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Evaluation of the Effectiveness of *Azadirachta Indica* Leaves Against Tooth Decay Microbes in Kirkuk City

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ABSTRACT: 100 samples were collected from people suffering from tooth decay attending dental clinics in Kirkuk City. The results showed that 59(59%) of samples have positive growth on Mannitol salt agar, MacConkey agar, and blood agar. While the remaining samples had no growth. Isolates were identified using biochemical and microscopic tests, 20 samples of *Streptococcus mutans* bacteria were isolated, with the highest percentage reaching (33.9%), 16 *Staphylococcus aureus* isolates representing (27.1%), followed by 13 (22%) *Candida albicans* isolates and 10 (17%) *Staphylococcus epidermidis* isolates. Aqueous and alcoholic extracts of the neem plant were prepared, the aqueous extract showed higher activity against the isolates than the alcoholic extract. Neem extracts also showed higher effectiveness against bacteria compared to fungi, Ciprofloxacin was used as a control agent for bacteria and fluconazole for fungi. *Streptococcus mutans* recorded the highest inhibition zone of 4.5±0.53 and 2.3±0.51 at 100 mg/mL concentrations for aqueous and alcoholic extracts, respectively, followed by the rest of the isolates with different inhibition zones. The minimum inhibitory concentration for the aqueous extract of neem was (40 mg/ml) and (60 mg/ml) for the alcoholic extract, While *C. albicans* recorded (20 mg/ml) and (40 mg/ml) for the aqueous and alcoholic extracts, respectively.

Keywords: dental caries, neem plant, bacteria, fungi.

1. INTRODUCTION

Azadirachta indica, is also known as neem, is a perennial tree from the *Meliaceae* family that is found in tropical regions. It flourishes in tropical and subtropical conditions, the tree exhibits quick growth and often reaches a height of 15-20 metres. Despite being evergreen, it may lose most or all of its leaves as a result of dehydration. For thousands of years, Indian tradition has recognised the therapeutic characteristics of neem [1, 2]. Various parts of *A. indica*, including the bark, stem, roots, seeds, leaves, fruits, and flowers, have been traditionally used as herbal remedies to cure human ailments. Additionally, neem twig chew sticks are popularly used for dental hygiene by millions of people [3, 4]. In addition, it has been employed for decades in traditional medicine as an anticancer, contraceptive, antacid, antimicrobial, antidiabetic, and for the treatment of certain skin and dental problems [5]. Besides being employed as a pesticide and fertiliser [6]. Tooth decay is a widespread oral health problem that affects a significant majority of people in all nations [7]. Initial clinical signs of caries show as a minuscule lesion on the surface of the tooth enamel and may occasionally be observed within interdental cracks. Dental caries spreads from the tooth root to the tooth crown via the alveolar bone. The enamel undergoes degradation and ultimately disintegrates, resulting in the formation of a hollow that subsequently leads to damage [8, 9]. Microorganisms possess the capacity to spread within pulp lining material and penetrate dentinal tubules, resulting in pulp irritation and the progression of caries [10]. Several researchers have established a correlation between oral bacteria and tooth decay, where the former causes harm to teeth and the latter is promoted by them [11]. The acids produced by bacteria through carbohydrate metabolism gradually destroy the calcified tissue of the tooth [12].

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Streptococcus mutans and *Streptococcus sobrinus* are highly significant bacteria in the progression of dental caries [13]. *Lactobacilli* and other bacteria also play a role in the development of the disease [14]. *Staphylococcus* spp. and *Candida* spp. are opportunistic bacteria that are known to be potential infections, particularly in individuals with systemic debilitations such as neutropenia, diabetes, AIDS, and agranulocytosis [15]. Staphylococci are considered temporary inhabitants of the oral cavity [16], but they are also recognised as possible pathogens in several oral diseases, such as tooth decay [17]. The antimicrobial effects of neem leaf extract were found to be effective against oral pathogens [10]. Neem prevents bacteria from sticking to saliva-treated hydroxyapatite, which is a mixture of enamel and bone. Neem extract additionally inhibited the creation of insoluble glucans, resulting in a reduction in streptococci attachment to tooth surfaces. The antibacterial effects of neem are mostly attributed to azadirachtin, the main terpenoid compound found in neem [18]. The main objective of this study is to determine the antimicrobial activity of neem plant against bacteria and fungi isolated from dental caries.

2. MATERIALS AND METHODS

The study was performed in Kirkuk City from November 2023 until February 2024.

2.1 Samples collection and identification

100 samples were obtained from patients. Some of the samples were obtained using transfer swabs, which involved gently stroking the affected area or the gum connected to the teeth. The remaining samples were obtained from tooth extractions in dental clinics. The samples were cultured on blood agar, MacConkey agar, and mannitol salt agar at 37°C for 24 to 48 hours. Organisms were identified through the utilisation of microscopic and biochemical tests [19, 20].

2.2 Preparation of the Plant Extracts

A. indica Leaves were obtained from local markets, washed, and air-dried for 7 days, after which they were pulverised. A total of 50 gm. of powder was extracted using either 750 millilitres of 95% ethanol or distilled water. The extraction process involved shaking the mixture intermittently for 3 days. The mixes have been purified by filtration using Whatman ® Number (1) filter paper to achieve transparent filters. The filtrates obtained were further dehydrated in beakers utilising a water bath set at a temperature of 60 °C for an approximate duration of 12 hours, resulting in the production of 5.60 gm. of ethanol-alcohol extract and 13.2 gm. of aqueous extract. The extracts were subsequently stored in the refrigerator until they were used [21].

2.3 Inoculum Preparation

Bacteria and fungus were transfected in sterile 0.9% saline solution and adjusted to a standard of 0.5 McFarland each. The inoculum was then standardised to include around 108 CFU/ml for bacteria and 107 CFU/ml for fungus [22, 23].

2.4 Disk diffusion method

The method was used to measure the antimicrobial effect of *A. indica* extracts. Concisely, paper discs (6 mm) were soaked with 10 µl of each concentrated extract (10, 20, 40, 60, 80, and 100) mg/mL, then placed on prepared inoculation plates (nutrient agar for bacteria and Sabouraud agar for fungus). The plates containing bacteria were incubated at 37°C for 24 hours, while those containing fungi were incubated at 27°C for 48 hours. The diameters of the zone that inhibits the growth of microbial cells were measured in millimetres. Fluconazole (25 µg/disc) and Ciprofloxacin (5 µg /disc) have been positive controls for fungi and bacteria, respectively. Each test was carried out three times [24, 25]. The minimum inhibitory concentration (MIC) of the extracts was evaluated using the disc diffusion method. The minimum inhibitory concentration is defined as the lowest concentration at which the growth of microbes is inhibited [26].

2.5 Phytochemical Screening of *A. indica* Extracts

The phytochemical components of extracts of *A. indica* were analysed utilising the methodologies described by Soundararajan *et al*., [27], and Kumar *et al*., [28]. The phytochemical components that were analysed include phenolics, alkaloids, tannins, saponins, steroids, flavonoids, and terpenoids.

2.6 Statistical analysis

The percentages, mean, and standard deviation were calculated using SPSS version 18 and Microsoft Excel 2013.

3. RESULTS AND DISCUSSION

100 samples were taken from patients of all ages who visited private dental clinics in Kirkuk City. Out of these samples, 59 (59%) showed microbial growth, indicating tooth decay. Out of the remaining 41 samples, no microbial growth was observed, possibly due to their anaerobic nature, which made them ineligible for the present study. Variations in the prevalence of dental decay are common. Several variables impact the likelihood of tooth decay, such as immunity, age, overall health, the kind of food consumed, sugar intake, and daily dental hygiene practices. In addition, several additional factors significantly influence the occurrence of tooth decay, including the quality of food [29, 30]. According to our research, *Streptococcus mutans* was the most common bacteria found in infected people, accounting for 33.9% of cases. This was followed by *Staphylococcus aureus*, which was isolated in 27.1% of cases, as indicated in Table 1. *Candida albicans* and *Staphylococcus epidermidis* were found at frequencies of 22% and 17%, respectively.

Table 1. Numbers and percentages of microbes isolated from tooth decay

Isolated sample	number	percentage
Streptococcus mutans	20	33.9%
Staphylococcus aureus	16	27.1%
Candida Albicans	13	22%
Staphylococcus epidermidis	10	17%
	59	100%

The results of our study isolates were similar to those of Rana *et al*., They isolated *S. aureus*, *Streptococcus* spp. and *Candida albicans* at rates of 62.29%, 26.22%, and 11.47%, respectively [31]. The results of our research also agreed with the findings of studies Abdulhadi & Nijris [32], Jassam *et al*., [33], and Abd Al-Zahra & Saleh [34], which reported the highest percentages of *Streptococcus mutans* isolation at 33%, 26.31%, and 41%, respectively. The ability of Streptococcus to produce a sticky sugar matrix around bacterial cells is associated with its ability to cause smooth surface degeneration. This matrix shows many attributes associated with adhesion, bacterial defence, and degradation. Streptococcus is considered to be the main factor behind tooth decay and has distinct carcinogenic properties [35].

3.1 Antimicrobial Activity of *A. indica* Extracts (Aqueous and Alcohol)

The disc diffusion technique tested the organisms' susceptibility to *A. indica* extracts at concentrations (10, 20, 40, 60, 80, and 100) mg/mL. Table 2 shows the inhibition zones (mm) for *A. indica* aqueous extract at different concentrations (mg/mL). *Streptococcus mutans* had the highest susceptibility (4.5 ±0.53) at 100 mg/ml, followed by *S. aureus* (2.6 ±3.30), *S. epidermidis* (2.4 ±2.43 mm), and *C. albicans* (2.1 ±0.61 mm).

Table 2. Mean zone of inhibition (mm) of *Azadirachta indica* aqueous extract against isolates.

Bacterial Isolates	**(10) mg/mL**	**(20) mg/mL**	**(40) mg/mL**	**(60) mg/mL**	**(80) mg/mL**	**(100) mg/mL**	**Control Ciprofloxacin (5 µg/disc)**
Streptococcus mutans	0	0	1.4±0.57*	2.5 ±0.61	3.7 ±1.50	4.5 ±0.53	38.50 ±0.54
Staphylococcus aureus	0	0	1.5±0.50*	1.8±2.51	2.1±3.50	2.6±3.30	36±2.43
Staphylococcus epidermidis	0	0	1.0±0.44*	1.4±2.23	1.9±3.52	2.4±2.43	38.23±4.10
Fungi Isolates	**(10) mg/mL**	**(20) mg/mL**	**(40) mg/mL**	**(60) mg/mL**	**(80) mg/mL**	**(100) mg/mL**	**Control fluconazole (25 µg/disc)**
Candida albicans	0	0.5±2.43*	0.8±0.54	1.4±0.13	1.7±0.53	2.1±0.61	26±0.52

*- MIC of aqueous extract against isolates

And in Table 3, *S. mutans* also recorded the highest zone of inhibition at a concentration of 100 mg/mL for the alcoholic extract, with an inhibition zone of 2.3 ± 0.51, followed by *Staph epidermidis* (2.2 ± 0.32), *Staph aureus* (2.0 ± 0.52), and *C. albicans* (1.5 ± 0.50).

Table 3. Mean zone of inhibition (mm) of *Azadirachta indica* alcohol extract against isolates.

Bacterial Isolates	**(10) mg/mL**	**(20) mg/mL**	**(40) mg/mL**	**(60) mg/mL**	**(80) mg/mL**	**(100) mg/mL**	**Control Ciprofloxacin (5 µg/disc)**
Streptococcus mutans	0	0	0	0.6 ±0.23*	1.4 ±0.50	2.3 ±0.51	38.50 ±0.54
Staphylococcus aureus	0	0	0	0.5±3.52*	1.0±0.53	2.0±0.52	36±2.43
Staphylococcus epidermidis	0	0	0	0.6±2.52*	1.5±2.50	2.2±0.32	38.23±4.10
Fungi Isolates	**(10) mg/mL**	**(20) mg/mL**	**(40) mg/mL**	**(60) mg/mL**	**(80) mg/mL**	**(100) mg/mL**	**Control fluconazole (25 µg/disc)**
Candida albicans	0	0	0.5±1.50 *	1.0±0.53	1.3±2.54	1.5±0.50	26±0.52

*- MIC of aqueous extract against isolates

The results of the present study agreed with Bansal *et al* findings, showing that neem had the highest activity against *S. mutans*, as shown by an average inhibition zone of (11.4 ± 4.03 mm) [36]. The neem's aqueous extract exhibited higher antibacterial effects compared to the alcoholic extract. The findings of the present study agreed with Hikaambo *et al*., study as the aqueous extract exhibited more antibacterial activity in comparison to the alcoholic extract [37]. The variation in inhibiting zones can be due to variances in the phytochemical contents of the extracts, hence impacting the therapeutic benefits of the plant extract. Moreover, the differences noted in the published research could be attributed to changes in geographical regions, harvesting periods, and rainfall patterns [37]. The activity of *A. indica* is substantially influenced by the extraction solvent polarity. Subramaniam *et al*., study revealed that neem leaf extract possesses antibacterial properties against *Streptococcus mutans* [38]. Asif's study showed the efficiency of *A. indica* extract against *Staph aureus* and *C. albicans* [39], and Yaseen's study also recorded the same extract against *Staph aureus* and *Staph epidermidis*, which is agreed with the results of our research [40]. *Streptococcus mutans*, *Staph aureus*, and *Staph epidermidis* showed the same minimum inhibitory concentration. The MIC for the aqueous extract of *A. indica* was 40 mg/ml and 60 mg/ml for the alcoholic extract, while *C. albicans* recorded (20 and 40) mg/ml for aqueous and alcoholic extracts, respectively. The extracts had more impact on bacteria than on fungus, as all the data support the hypothesis that the phenolic group of chemicals that makes up neem's active ingredient can break down bacterial cell walls, hence inhibiting the growth of bacteria. Osmotic pressure changes and cell death results from cell wall collapse [41].

3.2 Phytochemical screening test

Phytochemical screening tests were conducted for aqueous and alcoholic extract of *A. indica*. The aqueous extract showed positive results in flavonoids, Saponins, phenolic, and tannins. While the alcoholic extract showed positive results for Phenolic, Tannins, and Steroids as shown in Table 4

Table 4. Phytochemical analysis of *Azadirachta indica* extracts.

Phytochemicals tested	**Aqueous extract**	**Alcohol extract**
Saponin	+	-
Phenolic	+	+
Tannin	+	+
Flavonoid	+	-

Alkaloid	-	-
Terpenoid	-	-
Steroid	-	+

+ (Present), _ (Absent)

The results of the present study agreed with the research done by Sharma *et al*., which showed the existence of tannins, and phenolics in the alcohol extract, and saponin in the aqueous of *A. indica* extract [42]. Moreover, the results also agreed with Hikaambo *et al*., except that the aqueous extract did not contain alkaloids [37]. The alcoholic extract contained both steroids and tannins, which agrees with the findings of Susmitha *et al*., who also observed the presence of tannins and steroids in their alcoholic extract [43]. The observed changes in phytochemical content can be related to solvent polarity. Moreover, depending on the geographic and environment conditions that the plant was exposed, research carried out in different areas may indicate the existence or lack of phytochemicals found in our study[37].

4. CONCLUSIONS

The results of this study indicated that neem leaves can be used as an antimicrobial agent against pathogens that cause tooth decay. The aqueous extract shown more efficacy than the alcoholic extract against the isolated microorganisms. Neem leaves also recorded more activity against bacteria than isolated fungi, and the antimicrobial activity of neem leaf extracts was concentration dependent, as evidenced by different zones of inhibition at different concentrations. *Streptococcus mutans* recorded the highest isolation rate in dental caries cases. More research is required to evaluate bio compatibility and safety before recommending neem for its antimicrobial properties as a mouthwash.

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