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Antimicrobial Efficiency of Three Herbal Leaf Extracts Against *Streptococcus mutans*

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Bacteria are the most common cause of pulpal and periapical infections. In particular, *Streptococcus mutans* is one of the pathogens responsible for early pulpal infection, eventually leading to other pulpal diseases. In endodontic treatment, therapeutic agents from medicinal plants, including *Murraya koenigii* (L.) Spreng, *Phyllanthus niruri* L., and *Tinospora cordifolia* (Willd.) were used as a natural remedy as they possess antibacterial properties. The purpose of this research was to evaluate the efficacy, Minimum Inhibitory Concentration (MIC), and Minimum Bactericidal Concentration (MBC) of three herbal leaf extracts against *S. mutans*. Kirby-Bauer disc diffusion method was employed to determine the Zone of Inhibition (ZOI) of three groups of ethanolic herbal extracts, including *M. koenigii* (Group 1), *P. niruri* (Group 2), and *T. cordifolia* (Group 3) against *S. mutans* with Triple Antibiotic Paste (TAP) (Group 4) as a positive control. All the experimental groups were further evaluated for their MIC and MBC using the serial dilution method. Group 2 demonstrated a higher ZOI of 17.46 ± 4.95 mm compared to Groups 1 and 3. However, it was less than the control group ($p < 0.001$). Meanwhile, Group 2 exhibited bacteriostatic potential with its MIC compared to Groups 1 and 3, which demonstrated bactericidal potential. Although *P. niruri* presented better ZOI, the other two extracts have exhibited potential bactericidal activity against *S. mutans*. Hence, they could be beneficial in reducing root canal pathogens as medicaments.

Keywords: Antimicrobial, Herbal extract; *Streptococcus mutans*; Triple antibiotic paste

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Extensive tooth decay, progressing to the dental pulp in the primary dentition, is still a significant issue in paediatric dentistry. Regardless of advancements in understanding the incidence, etiology, preventive strategies, and minimally invasive therapy of the carious process, the disease and its sequelae remain the main clinical concern [1]. Bacteria are the most common cause of pulpal and periapical infections. As a result of infections, infected root canals are a prevalent concern in both primary and permanent dentition. As a complication, it may lead to severe pain, swelling, abscesses, and even tooth loss [2]. It has been proven that endodontic infection is caused by a complicated mixture of different kinds of bacteria. *Porphyromonas gingivalis, Treponema denticola, Enterococcus faecalis*, and *Prevotella intermedia* were some of the frequent bacteria reported in symptomatic deciduous teeth with periapical disease. At the same time, *Streptococcus sobrinus*, *Streptococcus mutans*, and *Lactobacillus acidophilus* have been associated with the early stages of dental caries, including colonisation on the pulpal surface [2, 3].

The bacteria that persist in infected root canals is *E. faecalis*, while *S. mutans* is a significant pathogen in early lesions of the pulp and future pulpal diseases [4]. The decrease or complete removal of bacterial infection is one factor determining the outcome of endodontic treatment. The procedure involves chemo-mechanical instrumentation using chemically active solutions. However, the canals are not fully devoid of microbes. Therefore, the intracanal medicament plays a key role by killing all the possible pathogens and preventing their regrowth in the root canals [5]. One of the most often utilised intracanal medications is calcium hydroxide. Its high alkaline pH, antibacterial activity, and capacity to promote pulp and periapical tissue healing contribute to its antimicrobial efficacy. It was discovered that although it exhibits antimicrobial activity against various microflora, it is less efficient against organisms. The drawback of calcium hydroxide is that it promotes antibiotic use inside root canals [6]. Therefore, lesion sterilization of tissue repair (LSTR), an alternative method with three mixed antibiotics pastes, was introduced as a non-invasive, less time-consuming, non-instrumentation endodontic treatment

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option for infected teeth. It has been reported that the mixture of three antibiotics, including minocycline 100 mg, metronidazole 500 mg, and ciprofloxacin 200 mg in the ratio of 1:1:1 as a Triple Antibiotic Paste (TAP) can sterilize infected root dentin with necrotic pulp [7]. However, the drawbacks of TAP include discolouration of teeth, changes in the mechanical property of the dentin that lead to tooth fragility and reduced dentin microhardness [8]. Hence, to address the shortcomings of conventional endodontic treatment with these existing medicaments, the use of natural medication derived from plants has been expanded as a natural remedy in the last few decades due to their increased tolerance in the body and occurrence of lesser adverse effects and their easy availability [9]. In the field of dentistry, over the last few years, there has been an increase in the utilization of herbal plants for the treatment of many infectious diseases as they possess good antibacterial, anti-inflammatory, analgesic, and bioactive properties. A huge number of medicinal plants are used in Ayurveda. Among them, *Murraya koenigii, Phyllantus niruri*, and *Tinospora cordifolia* are widely used herbs in Ayurveda. As these extracts from medicinal plants are economical with greater safety margins, they are effectively employed in various dental treatments. *M. koenigii* is a *Rutaceae* family plant known as Curry Leaf. It is a popular leafy vegetable that originated in India and Southeast Asia [10]. The leaves have a flavor that is bitter, slightly spicy, and acidic; this flavour and other characteristics are retained even after drying [11]. *P. niruri*, a member of the *Euphorbiaceae* family, is native to India and may be found in subtropical and tropical areas. It is employed in all types of traditional medicine, including Ayurvedic, Chinese, and Unani. The presence of the secondary metabolites contributed to the medicinal activities of the plant [12]. Similarly, *T. cordifolia* is one of the most extensively utilised medicinal plants in the domains of Ayurveda and natural medicine [13]. *T. cordifolia*, a deciduous shrub from the *Menispermaceae* family, is commonly known as the heart-leaved moonseed plant. Several phytochemical compounds and numerous other biochemical components affecting microorganisms were discovered during the phytochemical analysis of the plant's extracts [14]. As a result of its medical qualities, the plant is of significant interest to researchers [15].

In the literature, several studies have reported the activities of extracts from medicinal plants against various pathogens. However, no studies have compared the antimicrobial efficiency of these three herbal plant extracts against *S. mutans*. Hence, the current investigation evaluated and compared the effects of these herbal plant extracts with TAP against *S. mutans*.

EXPERIMENTAL

The experiment was performed in a laboratory with an in vitro experimental study design at Universiti Teknologi MARA. The ethics committee of the institution approved the study (REC/10/2022 (PG/MR/251).

Preparation and Culturing of Bacteria

A bacterial strain of *S. mutans* 25175 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany (DSMZ). Brain Heart Infusion (BHI) agar was used to culture *S. mutans* with incubation at 37°C for 24 h. The standard bacterial suspension was prepared by adjusting the turbidity of an overnight culture of *S. mutans* to 0.5 McFarland standards, resulting in a final inoculum of 1.5 × 108 CFU/ mL.

Preparation of Ethanolic Extract from Herbal Leaves

The mature leaves from the plants *M. koenigii, P. niruri* and *T. cordifolia* were collected from the nursery and sent to the Herbarium Universiti Kebangsaan Malaysia to identify the species. Confirmation of the species was received with the voucher number for *M. koenigii* (ID064/2023), *P. niruri* (ID066/2023) and *T. cordifolia* (ID067/2023). Accordingly, 500 mL of 99.8% ethanol was used to mix with 50 g of individual herbal leaves powder. A method by Troung et al. was used to prepare the ethanolic extract of all the herbal plant leaves with minor modifications [16].

Preparation of Triple Antibiotic Paste

Initially, the coating on the pills containing ciprofloxacin (Ciprodac ® 500 mg, Cadila Pharmaceuticals Ltd.), metronidazole (Axcel ® 400 mg, Kotra Pharma), and doxycycline (100 mg, Duopharma) were eliminated. Afterwards, the tablets were powdered in sterile mortars. A total of 1.0 mg/mL was achieved as the final concentration by mixing all the powdered antibiotics evenly in a 1:1:1 ratio with sterile water [17].

Disc Diffusion Method

The disc diffusion method was used to examine the antibacterial effects of the herbal plant extracts [18]. To achieve a concentration of 250 mg/mL, sterile distilled water and Dimethyl sulfoxide (DMSO) were mixed with the extracts, ensuring that the final concentration of DMSO did not exceed 1% and passed through a Millipore filter. Then, 20 µL of leaf extract of *M. koenigii* (Group 1), *P. niruri* (Group 2), and *T. cordifolia* (Group 3) were loaded onto 6 mm diameter blank antimicrobial susceptibility discs (Oxoid TM). Meanwhile, TAP (Group 4) and a disc of sterile water (Group 5) were used as positive and negative control groups, respectively. The standard bacterial suspension was evenly spread across the surface of each agar plate. Subsequently, discs containing different plant extracts, as well as negative and positive controls, were placed on the agar. After incubating plates for 24 h at 37°C,

the Zone of Inhibition (ZOI) was measured in millimeters using a digital caliper (Figure 1).

Minimal Inhibition Concentration (MIC)

Minimal Inhibition Concentration (MIC) of *M. koenigii, P. niruri* and *T. cordifolia* was determined against *S. mutans* using the microdilution method. Each well in rows A, B and C of the 96-well plate received 100 µl of BHI broth, up to 10 wells. Next, 100 µl of plant extract (initial concentration 500 mg/mL) was placed in the first well of rows A, B and C. Two-fold serial dilutions were performed from well 1 to well 10, resulting in a final concentration range of 0.49 to 250 mg/mL. Finally, 10 µl of bacterial culture was added to each well. Serial dilution of extract in broth (without bacteria) was performed as a blank control in row E. Triplicate wells containing only BHI broth served as the sterility control, while triplicate wells containing BHI broth with bacteria served as the growth control. Additionally, triplicate wells containing TAP with the addition of bacteria act as the positive control. After completion of the two-fold serial dilution method, 24 h of incubation was done at 37°C. The same procedure was repeated for the other two leaf extracts. After 24 h of incubation, the Optical Density (OD) value of the wells was determined at 600 nm using a microplate reader. The MIC was defined as the minimum extract concentration with a difference in OD value (experimental group-blank control group) of less than 0.05 [19].

Minimal Bacterial Concentration (MBC)

The determination of MBC was performed after the MIC assessment. Accordingly, 10 mL were collected from the wells preceding the extract's MIC and inoculated into BHI-agar plates. Next, the plates were incubated for 24 h at 37°C. After 24 h, observation was made for colonies' formation. The MBC refers to the lowest concentration of the extract where no bacterial colonies are detected on the agar plate, suggesting complete bacterial eradication rather than just inhibition.

Statistical Analysis

To conduct statistical analyses, the Statistical Package for Social Sciences (SPSS) for Windows, Version 22.0 (launched in 2013) Armonk, NY: IBM Corporation was used. For organisms, the expression of the inhibitory zone in millimetres, together with each group's mean and standard deviation, are included in the descriptive analysis. One-way Analysis of Variance (ANOVA) and Tukey's post hoc test were used to compare the mean ZOI across the five groups. The statistical significance level was set at $P < 0.05$.

RESULTS AND DISCUSSION

In Group 1, the mean ZOI was 9.13 ± 0.45; Group 2 was 17.46 ± 4.95; Group 3 was 12.68 ± 1.26; Group 4 was 59.00 ± 1.22 and in Group 5 was 0.00 ± 0.00. Table 1 and Figure 2 depict that the mean ZOI (in mm) for *S. mutans* varied statistically significantly between the five groups at $p < 0.001$.

When the mean differences between the groups were evaluated, a multiple comparison demonstrated that Group 4 had the significantly greatest mean ZOI for *S. mutans* when compared to Groups 1, 2, 3, and 5 and that the mean differences were statistically significant at $p < 0.001$, respectively. Subsequently, the mean ZOI for *S. mutans* in Group 2 was considerably greater in comparison to Groups 1, 3, and 5, with a statistically significant difference at $p \leq 0.001$. Next, Group 3 demonstrated a substantially larger mean ZOI for *S. mutans* in comparison to Groups 1 and 5, with statistically significant mean differences ($p = 0.02$ and $p < 0.001$, respectively). Group 1 had a considerably greater mean ZOI than Group 5, with a significant difference ($p < 0.001$). This implies that in Group 4, the mean ZOI for *S. mutans* was significantly higher, followed by Groups 2, 3, and 1, and lowest in Group 5 (Table 2).

Table 1: A One-way ANOVA test showed the mean Zone of Inhibition (in mm) for *S. mutans* across five groups.

Groups	N	Mean (mm)	SD	Minimum	Maximum	p-value
Group 1	9	9.13	0.45	8.4	9.7	<0.001*
Group 2	9	17.46	4.95	12.2	25.8	
Group 3	9	12.68	1.26	10.4	14.7	
Group 4	9	59.00	1.12	57.0	60.0	
Group 5	9	0.00	0.00	0.0	0.0	

* - Statistically Significant

Table 2. Multiple comparisons of mean difference in the Zone of Inhibition (in mm) for *S. mutans* b/w groups using Tukey's Post hoc Test.

(I) Groups	(J) Groups	Mean Diff. (I-J)	95% CI for the Diff.		p-value
			Lower	Upper	
Group 1	Group 2	-8.32	-11.48	-5.16	<0.001*
	Group 3	-3.54	-6.70	-0.39	0.02*
	Group 4	-49.87	-53.03	-46.71	<0.001*
	Group 5	9.13	5.98	12.29	<0.001*
Group 2	Group 3	4.78	1.62	7.94	0.001*
	Group 4	-41.54	-44.70	-38.39	<0.001*
	Group 5	17.46	14.30	20.61	<0.001*
Group 3	Group 4	-46.32	-49.48	-43.16	<0.001*
	Group 5	12.68	9.52	15.84	<0.001*
Group 4	Group 5	59.00	55.84	62.16	<0.001*

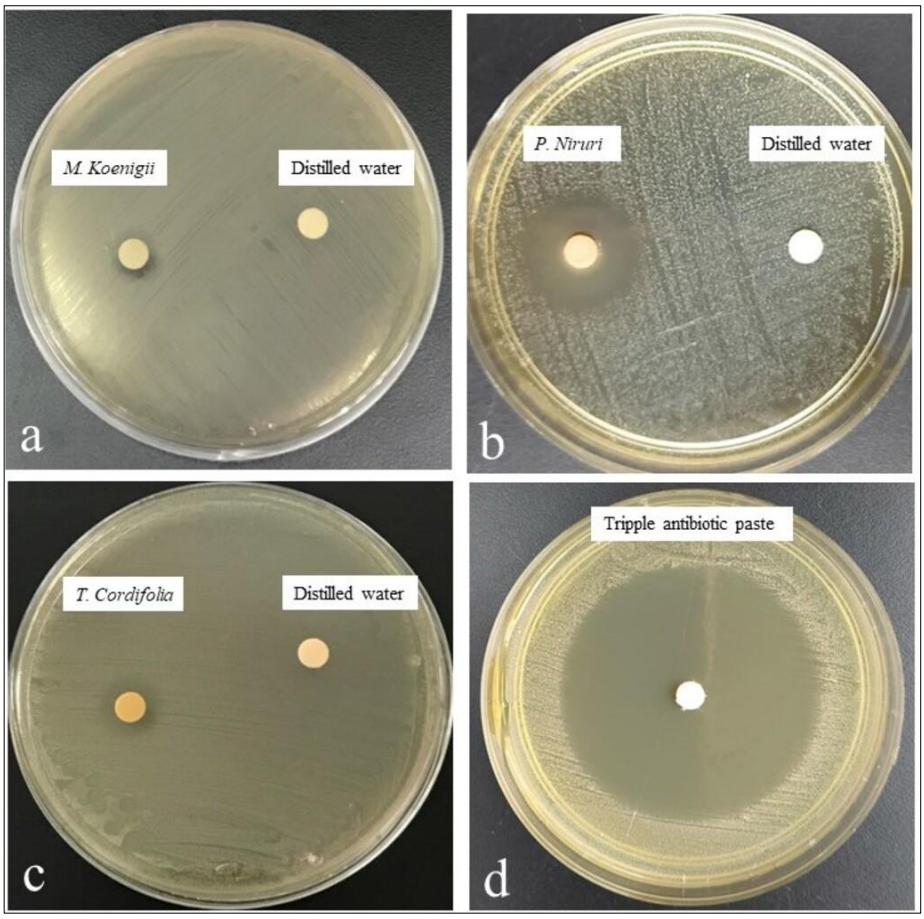

Figure 1 (a, b, c, and d). The antibacterial activity of *Murraya koenigii*, *Phyllanthus niruri*, *Tinospora cordifolia* and Triple Antibiotic Paste respectively. Distilled water was as negative control against *Streptococcus mutans.*

Table 3. Minimum inhibitory and bacterial concentrations for *Murraya koenigii*, *Phyllanthus niruri*, and *Tinospora cordifolia* against *Streptococcus mutans.*

Groups	MIC (mg/ml)	MBC (mg/ml)	MBC/MIC Ratio
Group 1	7.81	7.81	1
Group 2	7.81	250	32.0
Group 3	31.25	62.50	2

In the current experiment, the antibacterial effectiveness of three different plant extracts was assessed using an ethanol solvent. *P. niruri* plant extract demonstrated better ZOI than *M. koenigii* and *T. cordifolia* against *S. mutans.* Similar to the current experiment results, the ethanolic extract of *phyllanthus* demonstrated antibacterial effects against *S. salivarius and S. mutans* due to its components of cardiac glycosides, alkaloids, saponins and tannins [20]. *P. niruri's* ethanolic extract was beneficial as an intracanal medication for teeth with persistent apical periodontitis in a different in vivo study conducted on Wistar rats [21]. Whereas, in another study, results except for *C. albicans, P. niruri*'s ethanolic, methanolic and aqueous extracts suppressed the growth of *B. subtilis*, E. coli and *S. aureus*. This explains that the antimicrobial peptides called Thionins peptide compounds present in the plant cause toxicity by attaching themselves to the membranes of bacteria and fungi. Other secondary metabolites found in the *P. niruri* plant include acidic diterpenes, alkaloids, flavonoids, hypophyllanthin, lignans, arabinogalactan, phyllanthin, phenols, proteins, amino acids, tannin, and carbohydrates [12]. In another study, results, the antimicrobial activity of *P. niruri* against *S. aureus, E. coli, B. cereus, P. aeruginosa,* and *B. subtilis* was higher in methanolic extract than aqueous and ethanolic extracts. This might be attributable to the fact that the active chemicals in this solvent were more soluble, and methanol was more effective at extracting the plant's potent antibacterial components [22]. Another *in vivo* clinical study demonstrated the successful effect of *P. niruri* extract in combination with antiretroviral therapy in boosting the absolute CD4 cell count in HIV patients. compared to the administration of antiretroviral medication alone [23].

Although in the present study, *P. niruri* exhibited better ZOI, *M. koenigii*, and *T. cordifolia* herbal extracts have exhibited bactericidal activity against *S. mutans* as their MBC/MIC ratio is ≤ 4 (Table 3). Similarly, *Murraya's* ethanolic extract exhibited good antimicrobial efficacy with a MIC of 0.625 against *S. mutans*. Its antibacterial properties could originate from the presence of gallic acid and other phenolic compounds [24]. However, similar activities were reported in the literature against several other microorganisms [25-28]. Some *in vivo* studies demonstrated its beneficial effects in preventing dental diseases caused by plaque and dental caries when used as a polyherbal mouthwash and other herbs [29]. In treating plaque-induced gingivitis, *M. koenigii* mouthwash had the same effectiveness as chlorhexidine [30]. The ethanolic extract of *T. cordifolia* also exhibited its antibacterial activities in the present study as it contains compounds such as flavonoids, phenolics, terpenoids, alkaloids, steroids, glycosides, diterpenoid lactones, aliphatic compounds, sesquiterpenoids, tinosporin, tinosporic acid, essential oils [31,14]. Studies have reported the antimicrobial activities of *T. cordifolia* against various pathogens [31-33]. In the present study, TAP was considered a positive control. Compared to all the extracts, it exhibited the highest inhibition of 59 mm. However, its disadvantages in the form of reduced dentin microhardness might restrict its long-term application and difficulty of paste removal from the root canal space as it penetrates and adheres to the structure of the dentin and discolouration of teeth [8,17]. However, clindamycin might have been used as an antibiotic replacement for minocycline as it is efficient against the *streptococci* group of microorganisms as well as anaerobes [34]. According to the data provided on ZOI, MIC and MBC values in Tables 1 and 3, these ethanolic plant extracts demonstrated various degrees of inhibition against *S. mutans*. The values obtained for ZOI mainly depend on the characteristics of medicinal plants, such as their components and their ability to dissolve. Discrepancies in the genetic makeup and/or cell wall composition of bacteria, as well as variations in the quantity, type and mode of action of the bioactive chemicals found in plant extracts, are the primary causes of variations in bacterial susceptibility to the extracts [33].

In the current investigation, the ethanolic extract of the herbal leaves was used without phytochemical analysis to determine their bioactive components. Furthermore, this study will continue investigating the precise mechanism of action by which they exert their antibacterial properties. However, further research in clinical trials is required to assess their antibacterial properties and potential long-term harmful effects on the complex environment of the oral cavity.

CONCLUSION

Although the findings from the present investigation suggested that the 250 mg/mL ethanolic extract of *P.*

niruri has better ZOI, the other two herbal extracts of *M. koenigii* and *T. cordifolia* have exhibited potential bactericidal activity against *S. mutans*. As such, they could be beneficial in reducing root canal pathogens when used as medicaments. Thus, it is recommended that the synergistic effects of these herbal extracts be explored in future studies.

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