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ANTIMICROBIAL ACTIVITY OF A SELECTED MEDICINAL PLANT EXTRACTS MIXTURE USED IN THE MANAGEMENT OF CHRONIC SKIN WOUNDS IN AYURVEDIC MEDICINE

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ABSTRACT: The aim of the study was to determine the synergistic antimicrobial activity of a selected medicinal plant extract mixture composed of *Calotropis gigantean* (ElaWara, Family: Asclepiadaceae), *Datura metel* (Kalu Attana, Family: Solanaceae) and *Ricinus communis* (Erandu, Family: Euphorbiaceae) against selected gram positive and gram negative bacteria. In the study, antimicrobial activity was evaluated against two-gram positive organisms; *Staphylococcus aureus* (ATCC 95923) and group A *Streptococcus spp.* (ATCC 12384) and two gram-negative organisms; *Escherichia coli* (ATCC 95922) and *Pseudomonas aeruginosa* (ATCC 27853). Agar disc diffusion method was carried out to evaluate the antibacterial activity of the selected medicinal plant extract mixture, and the inhibition zone diameters were measured. Further, minimum inhibitory concentration (MIC) was evaluated using a broth microdilution method. The inhibition zones against *S. aureus* and group A *Streptococcus spp*. were observed. In contrast, inhibition zones were not demonstrated against selected gram-negative organisms. The MIC evaluated for *S. aureus* was 16 mg/mL by the broth dilution method. The results revealed that the selected medicinal plant extract mixture exerted synergistic antimicrobial activity against *S. aureus* and group A *Streptococcus spp*. However, antimicrobial activity against *E. coli* and *P. Aeruginosa* was not significant. Results were able to scrutinize the therapeutic benefits of the selected medicinal plant extracts mixture in the management of chronic skin wounds in Ayurvedic medicine. Also, the plant extract mixture may offer valuable natural drug leads for the development of a commercially viable herbal antimicrobial formulation in the future.

Keywords: Broth dilution method, Disc diffusion method, Medicinal plant extract mixture, Minimum inhibitory concentration, Sri Lankan Ayurveda medicine, Synergistic antimicrobial activity

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INTRODUCTION: Throughout history, man has had to contend with different kinds of skin wounds. Healing of chronic wounds, including pressure sores, leg ulcers, diabetic foot ulcers, and other kinds of wounds, by secondary intention, are common in the community and clinical settings [1]. The management of chronic wounds represents a significant proportion of health-care resources and makes up a substantial amount of patient contact time in a clinical setup. The treatment of chronic wounds includes many strategies, including the use of various wound dressings, antimicrobial agents

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such as antibiotics, footwear, physical therapies, and educational strategies. Use of antimicrobial agents for chronic wound care is promising than any other strategy [2, 3]. There is a wide range of antimicrobial agents with proven antimicrobial properties. In the past, acetic acid, chlorhexidine, honey, hydrogen peroxide, sodium hypochlorite, potassium permanganate, and proflavine have been used; however, the discovery and development of antibiotics during the 20th century has revolutionized the clinical therapy [4, 5]. Antibiotics are one of the most important weapons in fighting bacterial infections.

However, over the past few decades, these health benefits are under threat as many commonly used antibiotics have become less effective against certain conditions due to their toxic reactions and the emergence of drug-resistant bacteria [6]. The development of bacterial resistance is a huge problem since it is often not restricted to the specific antibiotic prescribed, but generally extends to other compounds of the same class [7]. Bacterial resistance and its rapid increase are the major concerns of global public health and are emerging as significant challenges to human health [8]. However, the relentless emergence of antibiotic-resistant strains of pathogens together with the retarded discovery of novel antibiotics has led to discovering antimicrobial agents specifically from the herbal origin. In this sense, natural antimicrobials seem promising to many of the problems associated with the currently used antimicrobial agents in the management of wound infections. Unlike synthetic drugs, antimicrobials of plant origin are unlikely associated with side effects and exert a sufficient therapeutic potential to cure many infectious diseases [9, 10].

Finding healing powers in plants has a long history that began with folk medicine, and through the years, it has been incorporated into traditional and allopathic medicine. The mystique surrounding concepts in those traditional remedies has already led to an explosion of potential drug targets, and numerous approaches have been developed to capture their intrinsic value. Among known plant species on the earth, only a small fraction has been investigated in the presence of antimicrobial compounds.

In fact, during the last few years, medicinal plants especially crude medicinal plant extract mixtures have attracted the attention of pharmaceutical and scientific communities, and evidence has demonstrated on promising potential of anti-microbial plant-derived substances [11]. In Sri Lanka, Ayurvedic physicians use various plants in the management of wound healing as individual plants or as combinations of several plants. “Roganikash / Rohanekash” is a commonly used plant combination in the management of chronic skin wounds in Ayurvedic medicine. It consists of the leaves of *Calotropis gigantean* (ElaWara, Family: Asclepiadaceae), *Datura metel* (Kalu Attana, Family: Solanaceae) and *Ricinus communis* (Erandu, Family: Euphorbiaceae) **Fig. 1-3**.

FIG. 1: *CALOTROPIS GIGANTEA* LINN.

FIG. 2: *DATURA METEL* LINN.

FIG. 3: *RICINUS COMMUNIS* LINN.

However, therapeutic applications in the management of chronic skin wounds have not been scrutinized scientifically. The present study was to determine the synergistic antimicrobial activity of the ethyl acetate extract of the above medicinal plant mixture composed of leaf extracts of *C. gigantean*, *D. metel* and *R. communis* against selected gram positive and gram negative bacteria.

MATERIALS AND METHODS:

Chemicals and Reagents: All chemicals and solvents were analytical grade and were used without purification.

Selection, Collection, and Authentication of Plant Materials: The medicinal plants selected for the investigation are listed below. All fresh plant parts were collected from the Southern region of Sri Lanka. Botanical identity was determined by the descriptions given by Jayaweera (1982) [12]. Voucher specimens were preserved at the Department of Medical Laboratory Science, Faculty of Allied Health Sciences, University of Ruhuna, Galle (FAHS/Piyumali/2017/1-3)

Extraction of Plants: The leaves of selected medicinal plants were cleaned and oven dried at 40°C until a constant weight was reached. Then, the dried plant materials were ground, and the powder form of all plant material was mixed according to the formulae used in traditional medicine. The same amount of each plant powder was mixed and dissolved in ethyl acetate. It was kept sealed in a conical flask and placed in a sonicator for 72 h. Then it was filtered, and the filtrate was evaporated to dryness using a rotary evaporator. The percentage yield of the plant extract was 4.74%.

Determination of Antimicrobial Activity:

Bacterial Strains, Culture Media and Growth Conditions: Antimicrobial effects were individually tested against four bacterial strains (two gram-positive organisms; *Staphylococcus aureus* (*S. aureus*, ATCC 25923) and group A Streptococcus (ATCC 12384), two gram-negative organisms; *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922).

The standard cultures were used to evaluate the antimicrobial activity of the plant extract mixture. The pure cultures were maintained on sheep blood agar medium for the entire study. Antimicrobial effect of the plant extract mixture was determined using agar disc diffusion method and broth dilution method according to the Clinical and Laboratory Standards Institute (CLSI) protocols [13]. The suspensions of the tested micro-organisms were prepared concerning the turbidity of the 0.5 McFarland standards.

Disc Diffusion Method: Filter paper discs were prepared from Whatman no.1 filter paper with a diameter of 6 mm. The discs were inserted into several small screw cap bottles and were autoclaved at 121 ºC, 15 lb/in^2 for 15 min. A concentration of 1000 mg/mL of plant extract was prepared by dissolving the plant extract in DMSO (Dimethylsulfoxide) to obtain the concentration of 5000 µg/disc. Then, a graded dose of dilutions (400, 600 and 800 mg/mL) was prepared using the above stock solution to obtain the concentrations, 2000 µg/disc, 3000 µg/disc, and 4000 µg/disc.

All solutions were well mixed using the vortex mixture. Filter paper discs were placed in sterile Petri dishes with 5 cm distance from each disc. A volume of 5 µL of each dilution of plant extracts mixture and DMSO for negative controls were dispensed carefully on filter paper discs. The Petri dishes were kept closed for 30 minutes for drying of filter paper discs. Petri dishes containing Muller Hinton agar (MHA) and Muller Hinton Blood agar (MHA + 5% human blood) for group A *Streptococcus spp.* were inoculated with relevant microbial suspensions. The filter paper discs impregnated with serial dilutions of plant extract solutions and with DMSO for negative controls were placed on the agar surface and were incubated overnight at 37 °C. The diameter of any resulting zone of growth inhibition was measured [13]. The clindamycin 2 µg and ciprofloxacin 5 µg were used as positive controls for gram positive and gram negative organisms, respectively. Antimicrobial activity was determined by measuring the diameter of the inhibition zone around the disc.

Broth Microdilution Method: The minimum inhibitory concentration (MIC) was determined for the organisms (except group A Streptococcus) which were sensitive to the plant extract mixture previously evaluated by the broth dilution method according to the guidelines of CLSI [13].

The serially diluted plant extract was added to sterile 96-well microtiter plates which contain bacterial suspension and Muller Hinton broth growth medium according to the standard CLSI protocol [13]. Muller Hinton broth was prepared and aliquoted into khan tubes. The broth volumes were prepared (1490 µL, 450 µL, and 2.5 mL) and autoclaved. A graded concentration of plant extract solutions, 480 mg/mL, 440 mg/mL, 400 mg/mL, 360 mg/mL, 320 mg/mL and 280 mg/mL were prepared. A volume of 50 µL of plant extract solution is mixed with 450 µL of Muller Hinton broth, which was previously prepared in khan tubes. It was well mixed, and 100 µL of the resultant was transferred to the first well of the microtiter plate. A volume of 50 µL of Muller Hinton Broth was added to the rest of the wells. Then 50 µL of first well was transferred to the second and well mixed again.

A volume of 50 µL was transferred from the second to the third. The same procedure was repeated except in the last well, which was considered as the negative control. Then a volume of 10 µL of organism suspension, which was adjusted to the turbidity of 0.5 McFarland, was added to 1490 µL of Muller Hinton broth to obtain the final inoculum size 106 CFU/mL. It was well mixed, and 50 µL was added to each well except the well before last well. The last well was remained un-inoculated, to identify the wells with complete growth inhibitions of the organism by comparing with it. The plate was mildly shaken carefully and was incubated at 37°C for 24 h. The minimum inhibitory concentration (MIC) is defined as the lowest concentration of antimicrobial agent that completely inhibits the growth of organisms in the micro-dilution wells as detected by the unaided eye.

The presence and inhibition of micro-organisms were identified by the turbidity in the broth. All dilutions in the wells were inoculated on a growth media to confirm the inhibition of the organism by the plant extract. Several concentrations of the plant extract mixture were evaluated for the MIC until success [14].

Statistical Analysis: Each test was performed in triplicates. Data will be expressed as mean ± standard deviation (SD).

RESULTS AND DISCUSSION: In the present study, the antimicrobial activity of a selected medicinal plant extract mixture which has been used as an antimicrobial medicine by Ayurvedic physicians was evaluated. The activity was tested against, wound associated pathogens; *S. aureus*, group A *Streptococcus spp*., *E. coli*, and *P. aeruginosa*. The plant combination was consists of three medicinal plants, namely *C. gigantea, D. metel*, and *R. communis*. The selected medicinal plant's parts were collected from July to August 2017, from their natural habitat.

Antimicrobial properties of medicinal plants are increasingly reported from different parts of the world. Accordingly, several single medicinal plants grown in Sri Lanka were subjected to determine the antimicrobial activity. However very few medicinal plant extract mixtures were used to determine the antimicrobial activity. However, the antimicrobial activity of the selected medicinal plant extract mixture was not investigated previously. This is the first report on scrutinization of the selected herbal mixture towards its antimicrobial activity.

The single medicinal plant extract of *C. gigantea* showed antibacterial activity against the number of organisms. The antibacterial activity of the methanol, petroleum ether, chloroform and ethyl acetate extract from the root bark of *C. gigantean* and its sub fractions were investigated using agar disc diffusion method. Its root bark extracts were active against *P. aeruginosa* and *E. coli* at 20, 30 and 40 µg/disc doses. The extracts showed inhibition zones more than 15 mm of diameter for both *P. aeruginosa* and *E. coli* [15]. In the present study, as shown in **Fig. 4**, **5**, **6**, **7**, **8**, there were no inhibitory zones for *S. aureus Pseudomonas aeruginosa* and *E. coli*. It implied that the individual plant extract of *C. gigantea* exerted a significant antimicrobial potential than that of in its combination. Another study showed that the crude extract of leaves of the *C. gigantea* exerted antimicrobial activity against *S. aureus*, *P. aeruginosa* and *E. coli.*

According to the previous reports, the MIC was evaluated by modified agar well diffusion method, and the MIC values for *S. aureus*, *E. coli*, and *P. aeruginosa* were 50 mg/mL, 12.5 mg/mL, 3.1 mg/mL respectively. In the present study, there was

no activity against *E. coli*, and *P. aeruginosa* and 16 mg/mL of MIC was determined for *S. aureus* **Table 1**, **2**, **3** and **Fig. 5**. However, the results showed that the plant combination, including the *C. gigantea* is more effective against *S. aureus* than in the single extract of *C. gigantea.* The antimicrobial activity of leaf, stem bark and root extracts of *D. metel* was evaluated by agar well diffusion method, against β hemolytic *Streptococcus*, *E. coli*, *P. aeruginosa*, and *S. aureus*.

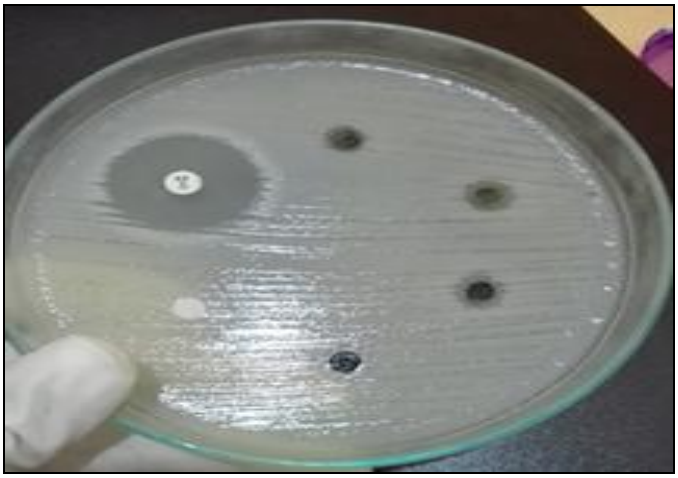

FIG. 4: ZONES OF INHIBITION AT VARIOUS CONCENTRATIONS OF PLANT EXTRACT MIXTURE AGAINST *S. AUREUS* IN DISC DIFFUSION METHOD

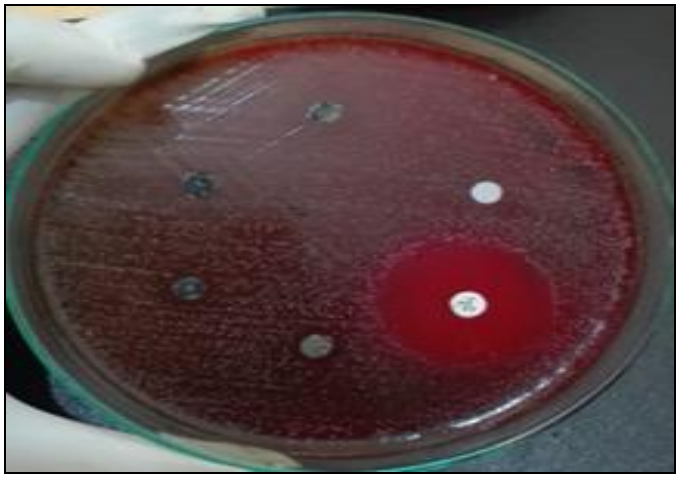

FIG. 5: ZONES OF INHIBITION AT VARIOUS CONCENTRATIONS OF PLANT EXTRACT MIXTURE AGAINST GROUP A *STREPTOCOCCUS* IN DISC DIFFUSION METHOD

FIG. 6: ZONES OF INHIBITION AT VARIOUS CONCENTRATIONS OF PLANT EXTRACT AGAINST *E. COLI* IN DISC DIFFUSION METHOD

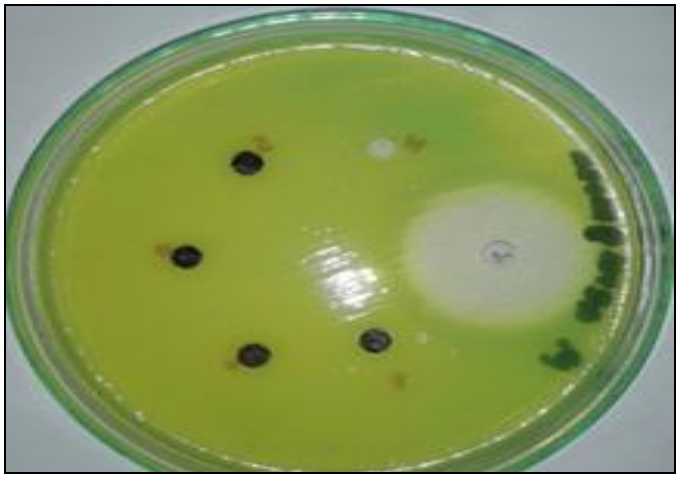

FIG. 7: ZONES OF INHIBITION AT VARIOUS CONCENTRATIONS OF PLANT EXTRACT AGAINST *P. AERUGINOSA* IN DISC DIFFUSION METHOD

TABLE 1: INHIBITORY ZONE DIAMETERS OBTAINED IN DISC DIFFUSION METHOD

Plant concentration (µg/disc)	The diameter of the inhibitory zone, M ± SD (mm)				
	2000	3000	4000	5000	Pos. control
Staphylococcus aureus	7.7 ± 0.6	7.7 ± 0.6	8.7 ± 0.6	8.7 ± 0.6	27.7 ± 1.5
Group A Streptococcus	8.3 ± 0.6	8.7 ± 0.6	9.7 ± 0.6	10.0 ± 1.0	32.7 ± 1.2
Escherichia coli	-	-	-	-	32.7 ± 0.6
Pseudomonas aeruginosa	-	-	-	-	32.0 ± 0.0

M ± SD: mean of the zone diameter ± standard deviation

They were sensitive to the ethanol and aqueous leaf and stem bark extracts of *D. metel*. The root extracts of the plants had no antibacterial activity. The leaf extracts exerted potential effects on above isolates. The crude ethanol extract exhibited an inhibitory zone of more than 30 mm against *P. aeruginosa*. It was found that the inhibitory zones were more than 20 mm for *E. coli*, *S. aureus*, and β hemolytic *Streptococcus* according to previous reports. The MIC evaluated using broth macro dilution method for these organisms were 20 mg/mL [16].

In the present study, inhibitory zones were not observed for *E. coli* and *P. aeruginosa.* As shown in **Table 2**, the plant extract showed an inhibitory zone at a diameter of (7.6 ± 0.6, 7.8 ± 0.6, 8.7 ± 0.6, 8.7 ± 0.6) mm for the concentrations, 400, 600, 800, 1000 mg/mL against *S. aureus* respectively. The deviations in results of the present investigation may be due to the difference of method used to evaluate the antimicrobial activity, method of extraction, extraction solvent and the difference in phytochemicals present in the plant extracts.

TABLE 2: MIC DETERMINATION USING THE PLANT EXTRACT AT THE CONCENTRATION OF 480 mg/mL

The concentration of plant extract in contact with bacteria in microtiter wells (mg/mL)	Clear or turbid (+/-)
48	+
24	+
12	-
6	-
3	-
0	-

(-): Clear, (+): Turbid

In the disc diffusion method, a small quantity of plant extract is saturated in a disc, and inhibitory zone surrounding the disc was observed. The MIC evaluated for *S. aureus* in the present study was 16 mg/mL. The value was quite smaller than the reported values in previous studies. It showed that the leaf extract of *D. metel* in combination is more effective than the individual leaf extract of it, against *S. aureus*. The antimicrobial activity was evaluated by agar well diffusion method in ethanol and ethyl acetate extracts of mature leaves of *D. metel* against *S. aureus, E. coli* and *P. aeruginosa*.

The ethyl acetate extract of *D. metel* (100 mg/mL) showed the maximum zone of inhibition of 19 mm against *E. coli*. There was no zone of inhibition demonstrated against *P. aeruginosa.* Inhibitory zone (13 mm diameter) was observed for *S. aureus.* The ethanol extract of the same plant, *D. metel* (100 mg/mL) showed the maximum zone of inhibition (26 mm) against *P. aeruginosa* and *E. coli*. *S. aureus* showed a relatively minor zone of inhibition (8 mm). This implied that the antimicrobial activity of the plant extracts depends on the solvent used for the extraction process [17]. In the present investigation, at the concentrations of 400, 600, 800, 1000 mg/mL, *S. aureus* exhibited inhibitory zones with (7.6 ± 0.6, 7.8 ± 0.6, 8.7 ± 0.6, 8.7 ± 0.6) mm of diameters respectively **Table 2**. The inhibitory zones were not obtained for *E. coli* and *P. aeruginosa*. However, the plant extract mixture was more powerful against *S. aureus*. The individual plant, *D. metel* was more active against *E. coli* than it's in the combination.

TABLE 3: DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION WITH TWO FOLD DILUTIONS

Original concentration of the plant extract solution (mg/mL)	Concentration of plant extract in contact with bacteria in microtiter wells (mg/mL)			
440	44	22	11	5.5
400	40	20	10	5.0
360	36	18	9	4.5
320	32	16	8	4.5
280	28	14	7	3.5

FIG. 8: MIC DETERMINATION IN A MICROTITER PLATE USING BROTH MICRO-DILUTION METHOD MIC OF *S. AUREUS* WAS 16 mg/mL

Methanol, ethanol and aqueous leaf extracts of *R. communis* had antimicrobial potential against *S. aureus* and *P. aeruginosa*. The inhibitory zones with diameters between 15 to 20 mm were obtained against *S. aureus* and *P. Aeruginosa* by agar well diffusion method [18].

In the present study, no inhibitory zones were obtained against *P. aeruginosa* and *E. coli.* The results could not be compared straightway since the antimicrobial activity was evaluated using two different methods as agar well diffusion and agar disc diffusion methods. However, the individual plant, *R. communis* as well as in combination, is active against *S. aureus.* The individual plant is more effective against gram-negative bacterial organisms than in its' combination.

While many studies have focused on the antimicrobial activity of individual plants, only a few have reported on plant-plant interactions when used in combination. Synergistic interactions noted for *Pentanisia prunelloides* combined with *Elephantorrhiza elephantina* validate an enhanced antimicrobial effect when used in combination [19]. Triphala which is commonly used Sri Lankan formulation of three medicinal plants, namely, *Terminalia chebula*, *Terminalia belerica*, and *Emblica officinalis* were subjected for the synergistic antimicrobial activity against common pathogens [20, 21]. The antimicrobial activity of essential oil of *Laurus nobilis* L. and *Myrtus communis* L. in combination has shown great potential with mild heat and high hydrostatic pressure to obtain a higher inactivation of foodborne pathogens [22]. According to the results of previous studies, individual medicinal plants among the plants selected in the present study, are potentially active against microbes than they are in combination. The leaf extract of *D. metel* is less susceptible to gram negatives, especially *P. aeruginosa*.

However, *C. gigantea* and *R. communis* are susceptible to gram-positive pathogens; *S. aureus* and group A *Streptococcus*, as well as gram-negative pathogens; *E. coli* and *P. aeruginosa*. So the activity of individual plants could be more susceptible than they were in combination. It is difficult to compare the results because most of the studies have done using agar well diffusion method in which a well contains a large quantity of the plant extract than in a disc diffusion method. However, the evaluation of the antimicrobial activity of individual plants as well as their combination, using the same methodology is important in the interpretation and comparison of antimicrobial activity more precisely. Ethyl acetate extract was used in the present study due to its high activity against microorganisms [22]. The medicinal properties of plant extracts normally depend upon the presence of active compounds possessing specific functional groups that are soluble only in solvents of particular polarity. Studies have shown that ethyl acetate extracts contain potent active compounds against microbes [22].

There was a significant difference in inhibitory zone diameters with the reference antibiotic. In the present study, clindamycin 2 µg and ciprofloxacin 30 µg were selected as the positive controls against gram-positive pathogens and gram-negative pathogens, respectively. Clindamycin is a lincosamide antibiotic that is active against many aerobic gram-positive *Cocci* and a range of anaerobic gram-positive and gram-negative bacteria. Ciprofloxacin is a fluoroquinolone antibiotic, with a broad spectrum of activity targeting aerobic gram-negative pathogens [23]. The diameters of inhibitory zones obtained positive controls were higher than the values obtained for antimicrobial discs prepared from the plant extract. It could be because the active compound was only a small percentage of the extract since no purification was done at this stage.

CONCLUSION: The ethyl acetate extract of the selected medicinal plant extract mixture composed of the leaves of *Calotropis gigantea, Datura metel*, and *Ricinus communis*, showed significant antimicrobial activity against gram-positive pathogens, *Staphylococcus aureus* and group A streptococcus spp. in agar disc diffusion method. The minimum inhibitory concentration for *S. aureus* was 16 mg/mL in the broth microdilution method. However, the plant extract mixture had no activity against the gram-negative pathogens, *Escherichia coli*, and *Pseudomonas aeruginosa*. Results were able to scrutinize the therapeutic benefits of the selected medicinal plant extract mixture in the management of chronic skin wounds in Ayurvedic medicine. Also, the plant extract mixture may offer valuable natural drug leads for the development of a commercially viable herbal antimicrobial formulation.

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