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Comparative Evaluation on Antimicrobial Activity of *Azadirachta indica* (Neem) Leaf Extract and Commercial Toothpaste (Spearmint), Against Clinical Isolates Dental Caries Bacteria in Sudan

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***Abstract*: Background: *Azadirachta indica* is an evergreen, temperature tolerant, flowering plant distributed in India and other continents of the world. It is among medicinal plants with a multipurpose and multiple health benefits both traditional and pharmaceutical industries. Objectives: To identify bacteria species that cause carious teeth, to verify and compare the activity of *A. indica* extract and commercial toothpastes against thirty clinical pathogenic bacteria. Material and Methods: thirty bacterial test strains: *Streptococcus spp, Lactobacillus acidophilus, Staphylococcus aureus, pseudomonas aeruginosa* and *Proteus vulgaris* was carried out in the study. Ethanolic extract of *A. indica* leaves and aqueous commercial toothpaste were prepared at varying concentrations which were applied on inoculated plates of Muller Hinton agar (MHA) using well diffusion method. Standardized discs of the synthetic antibiotics: ciprofloxacin and novobiocin were also applied on inoculated plates of (MHA). Results: *A. indica* leaf extract showed moderate antimicrobial activity against all bacterial species studied tested except *Pseudomonas aeruginosa*. It showed maximum inhibition against *Lactobacillus acidophilus* at 12.5 mg/ml concentration, when compared with commercial toothpaste (p = 0.004). Conclusion: *A. indica* leaves possess good antibacterial activity confirms the great potential of bioactive compounds and is useful for rationalizing the use of this plant in primary health care.**

***Keywords*: Azadirachta indica, commercial toothpaste, dental caries, pathogenic bacteria, well diffusion method.**

1. Introduction

Medicinal Plants are raw materials for drug production, Plants are rich in a wide variety of secondary metabolites such as tannins, alkaloids and flavonoids, which have been found in vitro to have antimicrobial properties (*Ncube et al*., 2008). The screening of plant extracts and plant products for antimicrobial activity has shown that plants represent a potential source of new anti-infective agents (Amani *et al* 1998) and (*Salvat et al*., 2001).

The use of plants for treating diseases is old as the human species, for thousands of years, natural products have been used in traditional medicine all over the globe and pre date the introduction of antibiotics and other modern drugs. The antimicrobial efficacy attributed to some plants in treating diseases has been beyond belief. It is estimated that local communities have used about 10% of all flowering plants on Earth to treat various infections, although only 1% have gained recognition by modern scientists. Owing to their popular use as remedies for many infectious diseases, researches for plants containing antimicrobial substances are frequent (Betoni et al., 2006). The human mouth serves as the best habitat for numerous bacterial species due to its alkaline condition, favorable to most bacteria and fungi. Many of these microbes are involved in developing oral diseases (Gholizadeh et al., 2016). Some species of bacteria, such as Pseudomonas aeruginosa, are opportunistic pathogens and cause disease, such as Infections derived from carious teeth (Entenza et al., 2014). Dental caries are one of the most common oral diseases and health problems in the world, is a chronic disease that destroys tooth tissue and that can adversely affect chewing and aesthetic appearance (Petersen, 2003).

Therefore, the aim of our study is to investigate the antimicrobial activity of Neem leaves against five clinical isolates dental caries bacteria and compare them with those of commercial toothpaste.

2. Materials and Methods

Fresh leaves of Neem (*A. indica*) were collected locally and were air dried in shade at room temperature (28–32 ◦C) for 10 days at the microbiology laboratory, faculty of pure and applied science, IUA. The A. indica leaf extract was then prepared by grounding 100 g of leaves using mortar and pestle.

Each of the coarsely powdered dried plant material (100 g) was exhaustively extracted for 8-12 hours with (80%) Ethanol using Soxhlet apparatus. Then the extracts were filtered and evaporated by air dried at room temperature (Harbone 1995).

The bacterial test strains used were 30 bacterial pathogens, isolated from dental caries infections. The clinical specimens were collected for microbiological testing at International University of Africa Dental Clinic (Khartoum). The organisms

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were purified by streaking on plates containing the appropriate selective and differential culture media. They were identified on the basis of the results of microscopical stain reaction (Gram stain), cultural characteristics and biochemical tests according to the standard microbiological techniques.

A. Bacterial Isolates Identification

1) Primary Identification

The primary identification was done by light microscope for Gram's stain and by examination with naked eye for colonial morphology e.g., appearance, motility, colony texture, lactose-fermenting etc.

2) Gram's Stain

In principle the bacterial film is fixed and stained with a Triphenylmethane dye such as Crystal violet, in conjunction with Iodine solution, and subsequently treated with an organic solvent such as Alcohol. Bacteria which retain the dye are designated Gram positive and other varieties of bacteria which lose the dye are termed Gram negative. Decolorized organisms are rendered visible by the application of a stain of suitable color e.g., Safranin red. Shapes and arrangements of the cells were also considered (Baker et al., 2008).

3) Biochemical tests for identification of the isolates

Species that cannot be distinguished by morphology and culture characters may exhibit metabolic differences that can be exploited. It is usual to test the ability of the organisms to produce acidic and gaseous end-products, when presented with individual carbohydrate (glucose, lactose etc.…) as the sole carbon source. Other tests determine whether the bacterium produces particular end-product (e.g., Indole) when grown in suitable culture media, and whether it possesses certain enzyme activities, such as Oxidase, Urease etc.

These bacterial test strains used were *Streptococcus spp* (11), *Lactobacillus acidophilus* (7), *Staphylococcus aureus* (6), *pseudomonas aeruginosa* (4) and *Proteus vulgaris* (2).

The antimicrobial sensitivity testing was conducted by the modified agar well diffusion method.

The sensitivity medium (Muller-Hinton agar) was prepared by adding Thirty-five grams of the powder in one liter of distilled water, boiled to dissolve the powder completely, and then sterilized in the autoclave at 121C for 15 minutes., and poured in sterile Petri plates up to a uniform thickness of approximately 4 mm and the agar was allowed to set at ambient temperature before use. The bacterial isolates were suspended in normal saline and incubated at 37° C for 1-2 hours before used as inocula. The turbidity of the normal saline culture was adjusted to 0.5 McFarland units. This gives a suspension containing approximately 1-2 x 106 colony forming units (CFU)/ml. A sterile cotton swab was inserted into the bacterial suspension, rotated, and then compressed against the wall of the test tube to express any excess fluid. The swab was then streaked on the surface of the Muller-Hinton agar plate.

To test antibacterial activity of Neem leaf extract, it was first dissolved in a methanol solvent, and then varying concentrations of the extracts (100mg/ml, 50mg/ml, 25mg/ml and 12.5mg/ml,) was prepared, Then standardized inoculum suspension of each bacterial strain was swabbed on the entire surface of Mueller-Hinton agar onto 9 cm diameter Petri dishes, then 20 µl of each extract was placed on each wells. The plates were incubated overnight at 37° C for 18-24 hours. The antimicrobial activity was detected by measuring zones of inhibition. To test antibacterial activity of the synthetic antibiotics, standardized discs of ciprofloxacin (5µg) and Novobiocin (105µg) were tested by the agar disc diffusion method by placing on a streaked Muller-Hinton agar plate surface. The antimicrobial activity was also detected by measuring zones of inhibition.

3. Results

A. Dental Caries Infections Were Isolated

The prevalence of five isolated bacteria from patients was, 11 (36.7%) of them *Streptococcus spp* and that was the most commonly isolated bacteria. *P. vulgaris* was the least commonly isolated bacteria from Dental caries of the study participants (6.7%) Table 1.

Table 1
Total number and percentages of the isolated thirty bacteria

Types of isolates	Total Number	Percentage
L.acidophilus	7	23.3%
S.aureus	6	20%
Streptococcus spp	11	36.7%
P.vulgaris	2	6.7%
p. aeruginosa	4	13.3%
Total	30	100%

Key: P.aeruginosa: Pseudomonas, S.aureus: Staphylococcus, S.trepto spp: Streptococcus spp, L.acidophilus: Lactobacillus acidophilus, P.vulgaris: Proteus.

Table 4. Exhibits the antibacterial activity of leaf extract against all clinical tested bacteria at all concentrations. As regard the lowest concentration (12.5 mg/ml) of the leaf extract, its highest Antibacterial activity was detected against *Lactobacillus acidophilus* (16±1mm inhibition zone) while detected inactive against *Streptococcus spp* and *Pseudomonas aeruginosa.*

Table 5. Exhibits the mean zones of inhibition (in mm) of the commercial toothpaste (Spearmint) with concentration of (100, 50, 25 and 12.5mg/ml) against five isolated bacteria using well diffusion assay.

Table 2
Microscopical stain reaction, cultural characteristics, and biochemical results for the dental caries pathogenic isolated bacteria

No.	Gram's Stain	Shape	K.I.A				Biochemical Tests					Remarks
			Slope	Butt	H_2S	gas	Ind	Cit	Ure	Cat	Mot	
1	+ve	Rods	Rods and non-spore-forming with rounded ends that occur singly									*Lactobacillus acidophilus*
2	+ve	Cocci	Clusters with catalase and coagulase positive									*Staphylococcus aureus*
3	+ve	Cocci	pairs or chains with catalase negative									*Staphylococcus aureus*
4	-ve	Rods	R	Y	+ve	D	+ve	D	+ve	+ve	+ve	*Proteus vulgaris*
5	-ve	Rods	R	Y	-ve	+ve	- ve	+ve	-ve	+ve	+ve	*Pseudomonas aeruginosa*

Key: D: different. -ve: Negative. Ind: Indole. Y: yellow. Ure: Urea. +ve: Positive. Mot: Motility. KIA: Kligler iron agar Cit: Citrate. R: Red. Cat: Catalase.

Table 3
The results of ciprofloxacin and Novobiocin antibiotic susceptibility

Organism/Statistical significance	Concentration (mg/ml)/mean zone of inhibition diameter (mm)(mean ±standard deviation)	
	Antibiotic	
	Ciprofloxacin (CIP)	Novobiocin (NV)
Lactobacillus acidophilus	46±1	36±1
staphylococcus aureus	37.7±2.5	0±0
Streptococcus spp	52.3±9.2	35.7±6.6
Proteus vulgaris	48±2	33.3±1.5
Pseudomonas aeruginosa	48.7±1.5	39±1

*Inhibition zones (mm) of the reference drugs (Novobiocin and Ciprofloxacin) against five isolated bacteria using disc diffusion assay**
**Values are given as mean ± standard deviation*

Table 4
Inhibition zones (mm) of the ethanol extracts of A.indica for different concentrations (100, 50, 25 and 12.5mg/ml) against five isolated bacteria using well diffusion assay*

Organism/Statistical significance	Concentration (mg/ml)/mean zone of inhibition diameter (mm)(mean ±standard deviation)			
	100	50	25	12.5
Lactobacillus acidophilus	22.3±2.5	19±3.6	15.3±5	16±1
staphylococcus aureus	25.3±2.5	21.7±2.9	17±1.7	13.3±3
Streptococcus spp	25.7±6	22±3.4	19.7±0.6	0±0
Proteus vulgaris	26.7±3.5	21±1.7	17.3±2.5	14.7±1.5
Pseudomonas aeruginosa	0±0	0±0	0±0	0±0

**Values are given as mean ± standard deviation*

Table 5
Inhibition zones (mm) of the commercial toothpaste (Spearmint) with concentration of (100, 50, 25 and 12.5mg/ml) against five isolated bacteria using well diffusion assay*

Organism/Statistical significance	Concentration (mg/ml)/mean zone of inhibition diameter (mm)(mean ±standard deviation)			
	100	50	25	12.5
Lactobacillus acidophilus	24±4.3	20±6	19±7	9.7±1.5
staphylococcus aureus	0±0	0±0	0±0	0±0
Streptococcus spp	0±0	0±0	0±0	0±0
Proteus vulgaris	34.7±1.5	23±1	17±2	9.3±1.5
Pseudomonas aeruginosa	29.3±2	23±1.7	0±0	0±0

**Values are given as mean ± standard deviation*

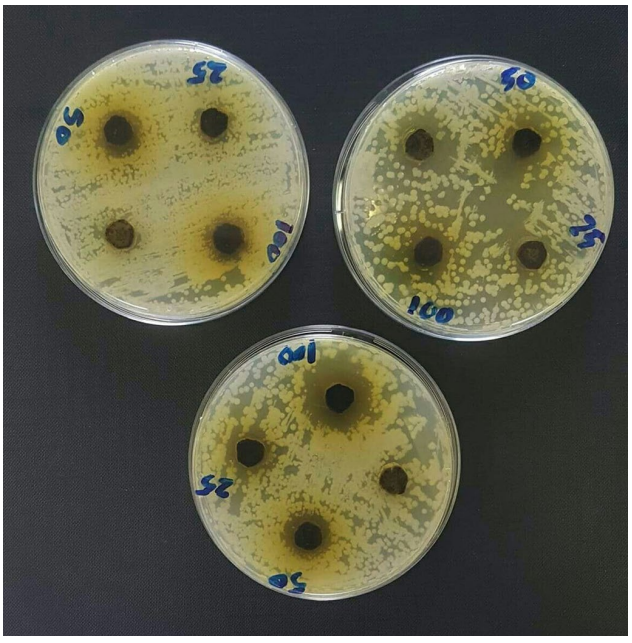

Fig. 1. Inhibition zones of ethanol extract of A.indica against L.acidophilus (Left up), S.aureus (Right up) and P.vulgaris (Down)

As shown the lowest concentration (12.5 mg/ml) of the commercial toothpaste, its highest Antibacterial activity was detected against *Lactobacillus acidophilus* (9.7±1.5 inhibition zone) while detected inactive against *Streptococcus spp*, *staphylococcus aureus* and *Pseudomonas aeruginosa*.

There was an insignificant difference (p >0.05) between the antibacterial activity of the leaf extract (Neem) and the commercial toothpaste (Spearmint) against *Lactobacillus acidophilus* at a concentration of 100, 50 and 25mg/ml (P=0.597, 0.819 and 0.485 respectively). Also, there was an insignificant difference between the antibacterial activity of the leaf extract and the commercial toothpaste against *Proteus vulgaris* at a concentration of 50 and 2525mg/ml (p = 0.158 and 0.866 respectively).

However, against *staphylococcus aureus, Streptococcus spp* and *Pseudomonas aeruginosa* there was a significant difference in the antibacterial activity of the leaf extract and the commercial toothpaste at a concentrations of 100, 50, 25 and 12.5mg/ml and commercial toothpaste (p =<0.05).

B. Discussion

Some synthetic drugs cause varying range of side effects, therefore there is need for the development of plant-based compounds which could be useful in meeting the demand for newer drugs with harmless or minimal side effects. (Srivastava et al., 2000). In the present study, it was found out that ethanol extract of the Neem plant had appreciable effect on the test organisms (Table 1). This study is in agreement with the work of Ugwu (2019) who reported that Neem leaf inhibited the growth of some bacterial pathogens.

Awasthy *et al*., (1999) reported that the ethanol extract of Neem is very useful orally to treat many diseases caused by bacteria. The high concentrations of azadirachtins, quercetin and β-sitosterol in *A. indica* leaves might be responsible for strong antibacterial and antifungal activity was findings of Subapriya and Nagini (2005).

4. Conclusion

This study showed that extract from *Azadirachta indica* possess antibacterial activity against bacteria present in the mouth. Thus, the results of this research have established the use of chewing sticks made from neem in maintaining oral hygiene. Also, the emerging healthcare products companies in Sudan can explore the option of using raw materials from neem in manufacturing newer products for oral application.

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