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Phytochemical Properties and the *in-vitro* Antibacterial Activity of Neem *(Azadirachta indica)* Twigs Extract on Bacteria Isolated from Human Mouth

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Abstract
The phytochemical properties and the antibacterial activity of aqueous extract of neem twigs (*Azadirachta indica*) on the bacterial species isolated from human mouth was investigated. The neem tree was observed to be devoid of any infection prior to cutting the twigs for laboratory analysis. The mouths of fifteen (15) students were swabbed using a sterile swab stick in the morning before each of them washes their mouth at Federal University Wukari. Bacterial isolation was done using nutrient agar. Identification of the bacteria isolates were done using standard techniques. Agar-well diffusion method was used to determine the antibacterial effect of the extract on a prepared nutrient agar. The twigs extracts showed antibacterial activity against all the test isolates at different concentrations. The zone of inhibition increased with increased concentrations of the extracts. Phytochemical extraction was done using GC-MS QP2010 PLUS machine. Polyphenols were extracted at a very large amount; Saponnins and Flavonoides were found to be in a large quantity while Glycosides and Alkaloids were found to be low. However, Phylobatanins, Anthraguinones, Hydroxymethyl Anthraguinones and Tannins were found to be absent. Most of these compounds extracted from neem twigs have antibacterial activity. Therefore, these results, further confirm the belief and other scientific studies on the importance of the traditional use of neem twigs in maintaining oral hygiene. Again, some of the isolates from the human mouth are potential pathogens and may pose a public health hazard to the community. Therefore, routine oral hygiene are strongly advocated to prevent oral diseases.

1. Introduction

Neem tree (*Azadirachta indica*) is a tall evergreen tree common with clear foliage. The tree was said to originate from India and with taxonomic grouping as thus: Order – Rutales; Sub order – Rutinae; Family - Meliaceae (mahogany family); Sub family-

Melioideae; Tribe – Melieae; Genus-Azadirachta and Species – indica [8, 9]. Amongst the plants introduced to Nigeria, it is one of the most prevalent species with a wide adaptive ability to various environments, even in the arid and sub arid regions. This plant species grows up to eighteen (18) to twenty four (24) meters tall with a fast-growing and robust twigs. Its leaves are separated into several parts, each of which looks like a typical leaf foliage with small white flowers which are assisting bunches with an average length of between 1.5cm to 2cm long, harboring green fruits with a seed in each when unripe and yellow fruits when ripe. Because of its strong adaptive ability to various environments, it has gained acceptance in the in arid and sub-arid regions in the prevention of erosion and afforestation programme [8, 9].

The importance of the plant extract cannot be over-emphasized. Studies have shown that extracts from every part of *Azadirachta indica* is a therapeutic agent [17, 18, 19]. At various concentrations, the plant has proven numerous promising positive results against bacteria, fungi and viruses [17]. According to [25], the antibacterial effect of the extracts from neem leaves and bark on *Pseudomonas aeruginosa, Corynebacterium diphtheriae* and *Bacillus* spp isolated from the mouths of adults is stronger at higher concentrations. Also, Several other in- vitro antibacterial studies of neem extract showed broad spectrum of antibacterial actions on *Mycobacterium. tuberculosis, Vibrio cholerae, Klebsiella pneumonia, Streptococcus faecalis, S.* spp and *M. pyogenes* [1, 2, 4, 22, 23]. In a report tittled "Neem: A tree for Solving Global Problems" published in 1992, the United States (U.S.) National Academy of Sciences enumerated many importance of neem which are but not limited to medicinal, pest control and environmental control [10, 23, 25]. Like most other therapeutic agents' sources, neem also was reported to have side effects. An oral administration of alcoholic extract of neem flower disrupted the estrous cycle in Sprague Dawley rats and causes a partial block in ovulation with the potential to cause infertility [11]. Phytochemically, varied compounds has been extracted form neem. From different parts of the tree, more than 135 compounds have been identified. These compounds are grouped into isoprenoid and non-isoprenoid [7, 14, 2]. The human mouth is an exclusive anatomical site in the human body with numerous epithelial and mucosal surfaces as well as calcified hard tissues [6]. These tissues are continually covered by the saliva and the constant moisture and presence of dissolved food as well as small food particles, make the mouth of human ideal environment for microbial growth. Although, the human saliva contains some antibodies and phagocytic cells that can destroy some microorganism in the mouth, while others get flushed into the stomach by constant saliva and get destroyed by the stomach's acid [21]. Yet, some of the microorganisms found in human mouth firmly sticks to various surfaces of the mouth such that they resist removal [21].

People from different parts of the world, such as India and Africa; regularly uses neem twigs as chewing stick and "tooth brush". This ancient practice has gained recognition by dentists that it was found to be effective in preventing periodontal disease. However, it is not clear whether the usage of neem twigs as tooth brush is due to regular gum massage or its antibacterial activity to prevent plaque or dental carries. This research sought to examine the phytochemical contents of neem twigs and test the antibacterial efficacy of the neem twigs extract on human mouth isolates at different concentrations among students of the Federal University Wukari, Taraba State, Nigeria.

2. Materials and Methods

2.1. Sample Collection and Preparation

Samples of the twigs of *Azadirachta indica* were collected from Wapan-Nghaku around the Federal University Wukari, Taraba State, Nigeria. The samples were immediately washed using tap water and crushed into a pieces using mortar and pestle. Exactly ten (20g) of the powder was weighed using an electronic weighing balance and dissolved into 1000mL of distilled water for each of the extracts. The same procedure was repeated for extracts of 40g, 80g, 100g, 200g, 400g, 600g, 800g and 1000g to obtain 2%, 4%, 8%, 10%, 20%, 40%, 60%, 80% and 100% concentrations accordingly [16].

2.2. Bacterial Analysis

Using a sterile swab stick, swab samples were collected from the mouth of students of Federal University Wukari, Taraba State, Nigeria at about 8:00hr before brushing their teeth. The swab sticks were immediately streaked on to a prepared nutrient agar and incubated at 37°C for 24h. The distinct colonies that emerged after incubation were identified using microscopy and then sub-cultured on to a prepared nutrient agar to obtain a pure culture. Then, the pure bacterial isolates obtained were Gram-stained and identified by comparing their characteristics with those of known taxa using the schemes of [5] and by the conventional bacteriological test methods and by reference to the keys outlined by [3]. See Table 1.

2.3. Antibacterial Analysis of the Sample

To determine the antibacterial activity of the aqueous extracts of the twigs *A. Indica*, an aliquot (0.1mL) of each of the prepared extracts of twigs *A. Indica* at the various concentrations (2%, 4%, 8%, 10%, 20%, 40%, 60%, 80% and 100%), were obtained using sterile syringe and inoculated onto prepared nutrient agar plates by agar well diffusion method, using a sterile cork borer of diameter size 6.00mm, to cut the solid Agar surface; (3 wells per 90 mm diameter plate) as carried out by [25]. Incubation was done at 37°C for 24 hours. Zone of clearance (inhibition) was determined and measured round the well. The minimum inhibitory concentration (MIC) was interpreted as the lowest concentration of the extract that did not show any visible growth when compared to control plates. See table 2

2.4. Phytochemical Analysis of *A. indica* (GC-MS Analysis)

The samples of the twigs of *Azadirachta indica* collected from Wapan-Nghaku around the Federal University Wukari, Taraba State, Nigeria were immediately washed up using tap water, crushed into a pieces using mortar and pestle. The pastes were sun-dried for 3 to 5 days. Using an electronic weighing balance, exactly 1000g was weighed and sent to the Department of Consultancy and Production, National Research Institute for Chemical Technology Zaria, Nigeria, for phytochemical analysis using a Gas Chromatography – Mass Spectrometry Analysis (GC –MS). GC-MS analysis was carried out using the GCMS-QP2010 PLUS SHIMADZU. The column used was Perkin Elmer Elite - 5 capillary column measuring 30m × 0.25mm with a film thickness of 0.25mm composed of 95% Dimethyl polysiloxane. Helium was the carrier gas used at a flow rate of 0.5mL/min. And the sample injection volume that was utilized was 1µL, maintaining 250°C inlet temperature. Initially, the oven temperature was programmed at 110°C for 4 min, then an increased to 240°C. And further programmed to increase to 280°C at a rate of 20°C ending with at 5 minutes. The total run time was 35 minutes. At a temperature of 200°C, the MS transfer line was maintained with the source temperature maintained at 180°C. The GC-MS was analyzed using an acceleration electron impact ionization at 70eV and data were evaluated using Total Ion Count (TIC) for compound identification and quantification. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS library. See Table 3

3. Results

Table 1. *Showing bacterial species isolated from human mouth.*

S/N	Bacteria Isolated
1.	*Lactobacillus* spp
2.	*S. aureus*
3.	*C. diphtheria*
4.	*Enterococcus feacalis*

Table 2. *Showing the zone of inhibition of A. indica twigs at varied concentrations.*

Concentrations of *A. indica* twigs extract (g/mL)	Diameter of zone of clearance (mm) on Bacterial Isolates				
	***Lactobacillus* spp**	***S. aureus***	***C. diphtheria***	***P. aeruginosa***	***Enterococcus feacalis***
2	06	2.5	02	06	1.5
4	6.1	03	3.5	06	1.6
8	08	3.8	06	09	03
10	8.8	05	07	10	4.2
20	9.3	06	09	11	07
40	11.2	7.3	11	14.7	7.3
60	13	7.7	13	18	08
80	16	09	13.8	19	8.7
100	19.2	11	15	23	9.2

The results of the Minimum Inhibition Concentration (MIC) of the aqueous extracts of *A. indica* twigs showed that *Lactobacillus* spp*, S. aureus, P. aeruginosa* and *E. feacalis* were inhibited at all the concentrations used. However the rate of inhibition differs. Some are highly sensitive at some concentrations while others are not. These results indicated that *P. aeruginosa* is the most sensitive at all concentrations while *E. feacalis* is the least sensitive.

Table 3. *Showing the Phytochemical properties of A. indica twigs aqueous extract.*

Phytochemical Composition of *A. indica*	Quantity
Saponnins	++
Phylobatanin	-
Anthraquinones	-
Hydroxylmethyl Anthraquinones	-
Polyphenol	+++
Tannins	-
Flavonoids	++
Glycosides	+
Alkaloids	+

Keys: (+ = Low amount, ++ = Large amount, +++ = Very large amount, - = Absent)

4. Discussion

Table 1 shows The bacterial species isolated from human mouth. Table 2 presents the zone of inhibitions (mm) of neem twigs against the test bacteria. Table 3 shows the phytochemical compounds extracted from the neem twigs extract.

The antibacterial activity of the neem twigs extracts showed growth inhibition at all the concentrations used. However, the results indicated that higher concentrations of the extract have higher zone of clearance (in millimeter). At a Lower concentrations of the extract has lesser effect on *S. aureus, Enterococcus feacalis* and *C. diphtheriae* but the effect was slightly high on *P. aeruginosa* and *Lactobacillus* species. However, at higher concentrations, there was considerably higher zone of inhibition on all the isolates. These effect are more pronounced on *P. aeruginosa*, *Lactobacillus* species and *C. diphtheria.* These results therefore, falls in alignment with several other similar studies [1, 2, 4, 18, 22, 23, 25]. This is evident on the successful wound treatment and in the deactivation of diphtheria toxins by [18] at higher concentration of the extract. The resistance at lower concentrations of the neem extracts, is most likely to the fact that *C. diptheriae* are spore – formers, as the spores shield the vegetative part of the microbial cell. This study although seems to show a higher zone of inhibition (mm) even with a slight increase in concentration compared to other relevant studies at different locations and time, the most probable reason that could be linked to this is most likely to be the

methods of processing the same. As their sample was sun-dried before the zone of clearance was determined. This study however, employed the use of fresh sample of *A. indica* twigs.

Several studies have extracted various essential extracts from different plants. These various extracts were applied to numerous aspects of life which are but not limited to pharmaceautical, food preservation and alternative source of natural medicine among others. The most essential of these substances include flavonoids, glycosides, tannins, alkaloids, polyphenols, saponnins and anthroquinones which are extracted from this studies and several other studies [12, 13, 17, 19, 20].

5. Conclusion

Conclusively, it is safe to say that these results, further confirmed the belief and other scientific studies on the importance of the traditional use of neem in maintaining oral hygiene. Again, some of the isolates from human mouth are potential pathogens and may pose a public health hazard to the community. Therefore, routine oral hygiene are strongly advocated to prevent oral diseases.

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