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Antimicrobial efficacy of *Tetracarpidium conophorum* extracts against oral pathogens

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Abstract

Background and Objectives: The escalating threat of antibiotic-resistant pathogens presents a pressing challenge in the battle against infectious diseases, particularly oral infections. Traditional medicine, with its long history of utilizing plant-based remedies for their therapeutic potential, offers a promising avenue. *Tetracarpidium conophorum* (commonly known as walnut) is a prime example. This study delves into the antimicrobial properties of *Tetracarpidium conophorum* extracts, a potential solution to the growing problem of antibiotic resistance.

Materials and Methods: The seeds, leaves, and roots of *Tetracarpidium conophorum* were meticulously collected from Adavi Local Government Area in Kogi State, Nigeria, and authenticated by a botanist at the University of Agriculture, Makurdi. The extracts were obtained using the cold maceration method with water, ethanol, and hexane as solvent, and the antimicrobial activities were rigorously assessed against a range of oral pathogens using standard susceptibility tests. The microbial isolates were sourced from a reputable institution, the Department of Microbiology, National Veterinary Research Institute, Vom, Plateau State, and confirmed through biochemical testing, ensuring the reliability of the results.

Results and Conclusion: The extracts from the seeds, leaves, and roots of *Tetracarpidium conophorum* demonstrated varying degrees of antimicrobial activity against the tested pathogens. Notably, the seed extracts exhibited the highest inhibitory effects, followed by the leaf and root extracts. The study identified significant differences in antimicrobial efficacy among the different plant parts, with potential implications for therapeutic use. These findings suggest that the plant of *Tetracarpidium conophorum*, particularly the seeds, could be harnessed as a valuable source of natural antimicrobial agents, offering a promising alternative to the conventional antibiotics in oral health management.

Keywords: Antibiotic resistance, Antimicrobial activity, Oral pathogens, Plant extracts, *Tetracarpidium conophorum*

1. Introduction

The emergence of antibiotic resistance among pathogenic microorganisms has become a pressing global health concern, underscoring the urgent need for alternative therapeutic strategies [1-2]. Historically, plants have been a cornerstone of traditional medicine, offering a vast repository of bioactive compounds with potential antimicrobial properties [3]. One such plant is *Tetracarpidium conophorum* (commonly known as walnut), which is native to Nigeria and is grown primarily for its fried nuts, which are eaten as snacks. This plant has been utilized in traditional practices for its di-

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verse medicinal benefits, including antimicrobial, anti-inflammatory, and antioxidant effects [4-7]. Figure 1 illustrates the seeds of *T. conophorum*, which are integral to its traditional use. Similarly, this plant's leaves (Figure 2) and roots (Figure 3) are also valued in traditional medicine for their therapeutic properties. Although, these plant's parts have been used to treat various ailments including infections, but their scientific validation for antimicrobial efficacy still needs to be explored.

Figure 1- Seeds of walnut (*Tetracapidium conophorum*) plant used for the current study

Figure 2- Leaves of walnut (*Tetracapidium conophorum*) plant used for the current study

Figure 3- Roots of walnut (*Tetracapidium conophorum*) plant used for the current study

Oral infections, driven by pathogens such as *Streptococcus mutans, S. sobrinus, S. salivarius, Candida albicans,* and *S. pyogenes* pose significant health risks leading to conditions such as dental caries, periodontitis, and systemic infections [8,9]. The effectiveness of conventional antibiotics is increasingly compromised by resistance, necessitating the exploration of novel antimicrobial agents [2]. Plant-based remedies have emerged as promising alternatives with unique mechanisms of action that could overcome the resistance issues [10-12]. Exploring the antimicrobial properties of *T. conophorum* could lead to the development of innovative, plant-based therapeutic agents for managing oral infections, just like some other phytocompounds [13-15]. This study aims to integrate traditional knowledge with scientific evidence, thereby supporting the development of alternative treatments and enhancing the role of herbal remedies in contemporary medicine. By validating the antimicrobial efficacy of *T. conophorum*, this research contributes to the broader goal of addressing the challenges posed by antibiotic resistance and improving oral health management.

Despite the traditional use of *T. conophorum* in managing infections, more scientific evidence is needed to demonstrate its efficacy against specific oral pathogens. This study has tried to fill this gap by evaluating the antimicrobial activities of aqueous, ethanolic, and hexane extracts of the seeds, leaves, and roots of *T. conophorum* against a range of oral pathogenic microorganisms. The primary objectives of our research are to extract and characterize the bioactive compounds present in the seeds, leaves, and roots of *T. conophorum*. Secondly, we assessed the antimicrobial efficacy of these extracts against *S. mutans, S. sobrinus, S. salivarius, C. albicans,* and *S. pyogenes*. Finally, the antimicrobial activity of different parts of the plant was compared to identify the most potent extract.

2. Materials and Methods

2.1. Collection and identification of plant materials

The seeds, leaves, and roots of walnut plant (*T. conophorum*) were collected from Adavi Local Government Area, Kogi State, Nigeria. After collection, the plant materials were packaged in sterile polythene bags, and transported to the Department of Biological Sciences, University of Agriculture, Makurdi. A botanist authenticated the plant species using identification keys. The collected plant parts were shade-dried at a temperature range of 20–27 °C for two weeks. Once adequately dried, they were crushed into powder using a clean mortar and pestle. The powdered plant materials were stored in sterile containers until extraction.

2.2. Extraction of plant materials

The plant extraction was conducted in the Chemistry Laboratory, Department of Chemistry, Benue State University, Makurdi. The cold maceration method described by Umeh et al. [16] was used for the extraction. A total of 50 g of the powdered plant material (leaves, seeds, and roots) was soaked separately in 500 ml of ethanol, hexane, and distilled water for 72 h, with intermittent shaking to facilitate extraction. After 72 h, the mixtures were filtered using Whatman no. 1 filter paper. The filtrates were concentrated using rotary evaporator at 40 °C for ethanol and hexane, while the aqueous extracts were dried in air-drying oven. The resulting extracts were stored at 4 °C in sterile labelled bottles for subsequent phytochemical screening and antimicrobial analysis.

2.3. Phytochemical screening

Qualitative phytochemical screening was carried out on the extracts. For determination of tannins by ferric chloride test, 10 ml of distilled water was added to 2 ml of the aqueous, hexane, and ethanol extracts in a test tube, and a few drops of diluted ferric chloride solution were added further. The presence of blue-black (dark) precipitate was given as an indication of the presence of tannin in the extract [17-19].

The combined frothing test was used for the presence of saponins. Two milliliters of the aqueous, hexane, and ethanol extracts was mixed with 5 ml of distilled water in a test tube. The mixture was

shaken vigorously. The formation of a stable froth (foam) was considered as positive result for the presence of saponins [20].

For flavonoids, 5 ml of the aqueous, hexane, and ethanol extracts was heated with 10 ml of ethyl acetate in a boiling water bath for 5 min. The mixture was then filtered and used for the following tests, in which 5 ml of the filtrate was shaken with 1 ml of dilute aqueous ammonia. It was left to allow the layers' separation. The yellow coloration in the aqueous ammonia layer indicated the presence of flavonoids [18,19].

For reducing sugar, three drops of Fehling's solutions I and II were added to 5 ml of the filtrate, and it was heated over a water bath to boil. A red precipitate indicates the presence of reducing sugar [18].

For phlobatannins, 2 ml of hydrochloric acid 5 M was added to 5 ml of the extract, and it was heated for 10 min. The presence of phlobatannins was indicated by the formation of red precipitate [18].

For detection of anthraquinones by Borntrager's test, 5 ml of benzene was added to 2 ml of the aqueous, hexane, and ethanol extracts. The mixture was shaken and filtered using Whatman filter paper no 1. Then, 10% ammonia solution was added to the filtrate, and shaken again. A pink precipitate (or violet) in the ammoniacal layer indicated the presence of anthraquinones [18].

For glycosides, 1 ml of the aqueous, hexane, and ethanol extracts was added to 5 ml of Fehling's solution I and II, and heated to boil on a water bath for 5 min. After boiling, the mixture was filtered. Then, 2 ml of diluted H_2SO_4 was added to the filtrate and reheated. It was allowed to cool and was neutralized with NaOH 2 M. Finally, 5 ml of Fehling's reagent was added, and the mixture was reheated on a water bath for 10 min. A brick red precipitate indicates the presence of glycosides [1,18].

For alkaloids, 5 ml of the aqueous, hexane, and ethanol extracts was added to a test tube. It was boiled with 2 ml of 1% HCl for 10 min on a steam bath. It was divided into two portions. To the first portion, three drops of Dragendorff's reagents were added, and an immediate precipitate indicates the presence of alkaloids. To the second portion, three drops of Meyers reagents were added, through which an immediate precipitate indicates the presence of alkaloids [1-3,18,19].

For determination of steroids by Salkwoski's test, 5 ml of the aqueous, hexane, and ethanol extracts was added to 2 ml of chloroform and filtered using Whatman filter paper no 1. In the filtrate, five drops of concentrated sulfuric acid were added. Then, a colorless layer below and a brownish layer above were observed while a reddish-brown ring coloration was observed in the interface [1-3,20].

Phenols content was determined by the addition of 10 ml distilled water to 2 ml of the aqueous, hexane, and ethanol extracts in a test tube followed by addition of a few drops of diluted ferric chloride solution. The presence of blue-black (dark) precipitate was given as an indication of the presence of phenols in the extract [16].

2.4. Preparation of microbial isolates

The microbial isolates used for this study included *S. mutans, S. sobrinus, S. salivarius, C. albicans*, and *S. pyogenes*. Stock cultures of these microorganisms were obtained from the Department of Microbiology, National Veterinary Research Institute, Vom, Plateau State. Single, well-isolated colonies of each microorganism were aseptically transferred into nutrient agar slants using a sterile inoculating loop. The cultures were incubated at 37 °C for 24 h. After incubation, the agar slants were stored at 4 °C for further use in antimicrobial susceptibility testing [21,22].

2.4.1. Antimicrobial susceptibility testing

The antimicrobial activity of the plant extracts was evaluated using the agar well diffusion method, as described by Bauer et al. [23]. Each microbial suspension was prepared in sterile saline solution, and the turbidity was adjusted to 0.5 McFarland standard (approximately 1.5×10^8 CFU/ml). Mueller-Hinton agar plates were inoculated with the microbial suspension using a sterile cotton swab. Wells of 6 mm diameter were created in the agar, and 100 µl of each extract (concentrations of 50,

100, 200, and 400 mg/ml) was introduced into the wells. Ethanol, hexane, and distilled water served as negative controls, while standard antibiotics (gentamicin for bacteria and nystatin for the fungus) were used as positive controls. The plates were incubated at 37 °C for 24 h for the bacteria and 15 °C for 3 to 5 days for the fungus, after which the inhibition zone around the wells was measured using a ruler. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC) were determined using the broth dilution method, as described by Aremu et al. [2].

2.5. Statistical analysis

The data are as mean ±standard deviation of triplicate experiments. Statistical analysis was performed using two-way ANOVA to determine the significant differences between antimicrobial activity of the extracts. The means were compared using Least Significant Difference (LSD). A p-value < 0.05 was considered as significant difference.

3. Results and Discussion

The phytochemical screening of walnut leaf extracts prepared by three solvents of ethanol, hexane, and water demonstrated the presence of several bioactive compounds (Table 1). The presence of alkaloids was confirmed in all solvents, suggesting that alkaloids in walnut leaves are readily extractable using both polar (ethanol and water) and non-polar (hexane) solvents. Saponins and tannins, other classes of secondary metabolites, exhibited a similar profile, indicating their solubility in a broad range of solvents. The absence of reducing sugars in all extracts suggests either their absence in the plant or the inefficacy of the solvents to extract these agents. Anthraquinones were only present in the ethanol extract of leaf and seed, indicating their moderate polarity and preferential extraction in ethanol. Moreover, it was likely absent in the root. Conversely, steroids were absent in the seeds, and just detected in hexane extract of leaf and ethanol extract of root, indicating their relatively non-polar nature. Flavonoids were present in both ethanol extract of seeds and leaves, and hexane extract of leaves, indicating their partial non-polarity but absent in the aqueous extract, suggesting limited solubility in water. In addition, it was not found in the extracts of roots. Interestingly, glycosides were just found in the hexane extract of leaves, a result that may indicate the presence of non-polar glycosides in the leaves. Phenolic compounds, known for their antioxidant properties, were detectable in the ethanol extract of roots, seeds, and leaves, and also hexane extract of roots, showing their partial non-polarity. Lastly, phlobatannins were absent in all solvents, implying their absence or low concentration in the walnut plant. Our investigation revealed that, except for reducing sugar and phlobatannins, ethanol was the most potent solvent for extraction of phytochemicals. On the other hand, leaves were the richest part of *T. conophorum* containing the most extractable phytochemicals.

Table 1- Phytochemical composition of *Tetracarpidium conophorum* leaf, seed, and root extracts

Plant parts	Media	Alkaloid	Saponins	Tannins	Reducing sugars	Anthraquinone	Steroids	Flavonoids	Glycosides	Phenols	Phlobatannins
Leaf	Ethanol	+	-	+	-	+	-	+	-	+	-
	Hexane	+	+	-	-	-	+	+	+	-	-
	Water	+	+	+	-	-	-	-	-	-	-
Root	Ethanol	+	+	+	-	-	+	-	-	+	-
	Hexane	+	+	+	-	-	-	-	-	+	-
	Water	+	+	+	-	-	-	-	-	-	-
Seed	Ethanol	+	+	+	-	+	-	+	-	+	-
	Hexane	+	+	+	-	-	-	-	-	-	-
	Water	+	+	+	-	-	-	-	-	-	-

+ present; - absent

Table 2 shows the antimicrobial activities of walnut leaf, root, and seed extracts in ethanol, hexane, and aqueous solvents against selected oral pathogens. The ethanol leaf extract (ELE) exhibited moderate antimicrobial activity, particularly against *C. albicans, S. salivarius,* and *S. sobrinus*. However, it showed no inhibitory effect on *S. pyogenes* and *S. mutans*. Same as hexane seed extract (HSE) and aqueous root extract (ARE), the hexane leaf extract (HLE) displayed no antimicrobial activity against any tested pathogens. The aqueous leaf extract (ALE) was only active against *S. pyogenes*, showing its selectivity in targeting this pathogen. The ethanol root extract (ERE) showed activity against *C. albicans* and *S. pyogenes*, while the hexane root extract (HRE) had a lower zone of inhibition against *S. pyogenes*. The ethanol seed extract (ESE) exhibited relatively strong activity against *C. albicans, S. pyogenes, S. salivarius,* and *S. mutans*. The aqueous seed extract (ASE) had the highest activity against *S. pyogenes*. Compared to the all extracts, ciprofloxacin exhibited significantly higher inhibition zones against all tested organisms, ranging from 15 to 29 mm, indicating its superior broad-spectrum antimicrobial activity. The larger inhibition zones observed for ESE and ELE, particularly against *S. pyogenes* and *C. albicans*.

Table 2- Zone inhibition (mm) of selected oral pathogens in the presence of *Tetracarpidium conophorum* leaf, root, and seed extracts prepared with ethanol, hexane, and water as solvent

Treatment	*C. albicans*	*S. pyogenes*	*S. salivarius*	*S. mutans*	*S. sobrinus*
ELE	9.67^{b}	0.00^{e}	8.33^{b}	0.00^{c}	10.67^{b}
HLE	0.00^{d}	0.00^{e}	0.00^{d}	0.00^{c}	0.00^{c}
ALE	0.00^{d}	10.00^{c}	0.00^{d}	0.00^{c}	0.00^{c}
ERE	7.67^{c}	7.67^{d}	0.00^{d}	0.00^{c}	0.00^{c}
HRE	0.00^{d}	7.33^{d}	0.00^{d}	0.00^{c}	0.00^{c}
ARE	0.00^{d}	0.00^{e}	0.00^{d}	0.00^{c}	0.00^{c}
ESE	7.33^{c}	10.33^{c}	7.67^{c}	8.33^{b}	0.00^{c}
HSE	0.00^{d}	0.00^{e}	0.00^{d}	0.00^{c}	0.00^{c}
ASE	0.00^{d}	11.67^{b}	0.00^{d}	0.00^{c}	0.00^{c}
DW	0.00^{d}	0.00^{e}	0.00^{d}	0.00^{c}	0.00^{c}
CP	16.33^{a}	20.67^{a}	15.00^{a}	29.00^{a}	22.33^{a}

*ELE=ethanol leaf extract; HLE= hexane leaf extract; ALE= aqueous leaf extract; ERE=ethanol root extract; HRE= hexane root extract; ARE= aqueous root extract; ESE=ethanol seed extract; HSE= hexane seed extract; ASE= aqueous seed extract; DW= distilled water; CP= ciprofloxacin

Table 3 shows the amounts of MIC for the leaf, root, and seed extracts against the oral pathogens. Due to the insufficient inhibition of hexane extract against the microorganism (Table 2), its MIC was not examined. For ELE, the MIC was achieved at 50 mg/ml for *C. albicans* and *S. salivarius*, while *S. sobrinus* showed a higher MIC at 200 mg/ml. Interestingly, *S. pyogenes* and *S. mutans* were inhibited even at lower concentrations so that no growth was observed in the presence of the least dilution. It indicates that the ethanol extract of walnut leaves could effectively inhibit these pathogens at relatively low concentrations. ALE had an MIC of 100 mg/ml for *S. pyogenes*, and other microorganisms were inhibited by the extract at concentrations lower than 50 mg/ml, indicating its strong ability to suppress the growth of these bacteria. ERE demonstrated an MIC of 200 mg/ml for *C. albicans*, while for *S. pyogenes*, it had a lower MIC at 25 mg/ml. Moreover, other microorganisms were more susceptible than *S. pyogenes*. ESE had notable MIC values across multiple organisms within the tested concentrations, including *C. albicans* and *S. pyogenes* (100 mg/ml) and *S. salivarius* and *S. mutans* (50 mg/ml). ASE exhibited its MIC for *S. pyogenes* at 200 mg/ml, and other microorganisms were in-

hibited at concentrations lower than 50 mg/ml. The significant no growth of the microorganisms at different concentrations of the extracts indicates the potent antimicrobial properties of *T. conophorum.*

Table 3- Minimum inhibitory concentration of the leaf, root, and seed extracts of *Tetracarpidium conophorum* prepared with ethanol, hexane, and water as solvent

Extract	Microorganism	Concentration (mg/ml)				
		400	200	100	50	25
ELE	*C. albicans*	-	-	-	*	+
	S. salivarius	-	-	-	*	+
	S. sobrinus	-	*	+	+	+
	S. pyogenes	-	-	-	-	-
	S. mutans	-	-	-	-	-
ALE	*C. albicans*	-	-	-	-	-
	S. salivarius	-	-	-	-	-
	S. sobrinus	-	-	-	-	-
	S. pyogenes	-	-	*	+	+
	S. mutans	-	-	-	-	-
ERE	*C. albicans*	-	*	+	+	+
	S. pyogenes	-	-	-	-	*
	S. salivarius	-	-	-	-	-
	S. sobrinus	-	-	-	-	-
	S. mutans	-	-	-	-	-
ARE	*S. pyogenes*	-	-	*	+	+
	S. sobrinus	-	-	-	-	-
	S. salivarius	-	-	-	-	-
	S. mutans	-	-	-	-	-
	C. albicans	-	-	-	-	-
ESE	*C. albicans*	-	-	*	+	+
	S. pyogenes	-	-	*	+	+
	S. salivarius	-	-	-	*	+
	S. mutans	-	-	-	*	+
	S. sobrinus	-	-	-	-	-
ASE	*S. pyogenes*	-	*	+	+	+
	C. albicans	-	-	-	-	-
	S. sobrinus	-	-	-	-	-
	S. salivarius	-	-	-	-	-
	S. mutans	-	-	-	-	-

+ = indicates growth; - = indicates no growth; * = indicates MIC; ELE = ethanol leaf extract; ALE = aqueous leaf extract; ERE = ethanol root extract; ARE = aqueous root extract; ESE = ethanol seed extract; ASE = aqueous seed extract

Table 4 shows the amounts of MBC and MFC of *T. conophorum* extracts against selected oral pathogens. The ELE showed bactericidal and fungicidal effects at different concentrations. For *C. albicans*, the minimum fungicidal concentration was 200 mg/ml, as indicated by the asterisk in the table.

However, microbial growth was observed at lower concentrations (100 mg/ml and below). Similarly, *S. salivarius* was eliminated at 100 mg/ml. In the case of *S. sobrinus*, ELE exhibited vigorous bactericidal activity at 400 mg/ml. *S. pyogenes* and *S. mutans* were inhibited at all concentrations of ELE. ALE demonstrated its minimum bactericidal concentration (MBC) against *S. pyogenes* at 200 mg/ml, and this extract could inhibit other microorganisms at all concentrations that show its potent antimicrobial potency even at low concentrations.

Table 4- Minimum bactericidal/minimum fungicidal concentration of the leaf, root, and seed extracts of *Tetracarpidium conophorum* prepared with ethanol, hexane, and water as solvent

Extract	Microorganism	Concentration (mg/ml)				
		400	200	100	50	25
ELE	*C. albicans*	-	*	+	+	+
	S. salivarius	-	-	*	+	+
	S. sobrinus	*	+	+	+	+
	S. pyogenes	-	-	-	-	-
	S. mutans	-	-	-	-	-
ALE	*S. pyogenes*	-	*	+	+	+
	S. salivarius	-	-	-	-	-
	S. sobrinus	-	-	-	-	-
	S. mutans	-	-	-	-	-
	C. albicans	-	-	-	-	-
ERE	*C. albicans*	*	+	+	+	+
	S. pyogenes	*	+	+	+	+
	S. salivarius	-	-	-	-	-
	S. sobrinus	-	-	-	-	-
	S. mutans	-	-	-	-	-
ARE	*S. pyogenes*	-	*	+	+	+
	C. albicans	-	-	-	-	-
	S. mutans	-	-	-	-	-
	S. salivarius	-	-	-	-	-
	S. sobrinus	-	-	-	-	-
ESE	*C. albicans*	*	+	+	+	+
	S. pyogenes	-	*	+	+	+
	S. salivarius	-	-	*	+	+
	S. mutans	*	+	+	+	+
	S. sobrinus	-	-	-	-	-
ASE	*S. pyogenes*	*	+	+	+	+
	S. salivarius	-	-	-	-	-
	S. mutans	-	-	-	-	-
	S. sobrinus	-	-	-	-	-
	C. albicans	-	-	-	-	-

+ = indicates growth; - = indicates no growth; * = indicates MIC; ELE = ethanol leaf extract; ALE = aqueous leaf extract; ERE = ethanol root extract; ARE = aqueous root extract; ESE = ethanol seed extract; ASE = aqueous seed extract

ERE could suppress *C. albicans* and *S. pyogenes* at 400 mg/ml, highlighting its efficacy at high concentrations. The ARE showed bactericidal activity against *S. pyogenes* at 200 mg/ml, similar to those observed for ALE, which shows their same antimicrobial potency within the tested concentrations against the oral pathogens.

ESE demonstrated various antimicrobial activity among the microorganisms. For *C. albicans*, it was achieved at 400 mg/ml, while *S. pyogenes* and *S. salivarius* were killed at 200 and 100 mg/ml, respectively. Additionally, for *S. mutans* the bactericidal activity was achieved at 400 mg/ml. These findings highlight the broad-spectrum antimicrobial potential of the ESE. ASE exhibited bactericidal activity against *S. pyogenes* at 400 mg/ml, while other microorganisms were killed at all concentrations. It indicates that aqueous extracts of walnut seeds possess potent bactericidal properties. Comparing the results of the extracts reveals the superior bactericidal/fungicidal activity of the aqueous extract of root and leaf followed by the seed rather than the other extracts.

The phytochemical screening of walnut (*T. conophorum*) plant extracts revealed the presence of multiple bioactive compounds in the leaves, roots, and seeds, which have the potential to influence their antimicrobial activities against oral pathogens. These findings are relevant in understanding how specific phytochemicals could contribute to the antimicrobial properties of the walnut plant, considering their known roles in various pharmacological activities. As mentioned earlier, alkaloids and saponins were detected in all parts of the walnut plant (leaf, root, and seed). Alkaloids are known for their antimicrobial properties, particularly in disrupting bacterial cell walls and inhibiting protein synthesis. The presence of alkaloids in all parts of the walnut plant contributes to its antimicrobial activity against oral pathogens [24-26]. Similarly, saponins have been reported to possess detergent-like properties that can disrupt microbial cell membranes, contributing to their antibacterial and antifungal actions [27-28]. The detection of saponins in all extracts indicates their potential role in the antimicrobial activity of walnut extracts. Tannins, the other prominent extractable component, play a crucial role in antimicrobial activity by precipitating the microbial proteins and inhibiting the bacterial adhesion to host tissues [29]. It is of great importance with respect to the oral pathogens, where adhesion is critical in forming dental biofilms [29-30]. Flavonoids have been widely recognized for their antibacterial, antiviral, and antifungal properties [31]. Their presence in both polar and non-polar extracts indicates that flavonoids in walnut may have a broad spectrum of antimicrobial activity, depending on their solubility and interaction with the microbial membrane [32]. The ability of flavonoids to inhibit bacterial growth and biofilm formation may be especially significant in targeting oral pathogens, which rely on biofilm structures for survival and proliferation [29]. In accordance, the phenolic compounds, abundant in ethanol extracts, are known for their potent antioxidant and antimicrobial properties [33-34]. They disrupt microbial cell walls, leading to cell lysis, and inhibiting the growth of various pathogens [35]. Given the role of oxidative stress in the pathogenesis of oral diseases such as periodontitis, the presence of phenolic compounds in walnut extracts may enhance their potential as therapeutic agents for oral infections. Their presence in the ethanol extract suggests that alcohol-based walnut extracts may be particularly effective in antimicrobial applications. The presence of anthraquinones in the ethanol extract of walnut leaves and seeds highlights their potential antimicrobial function. Anthraquinones are known to interfere in the microbial DNA replication, inhibiting microbial proliferation [36-38]. Specifically, steroids were not found in the seeds, which suggests that the antimicrobial activity may vary depending on the part of the plant used. Steroids are known to influence membrane permeability, which can enhance antimicrobial efficacy [39]. The absence and/or very low unextractable concentration of glycosides and phlobatannins in all parts of the plant by using the extraction solvents, except

for the HLE, suggests that these compounds may not contribute to the antimicrobial potency of the plant. It aligns with other studies where the absence of some compounds did not diminish the overall antimicrobial efficacy of the plant [40]. In comparison, alkaloids, saponins, tannins, flavonoids, and phenolics, that were found in the walnut extracts have demonstrated significant potential for antimicrobial activity against oral pathogenic microorganisms. These phytochemicals have well-documented effects on bacteria commonly found in the oral cavity such as *S. mutans*, *Porphyromonas gingivalis*, and *C. albicans*, which are associated with dental caries, gingivitis, and oral candidiasis [29]. The variety of phytochemicals across different solvents also suggests that polar and non-polar extracts could target a broad range of oral pathogens. For instance, the presence of flavonoids and tannins in the aqueous extracts provides a basis for using water-based formulations in oral healthcare products such as mouthwashes and toothpaste [29]. Meanwhile, the presence of phenolics and alkaloids in ethanol extracts points to the possibility of using alcohol-based tinctures as antimicrobial agent for treating infections and disinfecting surfaces.

The antimicrobial efficacy of walnut leaf, root, and seed extracts was measured using the zone of inhibition assay, where the results highlighted varying degrees of inhibition against selected oral pathogens. Ethanol and aqueous extracts of the walnut plant showed higher antimicrobial activity than their hexane counterpart. It suggests that ethanol and water are effective solvent for extracting bioactive compounds from the walnut plant. It aligns with previous studies demonstrating the superior efficacy of ethanol over hexane in inhibiting the microbial growth. The studies have shown that ethanol extracts often contain a higher concentration of phenolic compounds, flavonoids, and tannins, contributing to their antimicrobial activity [1-3,7,41]. The ethanol extracts are more potent in inhibiting bacterial growth, likely due to their ability to solubilize a broader range of antimicrobial compounds, including alkaloids, flavonoids, and phenolics, often present in walnut plants [42]. The ethanol extracts from the walnut plant possess significant bactericidal and fungicidal properties, especially against fungi and Gram-positive bacteria, both of which are of common oral pathogens.

The antibacterial effect of walnut extracts against *S. mutans* and *S. pyogenes* suggests their potential applications in dental care products, particularly at concentration of 400 mg/ml for the ethanolic extracts of the plant parts. These results are consistent with the antimicrobial activity of other plant-derived extracts used in traditional medicine for oral health, such as neem and green tea [43-45]. The favourable results of these extracts' antibacterial activity tests and bioactive components establish a potential basis for pharmaceutical research. The traditional usage of walnut in ethnomedicine has been validated by the association between its discovered bioactive components, including alkaloids, flavonoids, and steroids, and its demonstrated antibacterial efficacy against certain bacterial strains. Similarly, several chemical components in walnuts, such as tannins and phenols, corroborate their ancient medical usage by echoing their notable antibacterial effectiveness against various microorganisms, just like other plants of medicinal importance [1-3,46].

4. Conclusion

The screening of walnut (*T. conophorum)* plant extracts has revealed the presence of several bioactive phytochemical, including alkaloids, saponins, tannins, flavonoids, and phenolic compounds, which contribute to the plant's antimicrobial properties, especially against oral pathogens. Alkaloids and saponins disrupt bacterial cell walls and membranes, while tannins and flavonoids prevent bacterial adhesion and biofilm formation. Phenolic compounds, abundant in ethanol extracts, offer antioxidant and antimicrobial effects, and can be considered as therapeutic agents in treating oral infections like dental caries and gingivitis. Our findings emphasize the superior antimicrobial efficacy of ethanol and aqueous extracts over hexane extract, particularly in inhibiting the growth of key

oral pathogens like *C. albicans, S. pyogenes*, and *S. mutans*. It indicates that ethanol and aqueous extracts are more potent, likely due to their ability to dissolve a wide range of antimicrobial compounds, such as alkaloids and phenolics. The results also suggest that walnut plant extracts have significant potential for use in natural oral health care products such as mouthwash and toothpaste. Their efficacy against common oral pathogens makes them promising candidates for managing bacterial and fungal infections in the oral cavity. Moreover, the study highlights the growing importance of plant-based antimicrobials as potent and necessary alternatives to the conventional antibiotics, particularly in the context of rising antibiotic resistance. This underscores the significance of the research and its potential impact on the field of oral health. However, the current study identifies some limitations. The lack of specific identification of bioactive compounds responsible for antimicrobial activity calls for immediate and further research using advanced analytical techniques like high performance liquid chromatography and mass spectroscopy. Additionally, in vivo studies and the exploration of synergistic effects between walnut extracts and antibiotics could provide more comprehensive insights into their therapeutic potential. Expanding research into other extraction methods may also enhance the yield of bioactive compounds, improving the overall effectiveness of walnut plant extracts in oral healthcare.

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