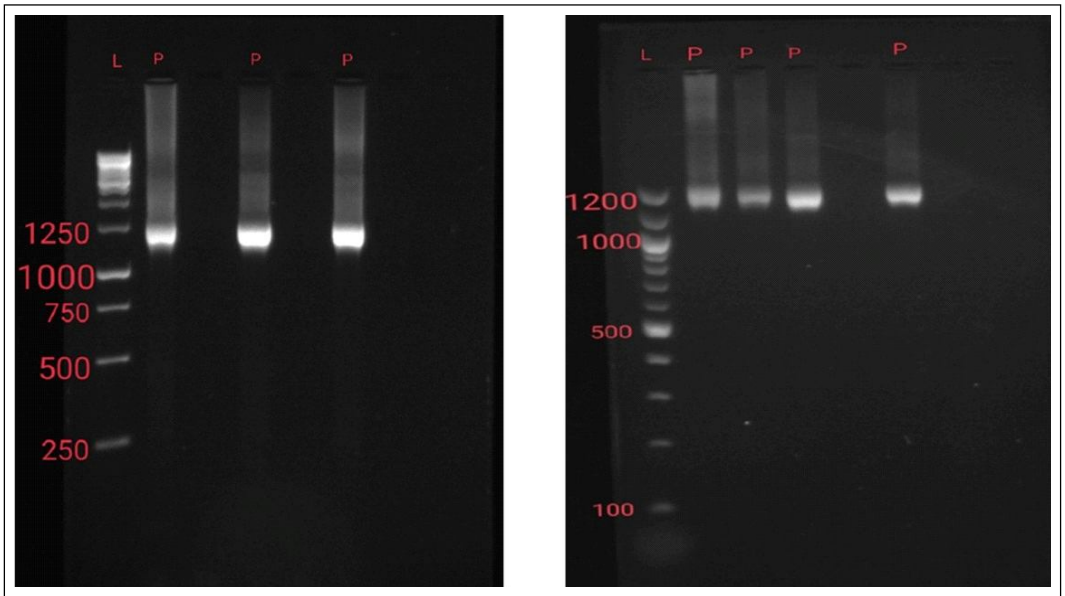
Results of the antibiotic sensitivity testing using disc diffusion technique. The isolate was sensitive for amoxicillin+ clavunic acid, co-trimaxazole, ceftriaxoneand ciprofloxacin but resistant for penicillin and cefixime. According to Kahsay *et al*. (2014), majority (>80%) of the *Staphylococcus aureus* isolates were resistant penicillin G, ampicillin, amoxicillin, gentamicin, erythromycin and cotrimoxazole antibiotics and less than 50% of isolates were resistant to vancomycin, oxacillin, tetracycline and clindamycin.

Extractability percentage of plant products

Aqueous, methanolic, ethanolic, chloroformand petroleum ether extracts of the plant materials were prepared following the methodology outlined in the methedology section. Among the five solvents employed, water proved to be the most effective extractant for the leaves of *C. decidua*, *A. javanica*, *C. gigentean* and *E. camaldulensis*, outperforming the other solvents. This finding is consistent with the results of previous research (Jahan *et al*., 2011; Al-Ghamdi, 2022), which similarly reported superior extraction efficiency of water for *A. javanica* and *E. camaldulensis*. Conversely, ethanol emerged as a more effective solvent for the extraction of bioactive compounds from the leaves of *L. pyrotechnica* and *A. vera* compared to the other solvents used.

Screening for antibacterial properties of plant extracts

The antimicrobial efficacy of several botanical extracts was evaluated by an agar-well diffusion assay conducted on Muller-Hinton agar (Fig 2). The process was repeated three times and the average (± standard error) diameter of the zone of inhibition for each herbal extract was computed (Table 1). Among the extracts tested, only the aqueous methanol, ethanol, chloroform and petroleum ether extracts from *E. camaldulensis*, as well as the methanol, ethanoland chloroform extracts from *A. indica*, exhibited activity against *S. aureus*. However, the aqueous and petroleum ether extracts of *A. indica* did not show any inhibition zone against this bacterium. In a previous study (Jahan *et al*., 2011) similar activity of methanolic, ethanolic and aqueous extracts of *E. camaldulensis* leaves is reported with smaller inhibition zones than the present study. Preliminary studies by other researchers have also shown significant antibacterial effects of Eucalyptus extracts against various bacterial strains (El-Mahmood, 2010; Shagal *et al*., 2012; Ishag *et al*., 2018; Ali *et al*., 2019). On the other hand, ethanolic and methanolic extracts of *A. vera* exhibited antibacterial activity against this bacterium, whereas petroleum ether, choloroform and aqueous extracts do not show antimicrobial activity. Similar results were achieved by other authors (Arunkumar and Muthuselvam, 2009; Bashir *et al*., 2011; Danish *et al*., 2020; Mehrishi *et al*., 2022). Different *A. vera* accessions exhibited the presence of phenolic compounds, alkaloids, glycosides, flavonoids, reducing sugarand tannins (Kumar *et al*., 2016). So, the absence of antimicrobial activity of chloroform, petroleum ether and aqueous extracts of *A. vera* leaf may be due to the lower amount of phytochemical extracted with these solvents. Likewise, all five extracts, *i.e*., aqueous, methanolic, chloroform and petroleum ether, of the leaves of *C. decidua, A. javanica, C. gigentean* and *L. pyrotechnica*, do not reveal antibacterial activity against *S. aureus*. Antibacterial activity

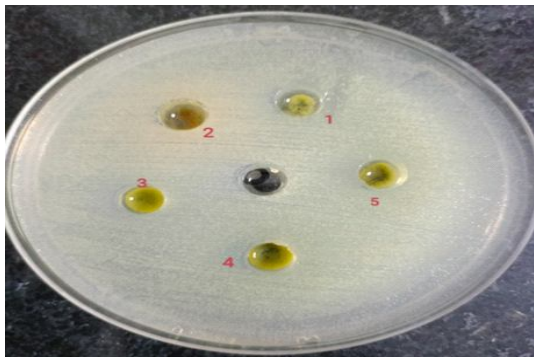


L- DNA ladder (1000 bp and 100 bp), P- Positive for bacterial isolate.

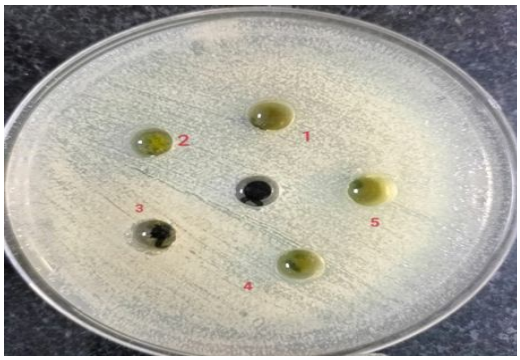
Fig 1: Electrophoretic pattern in 1.5% agarose gel showing the amplified product at 1250 bp (16s rRNA gene) for *Staphylococcus aureus*.

Table 1: Zone of inhibition and MIC of different plant extracts against *S. aureus.*

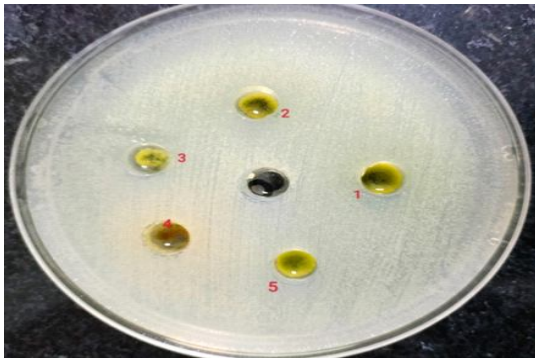
Plant	Solvent used	Zone of inhibition (mm)*	MIC (mg/ml)
A. indica	Chloroform	15±0.57	25 mg/ml
	Ethanol	17.33±0.33	6.25 mg/ml
	Methanol	17±0.57	6.25 mg/ml
A. vera	Ethanol	12.33±0.33	25 mg/ml
	Methanol	9.66±0.88	12.5 mg/ml
E. camaldulensis	Chloroform	21±0.57	1.56 mg/ml
	Ethanol	19.66±0.88	1.56 mg/ml
	Methanol	19.66±0.88	1.56 mg/ml
	Petroleum ether	18±0.57	3.13 mg/ml
	Aqueous	15±0.57	1.56 mg/ml



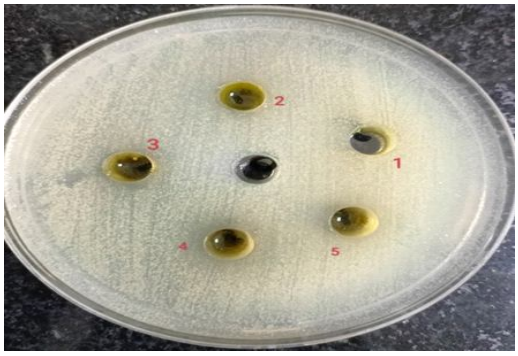
C. deciduas



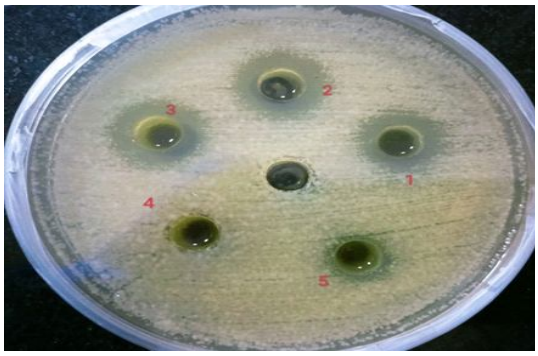
C. gigantean



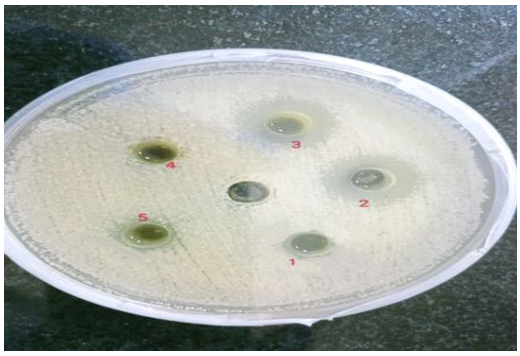
L. pyrotechnica



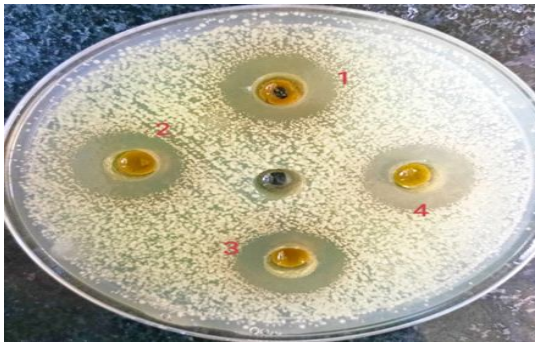
A. Javanica



A.indica



A.vera



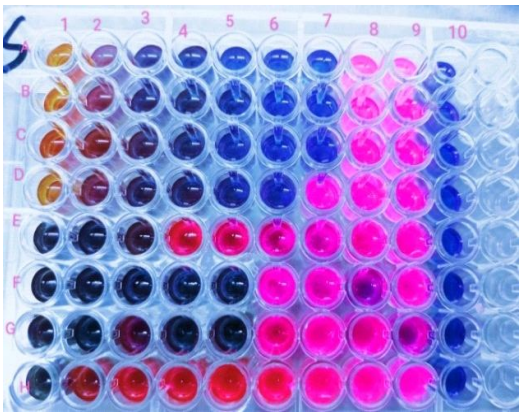
Eucalyptus camaldulensis



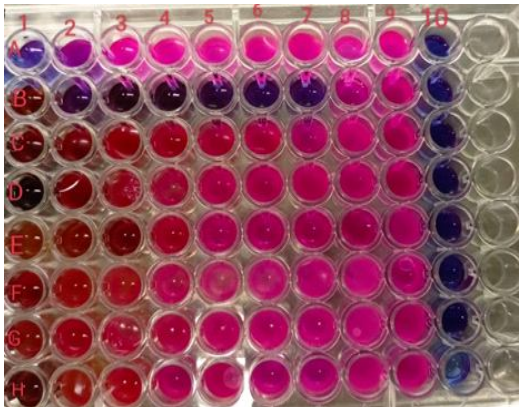
Aqueous extract *E. camaldulensis,* in centre

1: Chloroform extract; 2: Ethanolic extract; 3: Methanolic extract; 4: Petroleum ether extract
5: Aqueous extract.

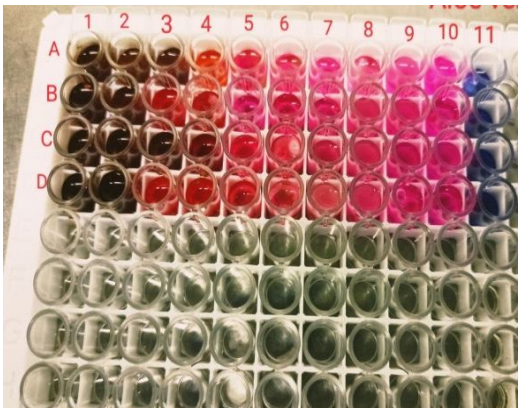
Fig 2: Assessment of antibacterial efficacy of plant extracts against *S. aureus* utilizing the agar well diffusion technique.



A: Chloroform extract of *E. camaldulensis*; B: Methanolic extract of *E. camaldulensis*; C: Ethanolic extract of *E. camaldulensis*; D: Petroleum ether extract of *E. camaldulensis*; E: Chloroform extract of *A. indica*; F: Methanolic extract of *A. indica*; G: Ethanolic extract of *A. indica*; H: Petroleum ether extract of *A. indica*



A: DMSO (100%); B: Aqueous extract of *E. camaldulensis*; C: Aqueous extract of *A. vera*; D: Aqueous extract of *A. indica*; E: Aqueous extract of *L. pyrotechnica*; F: Aqueous extract of *L. pyrotechnica*; G: Aqueous extract of *A. javanica*; H: Aqueous extract of *C. decidua*



A: Chloroform extract of *A. vera*; B: Methanolic extract of *A. vera*; C: Ethanolic extract of *A. vera*; D: Petroleum ether extract of *A. vera* Concentration of plant extract:- 100 mg/ml; 2. 50 mg/ml; 3. 25 mg/ml; 4. 12.5 mg/ml; 5. 6.25 mg/ml; 6. 3.125 mg/ml; 7. 1.562 mg/ml; 8. 0.7812 mg/ml; 9. 0.3906 mg/ml

Fig 3: Evaluation of MIC of different extracts of selected plants against *S. aureus.*

of plant extract depends on the presence of various phytochemicals such as flavonoids, ellagic acids, stilbenes, anthraquinones, chalcones, ellagitannins and phenolic acids in the extract and is affected by various factors such as extraction technique or solvent, growing conditions, germplasm, climatic factors, the part of the plant used and the time of collection (Gull *et al*., 2015; Alghamdi and Ababutain, 2019; Sharma *et el*., 2022; Kumar *et al*., 2023). It was observed in the present study that alcoholic (Ethanolic or methanolic) extracts of plants have more antibacterial activity than the petroleum ether or water extracts, it shows that antibacterial compounds have medium polarity like alcohols. Among all plants studied in the present study,most important is the antibacterial activity of the aqueous extract of the *E. camaldulensis*. So leaves of this plant have more potential to be used directly by the farmers for disinfection against *Staphylococcus aureus* and probabaly for other bacteria also. *Staphylococcus aureus* is also an important bacteria for the mastitis in cattle show leaves of *Staphylococcus aureus* can be utilized for the prevention of mastitis in cattle also. If further study on animal cells suggests that antibactyerial potential is being shown by non cytotoxic concentration for mammalian cells than potential of this plant can be used for wound management and skin infections.

Estimation of MIC of plant extracts

The MIC of different plant extracts showing antimicrobial activity in screening test were estimated using broth dilution technique using 96 well micro-titre plate (Fig 3). The MIC for all the extracts presented in (Table 1). The lowest MIC recorded for chlorofom, methanol, ethanol and aqueous extract of *Eucalyptus camaldulensis* and highest MIC recorded for chloroform extract of *Azadirachta indica* and ethanolic extract of *Aloe vera* against *Staphylococcus aureus*.

CONCLUSION

In vitro antibacterial activity shown by *E. camaldulensis, A. indica* and *A. vera* leaf extracts against *S. aureus* suggests there these plants have potential to be used therapeutically in horses for skin infection and wounds. So there is need to study the cytotoxicity of these plants on mammalian to decide non-cytotoxic concentrations for further *in vivo* study and therapeutic efficacy.

ACKNOWLEDGEMENT

This work is a part of the National Livestock Mission Project “Utilization of desert plants for the treatment of skin disease in horses.” We are grateful to the Department of Animal Husbandry and Fisheries, Govt. of India for providing funds to conduct our research work. We are also thankful to Dr. Praveen Malik, commissioner of the Department of Animal Husbandry and Fisheries, Govt. of India for valuable guidance and help. We would like to acknowledge the support provided by the National Research Centre on Equine, Equine Production Campus, Bikaner, Rajasthan, India.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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