Antibacterial efficacy and phytochemical properties of the bark extract of *Connarus monocarpus* (Fam: Connaraceae)

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Received: 13.09.2023 Revised: 26.10.2023 Accepted: 06.11.2023 Online: 15.12.2023

Abstract The Connaraceae family encompasses various plant species known for their antimicrobial properties found in their bark, leaves, and roots. Therefore, the present study was conducted to investigate the antimicrobial properties of *Connarus monocarpus* (L), a member of the Connaraceae family found in Sri Lanka which is traditionally used for therapeutic wound healing. The active phytochemicals were extracted from the barks, using the reflux method employed in an aqueous medium. The antimicrobial properties of plant extract were assessed using the disk (well) diffusion agar method against four common pathogens; *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Enterococcus.* Controls were run with double distilled water. All procedures were performed in accordance with aseptic methods. The diameter of inhibition zones was observed after the incubation period and statistical analysis was performed using SPSS software package. The independent t-test revealed a significant difference between the mean values of the control and bark extraction for *Staphylococcus aureus* ($p \leq 0.001$), *Pseudomonas aeruginosa* ($p \leq 0.001$), *Proteus mirabilis* ($p \leq 0.001$), *Enterococcus* ($p \leq 0.001$); revealing the antibacterial activity of the plant extract. Additionally, there was a statistically significant difference in antimicrobial activity among the four microbes according to the One-Way ANOVA test ($p \leq 0.0001$). The *Connarus monocarpus* plant's antibacterial activity is supported by the presence of phytochemicals such as alkaloids, steroids, flavonoids, phenol and tannins, which showed combination effects. The present findings highlight the potential of *Connarus monocarpus* bark extract as a valuable source of antimicrobial agents, warranting further investigation to elucidate its specific mechanisms of action and potential applications in the field of microbial control.

Keywords: *Escherichia*, control, disk-diffusion, microbial, phytochemical

Introduction

Antibiotics play a major role in treating bacterial infections, saving countless lives since their discovery. However, the widespread and indiscriminate use of antibiotics has led to the emergence of antibiotic-resistant strains of bacteria, posing a serious threat to human health. As highlighted by the World Health Organization (WHO), a post-antibiotic era in which common infections and minor injuries can kill far from being an apocalyptic fantasy, is instead a very real possibility for the twenty first century (WHO, 2014; Rönnerstrand et al., 2017) The rise of antibiotic resistance among clinically important pathogens has become a critical global health concern. This drawback of antibiotics has prompted researchers to explore alternative approaches that can address these challenges effectively. As the effectiveness of existing antimicrobial drugs diminishes, researchers and pharmaceutical companies are working diligently to develop new antibiotics to combat this alarming trend (Djeussi et al., 2013). The discovery and development of novel antibiotics have become paramount to ensure our ability to treat bacterial infections and safeguard public health. Several approaches are being pursued to address the challenge of antibiotic resistance and create new drugs with improved efficacy. These include phage therapy, probiotics, antimicrobial peptides, vaccines, nanoparticles, antibodies, cytokines and therapeutic medicinal plants (Joseph et al., 2022). Among these approaches, therapeutic medicinal plants with antimicrobial properties based on traditional medicine knowledge and practices are being explored to develop new drugs with improved

pharmacological activities (Kouakou et al., 2019). Throughout history, various cultures have harnessed the healing potential of plants to combat bacterial infections. These traditional remedies have gained renewed interest due to their potential to provide novel antibacterial agents while potentially circumventing the issues of antibiotic resistance. Furthermore, in developing countries, traditional medicine remains the favoured treatment option owing to economic and sociocultural factors. Therefore, innovative methods for assessing medicinal plants have been developed (Wen et al., 2012). Among these traditional remedies, which are abundant in biologically active compounds (Phytochemicals/Plant secondary metabolites) like alkaloids, flavonoids, tannins, and phenolic compounds (Edeoga et al., 2005; Ginovyan et al., 2017), therapeutic plants stand out. The use of phytochemical products and plant extracts as resistance-modifying agents (RMAs) represents an increasingly active research topic and phytochemicals frequently act through different mechanisms than conventional antibiotics and could, therefore be of use in the treatment of resistant bacteria (Aberu et al., 2017). Medicines derived from plants offer advantages like substantial therapeutic value, minimal to no adverse effects, affordability, and convenient availability. (Mothana & Lindequist, 2005; Selvamohan et al., 2012). Sri Lanka possesses significant biodiversity with around 3,300 flowering plant species while 830 (constituting 25%) of it are endemic (Hewage et al., 1998).

Upon discovering the antimicrobial properties present in plants, it became evident that extensive research in this area had been conducted on numerous medicinal plants in Sri Lanka (Jayasinghe et al., 2002; De zoysa et al., 2019). Consequently, a locally available medicinal plant *Connarus monocarpus* (L) (Sinhala=Radhaliya) was selected for the current investigation since the information on antimicrobial properties of *Connarus monocarpus* is not available to date. *Connarus monocarpus* boasts wound-healing properties in various folk medicines which has caught the attention of researchers and offers a fresh perspective in fighting against bacterial infections. Therefore, the present study was conducted to evaluate the potential antimicrobial efficacy of *Connarus monocarpus* L. (Indian Zebrawood/ Sinhala = Radhaliya). *Connarus monocarpus*, which is commonly known as the "Cluster fig" or "Marabou," is a tropical tree belonging to the Family Connaraceae. Further, despite its historical use, comprehensive scientific investigations on the antimicrobial potential of the plant remain scarce.

Methodology

Test plant material collection and preparation

Fresh and healthy barks from the *Connarus monocarpus* were collected from Wataddara, Sri Lanka (7.1558° N, 80.0493° E). Plants were confirmed to the species level with the identification keys available in reference books by comparing the morphological features of the plant (Wardah, 2003).

Bark samples from plants were washed well with tap water and then rinsed with distilled water. They were air dried for 1 week at room temperature (28 ± 2 ^{o}C) and were cut into pieces. Cut pieces were pulverized using an electrical blender to make a fine powder.

Aqueous crude extraction of plant materials

200g each of pulverized bark samples were placed in three bottom-rounded flasks (1000 mL). Distilled water was added to each bottom-rounded flask until pulverized leaves were covered. Then the soaked pulverized samples were refluxed separately in distilled water for 40 minutes at 40 °C using refluxing apparatus. Then the resulting solutions were filtered using a muslin cloth. Refluxed solutions were used for antimicrobial assays.

Antimicrobial susceptibility testing using the disc diffusion assay

The antimicrobial susceptibility test was conducted in accordance with the Kirby-Bauer Disk Diffusion Susceptibility Test Protocol (Hudzicki, 2009). The antibacterial properties of the aqueous bark extract were assessed for microbes; *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Enterococcus*. The disk diffusion susceptibility test was carried out.

Stock cultures of microorganisms were prepared using culti loops from ATCC Culture collection; *Staphylococcus aureus* (ATCC-25923), *Pseudomonas aeruginosa* (ATCC-27853), *Proteus mirabilis* (ATCC-12453) and *Enterococcus*

(ATCC-25922) and subcultures of microbes were maintained from the stock cultures using four-way streaking. Concurrently, a McFarland solution was employed per each test microbe, and the turbidity was compared and matched against the standard (Hudzicki, 2009). The microbe was inoculated on Muller-Hinton agar medium using a sterilized cotton swab. The designated microbes were introduced onto the agar surface and evenly spread. To establish the negative control, positive control and extract conditions, three wells were meticulously created within each petri dish. Distilled water was introduced into one well carefully, without overflowing to serve as the negative control, while the extracted solution was placed in the other well, again carefully, without overflowing. The positive control was run with a sensitive antibiotic for each test microbe.

An Antibiotic Sensitivity Test (ABST) was performed previously with antibiotic disk panels to identify the sensitive antibiotics for each test microbe (Figure 1). Accordingly, the most suitable sensitive antibiotic for each test microbe was used.

Four replica plates for each microbe were run. To facilitate the incubation process, all prepared plates were placed in an incubator. After the respective incubation time, the diameter of inhibition zones on each replica plate was carefully measured.

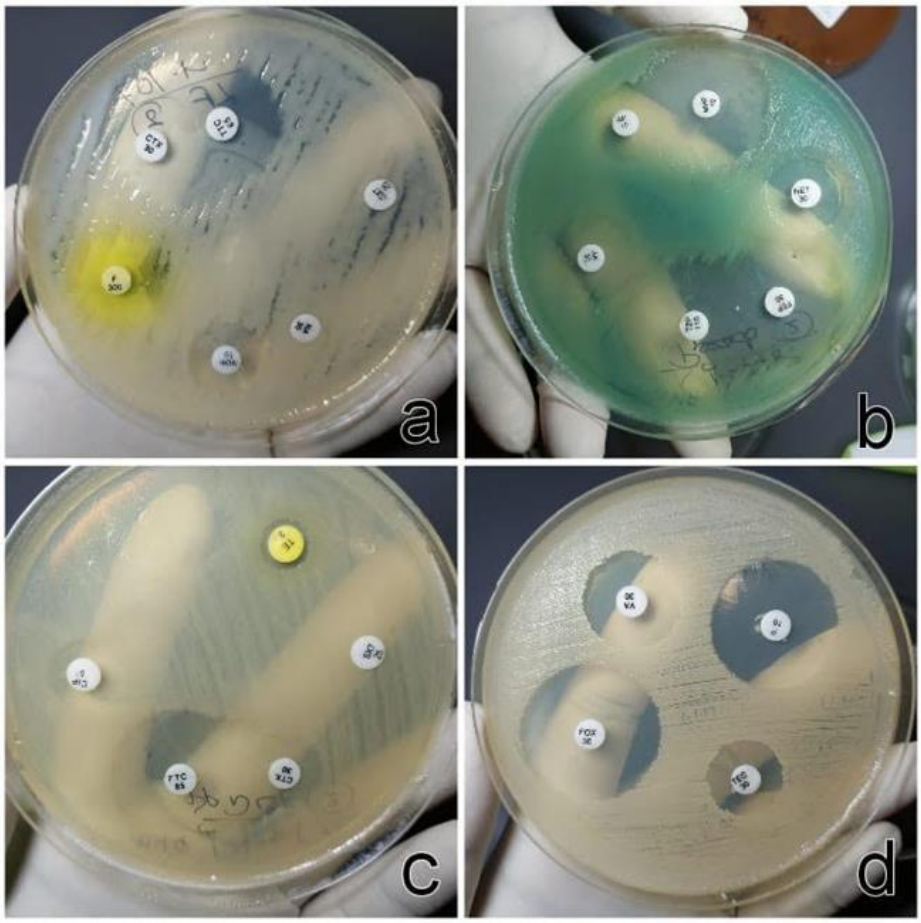

Figure 1: Antibiotic Sensitivity Test (ABST), a: *Enterococcus*, b: *Pseudomonas aeruginosa*, c: *Proteus mirabilis*, d: *Staphylococcus aureus*.

Phytochemical screening

The phytochemical tests were performed for each plant extract for the identification of, flavonoids, alkaloids, steroids, saponins, phenol, and tannins.

Test for flavonoids

Few magnesium turnings were added to the crude bark extract. Then conc. Sulphuric acid was dropped through the side of the test tube. The formation of a scarlet colour indicates the presence of flavonoids.

Test for alkaloids

Mayer's test was carried out for this. A few drops of Mayer's reagent were added to 1 mL of crude bark extract. A yellowish or white precipitate indicated the presence of alkaloids.

Test for steroids

Lieberman-Burchard's test was performed here. A few drops of acetic anhydride were added to the crude bark extract and mixed well. 1 ml of sulphuric acid was added from the side of the tube. The formation of a red ring at the junction of the two layers indicated the presence of the steroids.

Test for saponin

A drop of Na_2CO_3 solution was added to 5 mL of bark extract in the test tube. After shaking vigorously, it was left to rest for five minutes. The foam formation indicated the presence of saponins.

Test for phenol

Phenol test was performed. To the bark extract, 0.5mL of Ferric chloride was added. The formation of an intense blue-green colour indicated the presence of phenolics.

Test for tannins

A ferric chloride test was performed. 1 mL of 5% ferric chloride solution was added to the bark extract. The formation of greenish-black colour revealed the presence of tannins.

The test for the phenol and tannins

A ferric chloride test was performed here. 2 mL of 5% neutral ferric chloride solution was added to 1 mL of the crude bark extract. The dark blue colour indicated the presence of phenol and tannins.

Data analysis

Data analysis was performed SPSS Software package. Independent t-test was carried out to test whether there is a significant difference between the mean percentage diameter of inhibition zones of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Enterococcus* with control. One-way ANOVA was carried out to test whether there is a significant difference between the mean percentage diameter of inhibition zones of test microbes.

Results and Discussion

Antimicrobial activity of Connarus monocarpus aqueous bark extract

The inhibition zones were observed surrounding the wells in Muller Hinton Agar plates after the incubation period revealing the antimicrobial activity of the *Connarus monocarpus* bark extract against test microbes. The bark extract demonstrated notable activity against bacterial strains such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Enterococcus*, with clear zones observed. *Enterococcus* and *Pseudomonas aeruginosa* showed the highest and lowest mean inhibition zone diameters, respectively, among the test microbes (Figure 2).

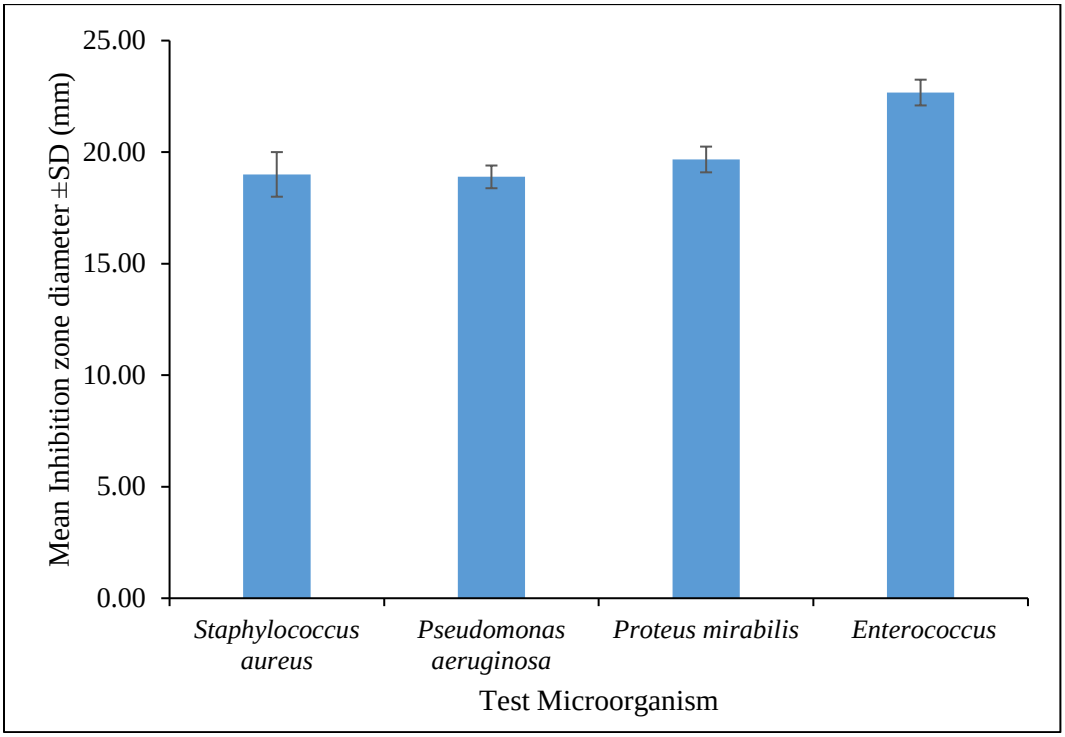

Figure 2: Mean inhibition zone ± SD of *Connarus monocarpus* bark extraction against test bacteria

Further, as per the statistical analysis of data, a significant difference in the mean inhibition zones between the bark extraction and the controls was observed (Figure 3) for *Staphylococcus aureus* (p= 0.001), *Pseudomonas aeruginosa* (p= 0.001), *Proteus mirabilis* (p= 0.001) *and Enterococcus* (p= 0.001) microbes (Independent t-test; $p<0.05$).

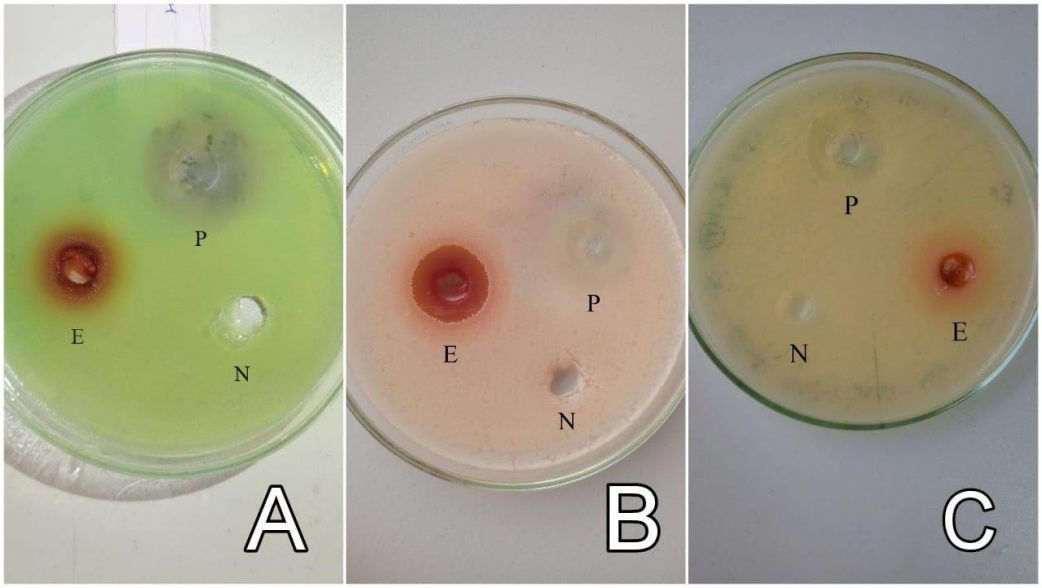

Figure 3: Inhibition Zones observed on Muller Hinton Agar plates for microbes (A- *Pseudomonas aeruginosa*; B- *Streptococcus aureus*; C- *Proteus mirabilis*. N- Negative control, P-Positive control and E- extraction.

Comparison of the efficiency of Connarus monocarpus on Streptococcus aureus, Pseudomonas aeruginosa, Proteus mirabilis and Enterococcus bacteria

The present study findings strongly reveal the microbial activity of bark extraction of *Connarus monocarpus* for test microbes. Interestingly there was a significant difference between the mean diameter of inhibition zones of microbes (One- way ANOVA; p= 0.001), revealing the equal efficacy of the antimicrobial activity of *Connarus monocarpus* bark extract against the different test microbes. Post-Hoc Analysis revealed that the mean percentage diameter of inhibition zones of *Enterococcus* with *Streptococcus aureus* (p=0.003) and *Pseudomonas aeruginosa* (p= 0.001) were significantly different (Table 1).

Table 1: Post-Hoc Analysis of inhibition zone diameters of *Streptococcus aureus*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Enterococcus*

Test Microbes	P value from Post-Hoc Analysis
Streptococcus aureus & *Pseudomonas aeruginosa*	0.883
Streptococcus aureus & *Proteus mirabilis*	0.946
Streptococcus aureus & *Enterococcus*	0.003
Pseudomonas aeruginosa & *Proteus mirabilis*	0.604
Pseudomonas aeruginosa & *Enterococcus*	0.001
Proteus mirabilis & *Enterococcus*	0.005

Phytochemical Screening of Connarus monocarpus bark extract

The antibacterial effect of *Connarus monocarpus* bark extract could potentially be attributed by the combined activity of specific phytochemicals present (Table 2). Specifically, the extract displayed reactivity towards flavonoids, alkaloids, steroids, phenols, and tannins (Figure 4). The present study was conducted to find an environmentally safe and eco-friendly solution for controlling pathogenic microorganisms. The controlling agent being a plant world comprises a rich untapped pool of phytochemicals that may be widely used in place of potential antibiotics. The antimicrobial activities arise from bioactive molecules present in the members of the Connaraceae family, with the most natural active biomolecule being the flavonoid (Roy et al., 2022). Flavonoids, known for their antioxidant and anti-inflammatory properties, are of particular interest due to their potential health benefits. Those flavonoids are involved in inhibiting enzymes such as DNA Gyrase and it leads to microbe destruction (Cushine & Andew, 2005). Further, another class of bioactive compounds, alkaloids often possess pharmacological activities that could contribute to the extract's observed effects. Steroids, phenols, and tannins are compounds with known biological activities that could further contribute to the overall therapeutic potential of the extract.

Table 2: Phytochemicals analyzed from bark extract of *C. monocarpus*

Phytochemical Tested	Present (+) / Not (-)
Flavonoid	+
Alkaloid	+
Steroid	+
Saponin	-
Phenol	+
Tannins	+

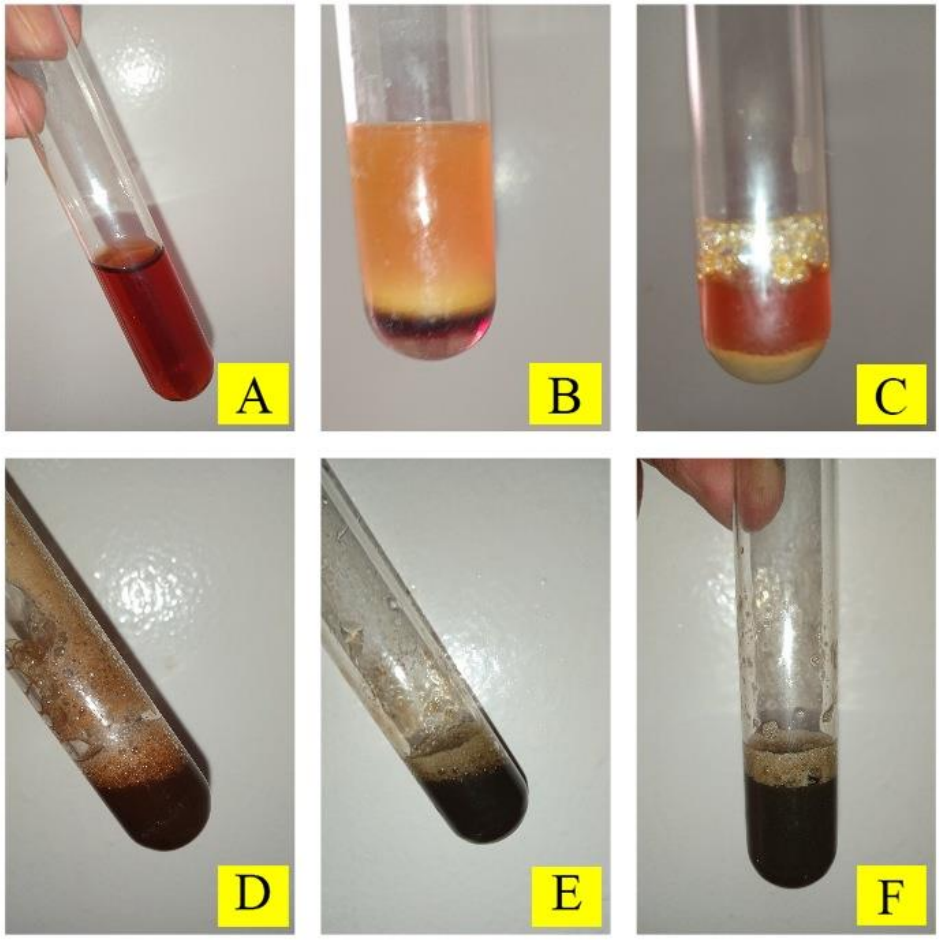

Figure 4: Saponin test (A-negative), Steroid test (B- positive), alkaloid test (C-positive), flavonoid test (D-positive), tannin test (E- positive), phenol test (F- positive)

The positive reactions observed with these phytochemicals suggest that *Connarus monocarpus* bark extract could be a promising source of bioactive compounds with potential applications in various fields, including medicine and natural product-based therapies. However, further research is warranted to characterize the potential mechanisms of phytochemical action on microbes.

Although several studies have documented the efficacy of plant extracts as the reservoir pool of bioactive toxic agents and pathogenic microbes, only a few have been commercially produced and extensively used commercially. The main reason behind the failure in the laboratory to land movements of bioactive toxic phytochemicals is poor characterization and inefficiency in the determination of the structure of exact active toxic ingredients responsible for antibacterial activity, which are essential for the development of specifications. In addition, the active ingredients can vary both in concentration and composition in the same plant species, in different clones, at different stages of plant growth and under different climatic and soil conditions. Adequate toxicological and eco-toxicological data are not available for many plant-based antimicrobial products. So, the scope for future research should be more focused on the isolation of toxic antibacterial active ingredients. However, the plant extracts may be more effective than the individual active antibiotic

compounds due to a natural synergism that discourages the development of resistance in microbes.

Conclusions and recommendations

The aqueous bark extract of *C. monocarpus* demonstrated notable antimicrobial activity against bacterial strains; *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Enterococcus*. A significant difference of the mean inhibition zones between the bark extraction and the controls was observed. Interestingly there was a significant difference between the mean percentage diameter of inhibition zones of test microbes (ANOVA P=0.001) revealing the unequal efficacy of the antimicrobial activity of *connarus monocarpus* bark extract. Post-Hoc Analysis revealed that the mean percentage diameter of inhibition zones of *Enterococcus* with *Streptococcus aureus* (p=0.003) and *Pseudomonas aeruginosa* (p= 0.001) were significantly different. The *C. monocarpus* plant's high antibacterial activity is supported by the presence of phytochemicals such as flavonoids, alkaloids, steroids, phenols, and tannins which showed combination effects in terms of antibacterial action. The activity may not be due to a single ingredient, but to a mixture of compounds, which may act synergistically. To discover the active ingredients responsible for the antimicrobial activity of these plants and to judge the in vivo actions of these constituents, additional scientific evaluation of these plants should be conducted.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article. Data will not be shared in any of the sources.

Competing interests

The authors declare that they have no competing interests.

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