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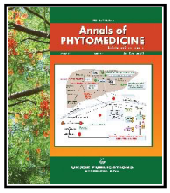

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Evaluation of the *in vitro* antibacterial activity of some plant extracts against *Dermatophilus congolensis* isolated from skin lesions of horses

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Abstract

The purpose of this research was to isolate and identify *Dermatophilus congolensis* from skin infections in horses in Rajasthan using conventional methods and polymerase chain reaction and to assess the efficacy of some selected plants against *D. congolensis* using agar well diffusion and broth microdilution techniques. The presence of *D. congolensis* was confirmed by the "Tram track" appearance of coccoid forms in culture-stained smears as well as colony characters and biochemical tests. The existence of *D. congolensis* was also confirmed by the resulting 500 bp PCR amplicon. The zone of inhibition ranged from 13 mm to 21 mm, and the minimum inhibitory concentration (MIC) ranged from 1.562 mg/ml to 3.125 mg/ml for all the extracts of *Eucalyptus camaldulensis* when tested against *D. congolensis*. Zones of inhibition of 15 mm to 21 mm (MIC-3.125 to 6.25 mg/ml) and 11 mm (MIC-25 mg/ml) were seen for *Azadirachta indica* (chloroform, methanolic and ethanolic extract) and *Aloe vera* (methanolic extract), respectively, when tested on this pathogen. To combat the issue of antibiotic resistance, the results of this study suggest that *E. camaldulensis*, *A. indica*, and *A. vera* can be used or incorporated into topical antibacterial medicines.

1. Introduction

Primary skin illness or a systemic ailment may be indicated by a lesion on the skin (Knottenbelt, 2012). The aesthetic value of horses is diminished due to the prevalence of skin lesions such as crusting, scaling, pruritis, photosensitization, and abscesses. *Dermatophilus congolensis*, particularly common in ruminants and horses, is a gram-positive filamentous actinomycete that causes exudative dermatitis in a wide range of species, causing opacified pustules (brush-like appearance) on the neck, body, and skin. Dermatitis, including alopecia that appears on the legs and death from severe envenomation, all of which are accelerated by prolonged wetting of the skin by ticks (Gitao *et al*., 1998; Origgi *et al*., 1999; Woldemeskel and Ashenafi 2003; Rad *et al*., 2004; Burd *et al*., 2007; Byrne *et al*., 2010.)

Researchers such as Pal (1995) and Awad *et al*. (2008) had previously reported finding *D. congolensis* in skin infections of Indian horses. Penicillin at 20000 units/kg body weight alone or in combination with streptomycin at 10 mg/kg body weight intramuscularly for 3 days or trimethoprim sulfa orally until 7 days past clinical remission, is the standard treatment for equine dermatophilosis (Anthony, 2016). The administration of such antibiotics parenterally for an extended period in horses has been linked to gut microbial disturbance and, ultimately colitis (Jimenez, 2021).

Currently, various phyoconstituents of plants are used for the treatment of multidrug-resistant (MDR) microbial pathogens (Shamna and Poyil, 2021; Seshadri, 2021). Horse farmers usually employ topical herbal medicines made from a wide variety of plants to treat skin infections, but no research has been done to test whether or not these plants have any antibacterial action against *D. congolensis*. Therefore, we chose to isolate and identify *D. congolensis* and evaluate the *in vitro* antibacterial activity of extracts from several plants (*Capparis decidua, Aerva javanica, Calotropis gigantea, Leptadenia pyrotechnica, Azadirachta indica, Aloe vera,* and *Eucalyptus camaldulensis*) against *D. congolensis*.

2. Materials and Methods

2.1 Sample collection and transportation

From June 2022 to October 2022, horses with clinical signs of hair knots and thick scabs on the back, legs, and face were collected from different geographical locations in Rajasthan, Haryana, and Gujarat, India. A total of 70 samples were collected under aseptic conditions using sterile cotton swabs placed in tubes containing phosphate-buffered saline and nutrient broth, followed by temperature maintenance at 4°C in an ice box. Ethical approval for this study was obtained from the Institutional Animal Ethics Committee of CVAS, Bikaner (Rajasthan) vide order no. CVAS/IAEC/2022-23/24.

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2.2 Identification and isolation of the bacterial pathogen

Because the motile zoospores of *D. congolensis* are chemotactic to carbon dioxide, they rise to the surface of the water after being ground up after 3 h. The test tube containing the skin scrapings is incubated in a candle jar for 30 min at room temperature, after which a loopful of fluid from the surface is inoculated in nutrient broth and incubated at 37°C for 72 h. Bacterial pathogens were assayed on a variety of selection and differentiation media, including 5% horse blood agar and brain heart infusion agar containing 0.25 g/ml malachite green, 10 g/ml nalidixic acid and 200 IU/ml nystatin, and their phenotype was analyzed.

After incubation, each culture was analyzed for signs of growth and recognized based on its unique morphology on the media, Gram, or Geimsa staining reactions, and the results of biochemical tests such as the carbohydrate fermentation test, catalase test, and indole test. The catalase and indole tests were conducted in accordance with Mannan *et al*. (2009), and the carbohydrate fermentation test was conducted using the HiCarbohydrate TM kit (KB009A). Polymerase chain reaction (PCR) was used to confirm the preliminary identifications of the species.

2.2.1 Extraction of bacterial genomic DNA

DNA of bacterial pathogen was extracted using commercially available DNA-Sure Blood Mini Kit NP-61107 Genetix Biotech Asia Pvt. Ltd (New Delhi) by following the manufacturer's instructions.

2.2.2 PCR amplification of 16S rRNA genes

The PCR reaction was performed using *D. congulensis* specific 16S rRNA primer (Forward strand primers 5'ACATGCAAGTCGAACG ATGA3' and reverse strand primers 5'ACGCTCGCACCCTACGTA TT3') for the identification of *D. congolensis* according to the method described by Chitra *et al*. (2017).

2.3 Antibiotic susceptibility assay

Clinical and Laboratory Standards Institute (CLSI) guidelines and the method described by Kahsay *et al*. (2014) were followed in conducting antibiotic sensitivity testing on *D. congolensis* by the disc diffusion method. In this analysis, we calculated antibiotic susceptibilities to amoxicillin/clavulanic acid (30 g), co-trimoxazole (25 g), ceftriaxone (30/10 g), ciprofloxacin (5 g), penicillin (10 g) and cefixime (10 g).

2.4 Preparation of plant extracts using various solvents

Capparis decidua (Kair), *Calotropis gigentea* (Milkweed), *Leptadenia pyrotechnica* (Khimp), *Aerva javanica* (Kapok bush), *Azadirachta indica* (Neem), *Aloe vera* (Gwarpatha) and *Eucalyptus camaldulensis* (Safeda) (all shown in Figure 1) were among the plants whose leaves were gathered. The gathered leaves were then rinsed thoroughly under running water to get rid of any remaining dirt and debris. All plant leaves were collected, let to air-dry at room temperature in the shade, then ground into a fine powder. For 72 h, 20 g of plant powder was submerged in 400 ml of distilled water, chloroform, petroleum ether, ethanol, and methanol. A sterilized sonicator was used to agitate each mixture once every 24 h. After the extraction was complete, the filtrate was concentrated in a vacuum using a rotatory evaporator after being filtered with Whatman No. 1 filter paper (Whatman, England). Each plant extract was then dissolved in sterile 10% DMSO to create a stock solution (10 mg/ml). However, water extract dissolves in distilled water.

Leaves of *C. decidua*

Leaves of *A. javanica*

Leaves of *L. pyrotechnica*

Leaves of *C. gigantea*

Leaves of *A. indica*

Leaves of *A. vera*

Leaves of *E. camaldulensis*

Figure 1: Leaves of different plants used in present study.

2.5 Screening of antibacterial activity of plant extract against isolated pathogen

After sterilizing the Petri dishes, around 20 ml of sterile Muller-Hinton Agar (MHA) or Nutrient agar was placed into each one. After the MHA plates had dried, a sterile cotton swab was used to inoculate them with a 0.5 MacFarland standard bacterial culture. Using sterile micropipette tips (6.0 mm in diameter), we drilled six wells into a plate and labeled them in preparation for the agar well diffusion procedure. Each well then had 200 µl of the plant extract added to it. The plates were then kept at room temperature for 1-2 h to allow the extracts to diffuse into the agar and then incubated at 37°C for 24 h. Thereafter, an inhibition zone around each well was measured throughout all the plates.

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

The minimum inhibitory concentration (MIC) for *D. congolensis* was determined by testing plant extracts using a modified broth micro-dilution method in a 96-well-containing microtiter plate. All of the wells in a microtiter plate except for column 1 were filled with 100 µl of medium (MHA broth) using a multi pipettor. The dish and its cover were clearly labeled. Column 1 (far left on the plate) received 200 µl of plant extract solutions at 100 mg/ml. From column 1, 100 µl of the plant extract solution was withdrawn and added to column 2 and pipetted 4-5 times to mix up. This effectively doubles the dilution of column 2. Following this, 100 µl of the solution in column 2 was moved to column 3. I did this until I reached the 10^{th} column and then stopped. The entire dilution series can be handled with the same set of hints. From column 10, throw away 100 µl of the solution instead of moving it to column 11. Then, columns 1 through 11 had 100 µl of an isolated bacterial culture (at the 0.5 McFarland standard) pipetted into each well using a multi pipettor. Column 12 (the blank for the plate scanner and the sterility control) did not have any bacteria culture put to it. The plates were then incubated at 37°C for 18-24 h. When turbidity appeared after the allotted incubation time, we added 40 µl of 0.001% resazurin dye to each well and put the plate back in the incubator for another 2 h. Seen the living bacteria cause a change in color from blue to pink in a well.

3. Results

3.1 Isolation and characterization of *D. congolensis* from skin lesions of horses

3.1.1 Bacterial culture

In the confirmed cases, nutrient broth became cloudy, and sediment formed. After anaerobic incubation of culture from nutritional broth at 37°C for 48-72 h under 5-10% CO_2, a whitish-gray colored, elevated, rough, and adherent colony developed on 5% horse blood agar with malachite green-0.25g/ml, nalidixic acid-10 g/ml, and nystatin-200 IU/ml (Figure 2). Then, the nutritional agar colony was transferred to brain heart infusion agar and broth for subculturing. After that, we confirmed the bacterium with gram staining and biochemical tests.

Figure 2: Growth of *D. congolensis* on 5% horse blood agar.

3.1.2 Morphological features

Giemsa's staining of *D. congolensis* revealed the presence of a typical tram-track appearance of the coccoid form depicted in Figure 3. In Gram's staining, these bacteria were less visible.

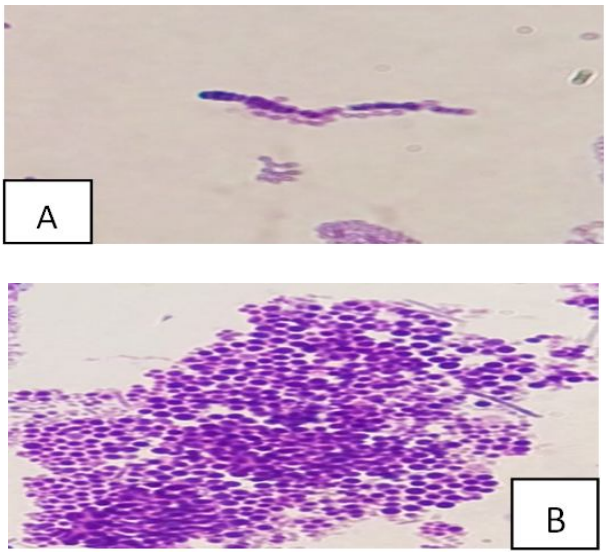
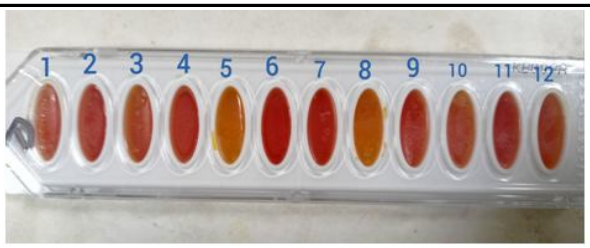
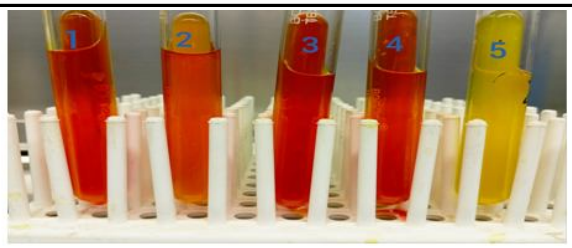


Figure 3: Typical 'tram track apearance of *D. congulensis* in Figure A and coccal form in Figure B.

3.1.3 Biochemical test results

Results of various biochemical tests shown in Figure 4, 5 and 6 revealed organism fermented dextrose and trehalose, but was unable to ferment sucrose, mannitol, fructose, arabinose, sorbitol, lactose, maltose, galactose, xylose, melibiose, and raffinose.





(1.Lactose, 2.Xylose, 3.Maltose, 4.Fructose, 5.Dextrose, 6.Galactose, 7.Raffinose, 8.Trehalose, 9.Melibiose, 10.Sucrose, 11.L-Arabinose, 12.Mannose) (1.Mannitol, 2.Sucrose, 3.Lactose, 4.Maltose, 5.Dextrose)

Figure 4: Carbohydrate fermentation test for *D. congolensis.*

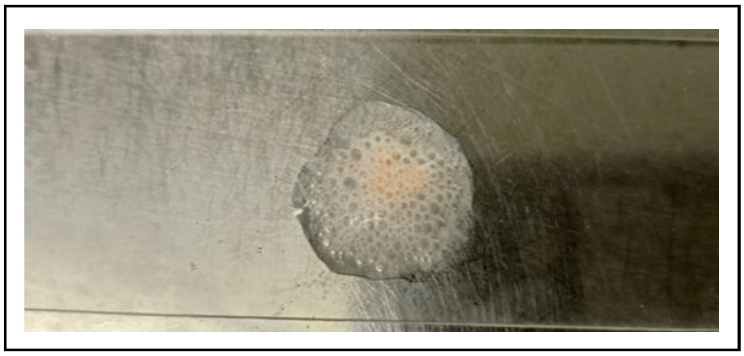


Figure 5: Catalase test for *D. congolensis*.

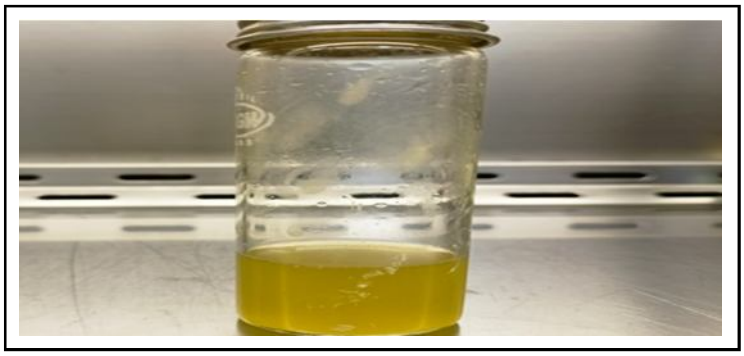


Figure 6: Indole test for *D. congolensis.*

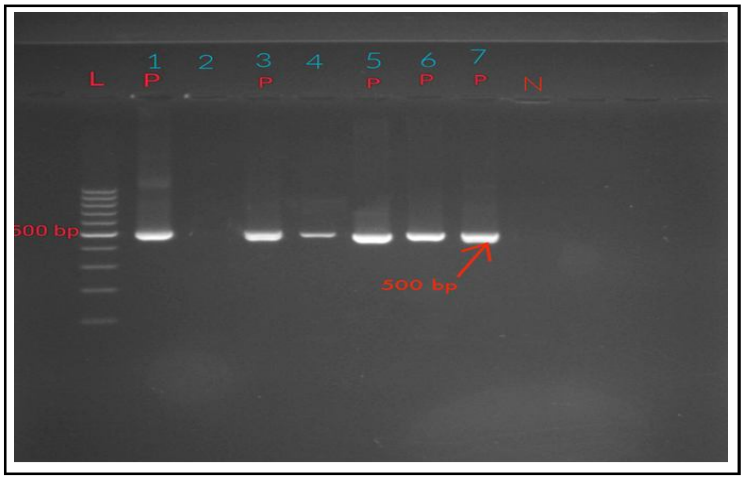


Figure 7: Electrophoretic pattern in 1.5% agarose gel showing the amplified product at 500 bp (16s rRNA gene) for *D. congolensis*.L-DNA ladder (100bp), P- Positive for bacterial isolate, N-Negative control.

3.1.4 Bacterial DNA isolation and PCR test

The results of PCR for *D. congolensis* isolated from horses are shown in Figure 7. This indicates the amplification of a partial 16S rRNA gene band of approximately 500 base pairs (bp).

3.2 Antibiotic sensitivity pattern

The results of the antibiotic sensitivity testing using the disc diffusion technique are depicted in Figure 8. The isolate was sensitive to amoxicillin + clavulanic acid, co-trimaxazole, ceftriaxone, and ciprofloxacin but resistant to penicillin and cefixime.

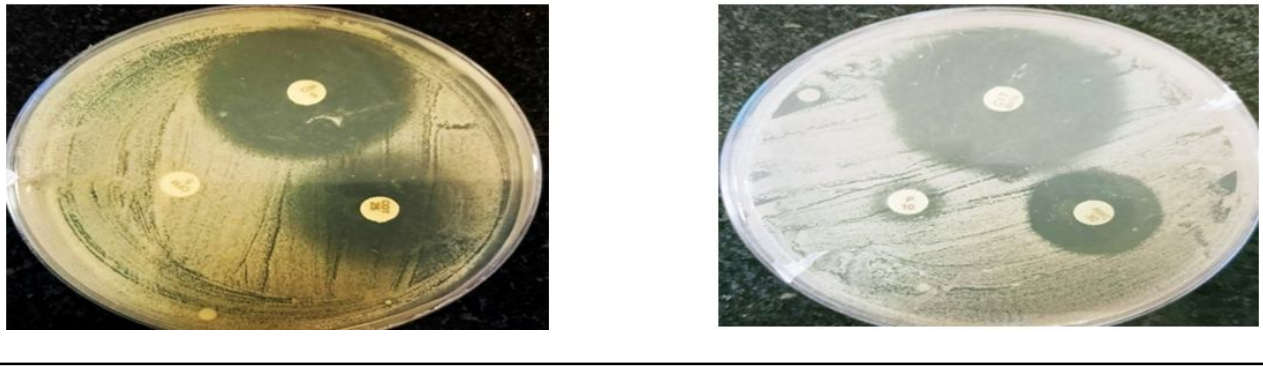
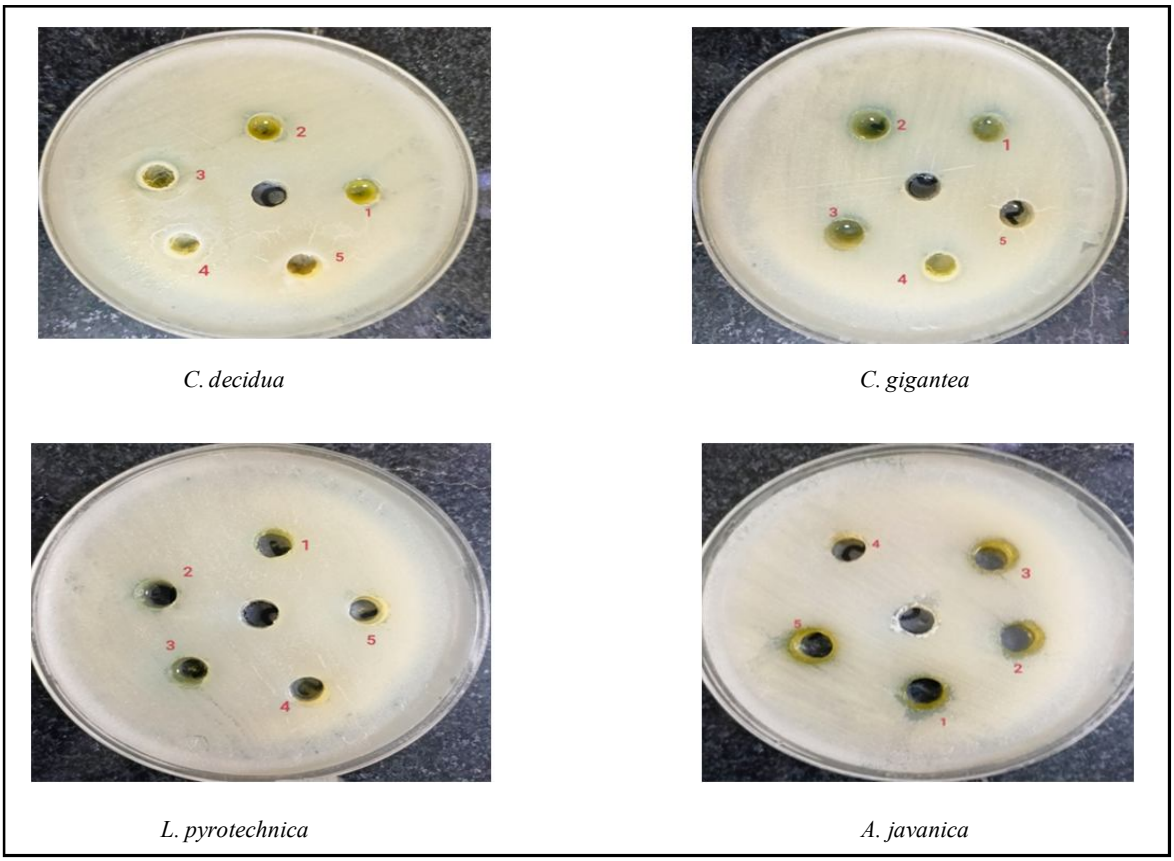
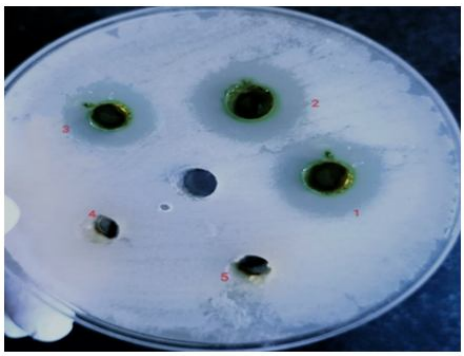


Figure 8: Antibiotic sensitivity pattern of *D. congolensis*.

3.3 Screening for antibacterial properties of plant extracts

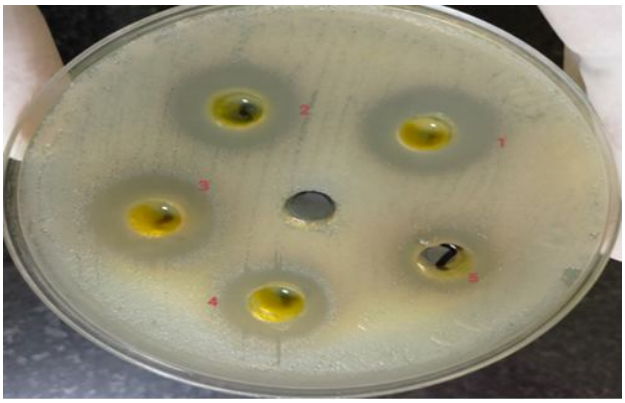
The agar well diffusion test on Muller Hinton agar was used to determine the antibacterial activity of various plant extracts (Figure 9). Three replicates were performed and the standard error of the mean diameter of the inhibitory zone for each herbal extract is shown in Table 1. Against *D. congolensis*, only methanolic and chloroform extract of *A. indica* and all extracts of leaves of *E. camaldulensis* showed activity, while ethanolic, petroleum ether, and aqueous extract of *A. indica* showed no zone of inhibition. Among all extracts of *A. vera*, only methanolic *A. vera* extract is the only one proven effective against *D. congolensis* in laboratory settings. Leaves of *C. decidua, A. javanica, C. gigantea*, and *L. pyrotechnica* were extracted in water, methanol, ethanol, chloroform, and petroleum ether, and none of them showed antibacterial action against *D.congolensis*.





A. indica

A. vera



E. camaldulensis

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

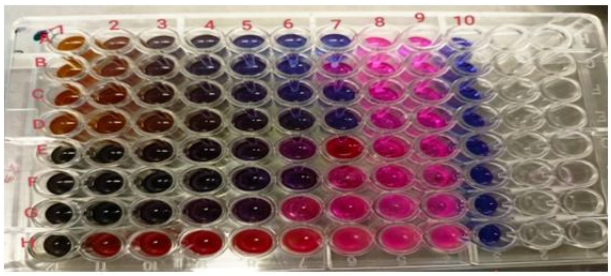
Figure 9: Determination of antibacterial activity of plant extracts against *D. congolensis* using agar well diffusion method.

3.4 Estimation of MIC of plant extracts

Figure 10 shows the results of a broth dilution assay utilizing a 96-well microtiter plate to determine the MICs of several plant extracts that showed antibacterial activity in a screening test. MIC values for all of the samples are shown in Table 1. Chloroform, methanolic, petroleum ether and aqueous extract of *E. camaldulensis* showed the lowest MIC against *D. congolensis*, while *A. vera* methanolic extract showed the highest MIC. The MIC has not been seen at concentrations of plant extract up to 100 mg/ml for any of the other plants tested (*C. decidua*, *A. javanica, C. gigantea*, and *L. pyrotechnica*).

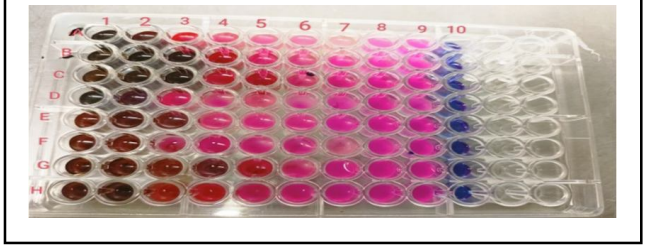
Table 1: Zone of inhibition and MIC of plant extracts against *D. congolensis*

S.No.	Plant	Solvent used	Zone of inhibition (mm)*	MIC (mg/ml)
1.	*A. indica*	Chloroform	20.66 ± 0.33	3.125 mg/ml
		Ethanol	22.00 ± 0.57	3.125 mg/ml
		Methanol	15.00 ± 0.57	6.250 mg/ml
2.	*E. camaldulensis*	Chloroform	19.33 ± 0.33	1.562 mg/ml
		Ethanol	17.00 ± 0.57	3.125 mg/ml
		Methanol	21.66 ± 0.33	1.562 mg/ml
		Petroleum ether	16.33 ± 1.20	1.562 mg/ml
		Aqueous	13.00 ± 0.57	1.562 mg/ml
3.	*A. vera*	methanol	11.00 ± 0.577	25.00 mg/ml



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 *C. gigentean*; 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* ; E: Chloroform extract of *A. javanica*; F: Methanolic extract of *A. javanica*; G: Ethanolic extract of *A. javanica*; H: Petroleum ether extract of *A. javanica.*

Concentration of plant extract: 1. 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.

Figure 10: Evaluation of MIC of different extracts of selected plants against *D. congolensis.*

4. Discussion

In the present study, the colonies of *D. congolensis* were confirmed microscopically by their typical "Tram-track appearance''. The results of microscopic examinations were very much similar to that reported and published in a study by Saravanan and Palanivel (2019) in which *D. congolensis* were demonstrated on the stained impression smear as gram-positive branching cocci as double rows of zoospores (tram-track appearance) and packets of coccoid forms and colony characteristics were similar to results of Amor *et al.* (2011) and Chitra *et al*. (2017). In agreement with the study by Mannan *et al*. (2009), our biochemical tests show that *D. congolensis* fermented dextrose and trehalose but not sucrose, mannitol, fructose, arabinose, sorbitol, lactose, maltose, galactose, xylose, melibiose, and raffinose. However, different researchers have reported varied findings when testing the same organism (*D. congolensis*) with maltose, galactose, sucrose, fructose, and other reagents (Shaibu *et al*., 2011). Microscopy, colony characteristics, and biochemical testing all pointed to the presence of the *D. congolensis* organism; this was later confirmed by polymerase chain reaction (PCR), which is widely regarded as a more reliable, sensitive, specific, accurate, rapid, and inexpensive method for isolating and identifying bacterial pathogens. Due to the highly conserved nature of the 16S rRNA gene in bacteria, it was used as a second confirmation method in the present investigation. This method yielded a band at 500 bp for *D. congolensis* but none for the negative control. All samples of scabs and cultures suspected of containing *D. congolensis* were amplified along a 500 bp section of 16S rRNA, as reported by Chitra *et al*. (2017). *D. congolensis* isolates were shown to be susceptible to amoxicillin + clavulanic acid, ciprofloxacin, co-trimoxazole, and ceftriaxone, but resistant to penicillin and cefixime, according to the antibiogram results of the current investigation. However, our results are not in agreement with Mannan *et al*. (2009), who observed that 70% of the isolates were resistant to ciprofloxacin, and are in agreement with Smsek *et al*. (2022) and Domingues *et al*. (2018).

There may be a connection between genomic changes, chromosomal mutation of the causal agent, and the frequent use of antibiotics, as suggested by the findings of Mannan *et al*. (2009).

Herbal medicines are gaining popularity for the treatment of a wide range of diseases and conditions, including skin disorders, as conventional antibiotics become less effective and antibiotic resistance among common bacteria increases. While these plants' antibacterial activity had previously been evaluated against other bacteria including *Staphylococcus aureus*, *E.coli*, *Streptococcus*, *etc.*, this was the first time it had been tested against *D. congolensis*. Only three of the seven plants tested in this study (*E. camaldulensis*, *A. indica*, and *A. vera*) showed antibacterial action against *D. congolensis*. Zone of inhibition values between 13 and 21.66 mm and MIC values between 1.562 and 3.125 mg/ml were observed for *E. camaldulensis* extracts in the current study, regardless of the solvent used to get the extract. This study's findings corroborate those of previous studies on the antibacterial properties of *E. camaldulensis* (El-Mahmood, 2010; Ali *et al*., 2020; Kumar *et al*., 2022), which found that ethanol, acetone, and water extracts of *E. camaldulensis* leaves exhibited antibacterial activity against isolates of *S. aureus*. Similar findings were reported by Shagal *et al*. (2012), who found that antibacterial activity was present in ethanol and water extracts of *E. camaldulensis* leaves against *S. aureus*, *Bacillus subtilis*, *E. coli*, and *Salmonella typhi*. Zones of inhibition ranging from 15 mm to 22 mm and minimum inhibitory concentrations (MICs) ranging from 3.125 to 6.250 mg/ml were observed for *A. indica* extracts used in this investigation when used against *D. congolensis*. The results of our findings are inconsistent with the various researcher (Francine *et al*., 2015; Pokhrel *et al*., 2015) who found that ethanolic and methanolic extract of *A. indica* showed antibacterial activity against *E. coli*, *Klebsiella*

pneumoniae, *S. typhi*, *Pseudomonas vulgaris*, *Pseudomonas aeruginosa,* and *S. aureus*. Similarly, Mankad *et al*. (2016) found that the synthesized zinc oxide nanoparticles from *A. indica* plant exhibited good antibacterial activity against plant pathogen, *Xanthomonas oryzae* pv. *oryzae*. Only the ethanolic extract of *A. vera* leaves demonstrated antibacterial action against this isolate, with a zone of inhibition between 9 and 12 mm and a minimum inhibitory concentration (MIC) between 12.5 and 25 mg/ml. In corroboration of the present findings, Danish *et al*. (2020) reported that ethanolic leaf extract of *Aloe vera* showed a zone of inhibition against gram-positive bacteria such as *B. subtilis, B. cereus, B. megaterium, Streptococcus pyogenes*, and *S. aureus* and against gram-negative bacteria such as *P. aeruginosa, E. coli*, and *Acinetobacter baumannii* at 30 µl volume. Plant extract's antibacterial activity is influenced by many factors, including the extraction technique, solvent used for extraction, germplasm, climatic variables, growing conditions, the part of the plant utilized, and the time of collection (Jahan *et al*., 2011; Gull *et al*., 2015).

5. Conclusion

Our research shows that the leaves of *E. camaldulensis, A. indica*, and *A. vera* have potent antibacterial activity against *D. congolensis*, suggesting that extracts from these plants may be used in topical antimicrobial preparations to treat bacterial infection caused by *D. congolensis*. The antibacterial action of these plants has been suggested, however, *in vivo* research is required to prove this.

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Conflict of interest

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

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