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Comparative Study of the Antibacterial Effect of Mouth Washes and *Vernonia amygdalina* (del.) on Some Tooth Decay Causing Bacteria

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Authors' contributions

All the authors have cordially supported the work and preparation of manuscript. Author BOO had designed the entire study and protocols with interpretations of the results and prepared the first draft of the manuscript. Authors LOE and AIA managed the analyses of the study, computational work/statistical analysis respectively. Author BJA guided in the entire research. All the authors have read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: To determine the antibacterial effect of the ethanol stem extract of *Vernonia amygdalina* (bitter-leaf) and some mouth washes against some bacteria that have been implicated in causing tooth decay so as to establish the role of herbal medicine and chemical compounds in oral hygiene.
Study Design: *In vitro* assay of antibacterial activities
Place and Duration of Study: Dental Department of the State Specialist Hospital, Akure, Ondo State, Nigeria and Department of Microbiology, Federal University of Technology, Akure, Nigeria, between October, 2012 and January, 2013.
Methodology: Bacterial isolates were collected, identified, standardized and the stem extract was prepared. Phytochemical screening of the extracts was carried out as well as the *in vitro* antibacterial assay using agar well diffusion technique. Minimum Inhibitory Concentration and antibiotics sensitivity test (disc diffusion assay) were also determined.
Results: The stem extract showed the presence of anthraquinone, alkaloid, saponin, steroid and cardiac glycoside. The ethanolic stem extract of *Vernonia amygdalina* inhibited

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all the test isolates at a concentration of 50 mg/ml with the highest zone of inhibition observed against *Staphylococcus aureus* (26.0 mm) while the least zone of inhibition of 14.0 mm was observed against *Streptococcus mutans*. Colgate mouthwash exhibited the highest zone of inhibition against *Staphylococcus aureus* while the least was recorded by Brett against *Staphylococcus epidermidis.* The antibacterial assay compared well with Ciprofloxacin, and in most cases higher zones of inhibition were recorded than the commercial antibiotics. The Minimum Inhibitory Concentration of the mouth washes ranged from 30 to 70% while it was 12.5 mg/ml for the stem extract.

Conclusion: Bioactive components of *Vernonia amygdalina* can be incorporated as ingredients in manufacturing mouthwashes and the plants' stem can be used in the form of chewing stick. Further purification of the extract is necessary to further enhance greater antibacterial activity.

Keywords: In-vitro; antibacterial; zone of inhibition; antibiotics; phytochemical screening; mouthwash, Kirby- Bauer test, Muslin cloth.

1. INTRODUCTION

The oral flora consists of a diverse and populous collection of bacteria, fungi, and transient viruses. Bacteria make up the largest number of varieties. More than 350 cultivable bacteria species have been identified in the mouth and molecular analyses suggest that an equal number of non- cultivable flora are also present [1]. Dental caries is largely due to the colonization of the teeth by a group of species of *Streptococci*, although both *Actinobacillus* and *Lactobacillus* have been implicated as well. Dental caries is readily prevented through oral hygiene after the morning meal and prior to going to bed [2]. It has been clearly shown in many *in-vivo* and *in-vitro* studies that a process starting with the colonization of *Streptococcus mutans* and continuing with the contribution of *Lactobacillus acidophilus* in susceptible individuals, leads eventually to caries initiation [3].

Mouthwash may be recommended as an antimicrobial, a topical anti-inflammatory agent, a topical analgesic, or for caries prevention [4]. They often contain antibacterial agents including cetylpyridinium chloride, chlorhexidine, zinc gluconate, essential oils, and chlorine dioxide. Zinc and chlorhexidine provide strong synergistic effect [5].

Before colonialism, herbal medicine was the major form of medicine in Nigeria. Although, orthodox medicine has overshadowed the development of herbal medicine in developing countries, it has proved ineffective in providing permanent cures to some tropical diseases [6]. The use of chewing sticks has been documented since ancient times, this kind of tooth brushing has been used by the Babylonians some 7000 years ago and its use ultimately spread throughout the Greek and Roman Empires. It is also used by Egyptians, Jews, Islamic Empires and presently in Africa, South America, the Middle East and Asia [7]. The plants used are very carefully selected for such properties such as foaminess, hardness, or bitterness and certain species are more popular than the others, in which a great number of these plant species have related medicinal properties that may be antibacterial and are as important today as they were thousands of years ago [8]. Extracts of these chewing sticks can be incorporated into tooth pastes or used as mouth washes [9].

Plants have the major advantage of still being the most effective and cheaper alternative source of drugs, one of such with promising medicinal principle is *Vernonia amygdalina,* a member of the family Asteraceae [10]. *Verninia amygdalina* is commonly called bitter leaf in

English because of its bitter taste, African common names include grawa (Amharic), ewuro (Yoruba), etidot (Ibibio), onugbu (Igbo), ityuna (Tiv), oriwo (Edo), chusar-doki (Hausa), muluuza (Luanda), labwori (Acholi), and olusia (Luo) [11-13]. *Vernonia amygdalina* have been in many homes in the eastern and Western parts of Nigeria as food especially in the preparation of soups, the characteristic bitter taste is believed to have after taste of sweetness and its peeled stem is often used as chewing stick for cleaning the teeth and is very effective as anticaries [14].

This research is therefore aimed at comparing the antibacterial activity of different brands of mouthwashes and ethanolic stem extract of *V. amygdalina* against bacteria that are associated with tooth decay.

2. MATERIALS AND METHODS

2.1 Collection and Identification of Bacterial Isolates

The 24 - hour grown bacterial isolates used for this study were collected from the Dental Department of the State Specialist Hospital, Akure, Ondo State, Nigeria and they include *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus mutans* and *Lactobacillus acidophilus* and their identities were confirmed using standard microbiological methods [15].

2.2 Standardization of the Test Organisms

A loop full of test organism was inoculated on nutrient broth and incubated for 24 hours. Exactly 0.2 ml from the 24 - hour grown culture of the organisms was dispensed into 20 ml sterile nutrient broth and incubated for 3 - 5 hours to standardize the culture to 0.5 McFarland standards (10^6cfu/ml) before use according to Oyeleke et al. [16].

2.3 Collection and Extraction of Plant Material

Vernonia amygdalina (bitter-leaf) was harvested on a farmland the Federal University of Technology, Akure, Ondo State, Nigeria, where it was found growing naturally. The plants were authenticated at the Department of Crop Science and Pest Management, Federal University of Technology, Akure, Nigeria. The harvested stems were air dried and ground to fine powder. Exactly 400 g of the ground bitter-leaf stem was soaked in 300 ml absolute ethanol and allowed to stand for 72 hours. The mixture was sieved using a clean muslin cloth. The filtrate was however passed through the Whatman No.1 filter paper. The filtrate obtained was passed through a rotary evaporator at 37ºC to recover a dry filtrate [17]. This was kept to be used for further studies.

2.4 Phytochemical Screening

The stem extract was screened for anthraquinone, alkaloid, tannin, saponin, phlobatannin, steroid, flavonoid, terpenoid and cardiac glycoside [18].

2.5 Experimental Procedure

2.5.1 Antibacterial activity

2.5.1.1 Determination of antibacterial activities of stem extract and mouth washes

The antibacterial activity of the plant extract was determined using agar diffusion method described by Madigan et al. [19]. About 1 ml of each of the standardized test organism was transferred into different sterile petri dishes. Mueller Hilton agar was then poured on these inocula and the plates swirled for even dispersion of the organism in the agar. After solidification of the agar, a 6 mm diameter cork borer was used to make wells in to each plate and the prepared extract was introduced. The concentration of the extract used was 50 mg/ml while the concentration of the mouth washes used was 100%. The plates were incubated at 37ºC for 24 hours. Clear zones around the bored holes are indicative of the inhibition of the organisms by the extract. 30% DMSO which is a reconstituiting solvent was used as negative control to determine if it has inhibitory effect on the test organisms.

2.5.1.2 Determination of the minimum inhibitory concentration

This was carried out using the agar dilution method described by Doughari et al. [20]. Different concentrations of the stem extract (30 mg/ml, 25 mg/ml, 12.5 mg/ml, 10 mg/ml, 6.25 mg/ml, and 5mg/ml) and mouth washes (70%, 50% and 30%) were used. Plates were incubated at 37ºC for 24 hours, after which they were observed for clear zones around the wells, indicating inhibition. The lowest concentration of the extract that caused complete inhibition of the test bacterial culture was taken as MIC.

2.5.1.3 Antibiotics sensitivity test

The Kirby - Bauer test also known as disc diffusion method was used to determine the effect of ciprofloxacin on the bacterial isolates as described by Willey et al. [21]. Standard antibiotic disc containing ciprofloxacin (30µg) was placed aseptically on agar plates already seeded with the test organisms using sterile forceps. The plates were then incubated at 37ºC for 24 hours. Zones of inhibition around the antibiotics disc were measured in millimeters.

3. RESULTS AND DISCUSSION

In this study, the mouthwashes and the ethanolic extract of the stem of *Vernonia amygdalina* contain active ingredients that are responsible for the antibacterial effects against the test isolates. The varying susceptibility of each of the organisms may be a function of the available binding sites on the bacterial cell walls [22].

The effects of the antibiotics and 30% DMSO used as positive and negative controls respectively on the test isolates were observed and recorded as zones of inhibition (mm). The 30% DMSO did not inhibit the growth of the organisms; however, ciprofloxacin had varying effects against all the test isolates except *Streptococcus pyogenes*. This is similar to the findings of Daniels et al. [23], wherein their work on the screening of *Daniella Oliveria* against three bacteria and one fungus, demonstrated *Streptococcus pyogenes* to be resistant to ciprofloxacin. The resistance might also be attributed to the clinical history of the organism. Moreover, at 100% concentration, all the mouthwashes inhibited the growth of the test isolates except for Brett which had no effect against *Staphylococcus aureus* and

Lactobacillus acidophilus. Colgate Plax was the most effective, followed by Dentiplus and Listerine while Brett was the least effective against the test isolates. The highest zone of inhibition (28 mm) was observed against *Staphylococcus aureus* by Colgate Plax while the least (6.0 mm) was against *Staphylococcus epidermidis* by Brett, as shown in Table 1.

Colgate Plax mouthwash displayed inhibitory activity against all the *Streptococci species* when compared to the other mouthwashes. This suggests that it plays a vital role in the prevention of dental caries since *Streptococcus species* have been implicated in its initiation [3].

The minimum inhibitory concentration (MIC) of the mouthwashes ranged from 30% to 70% with Colgate Plax inhibiting all the test isolates at a least MIC value of 30%, followed by Dentiplus and Listerine, with Brett having a MIC value of 50% as shown in Table 2.

All mouthwashes examined contain one or more different active agents. Thus the high efficacy of Colgate Plax and Dentiplus could be due to the presence of active ingredients like cetylpyridinium chloride and sodium fluoride. Listerine and Brett mouthwashes do not contain cetylpyridinium chloride and that reflected in the level of antibacterial activity exerted by them on the bacteria. Listerine and Brett mouthwashes both contain thymol and ethanol as their active ingredients.

Cetylpyridinium chloride (CPC) is a quaternary compound that reduces plaque and calculus. It is a cationic surface-active agent and has a broad antimicrobial spectrum and kills gram positive pathogens. Contact with bacteria occurs by the disturbance of the cell membrane, inhibition of cell function, seepage of cell contents, and eventually cell death [24]. Sodium fluoride is often present in toothpastes and mouthwashes to prevent dental caries [25]. It also has antibacterial properties and therefore has been explored in its use against periodontal diseases. It prevents pellicle formation and the adherence of bacteria to pellicle-covered teeth by competing for calcium ions known to be important in the bridging between acidic groups on the pellicle surface and the bacterial cell walls [26]. Phenolic compounds such as thymol in Listerine also have the ability to inhibit the accumulation of dental plaque; however, it has been reported as potentially irritant [27]. Thymol has been found to be more effective when used in combination with chlorhexidine or cetylpyridinium chloride to reduce dental plaque than when used alone [28].

Table 3 shows the antibacterial activity of the stem extract of *V. amygdalina* on the test isolates, the extract inhibited all the test isolates at a concentration of 50 mg/ml with the highest zone of inhibition observed on *Staphylococcus aureus* (26.0 mm) while *Streptococcus mutans* was the least inhibited (14.0 mm) which compared favourably well with the commercial antibiotics. Moreover, an inhibitory effect of the extract was observed against *Streptococcus pyogenes* (16.0 mm) which otherwise, was totally resistant to ciprofloxacin. It highlighted the importance of *V. amygdalina* stem extract to be used against test organisms which do not show any sensitivity even to standard antibiotics *i.e.* ciprofloxacin, thus indicating the better efficacy of plant extracts. The varying degree of sensitivity of all the bacterial strains to the extract might be due to the intrinsic tolerance of the bacteria, nature and combinations of phyto-compounds present in the extracts as observed by Suree and Pana [29].

Table 1. Antibacterial activities of different mouthwashes as compared with ciprofloxacin

Isolates	Zone of inhibition (mm) at 100% concentration					
	Brett	Colgate Plax	Listerine	Dentplus	Positive control (ciprofloxacin 30µg)	Negative control (30% DMSO)
Staphylococcus aureus	0.00±0.00^{a}	28.00±2.00^{c}	20.00±1.00^{c}	15.00±1.00^{b}	13.00±1.73^{d}	0.00±0.00^{a}
Staphylococcus epidermidis	6.00±1.00^{b}	26.00±1.73^{bc}	18.00±2.00^{c}	19.00±3.00^{c}	10.00±1.00^{c}	0.00±0.00^{a}
Streptococcus mutans	7.00±2.00^{c}	22.00±2.00^{ab}	23.00±1.73^{d}	10.00±1.73^{a}	3.00±1.00^{b}	0.00±0.00^{a}
Streptococcus pneumoniae	10.00±2.00^{d}	25.00±3.00^{bc}	12.00±1.73^{ab}	15.00±2.64^{b}	21.00±2.00^{e}	0.00±0.00^{a}
Streptococcus pyogenes	11.00±1.00^{d}	27.00±2.00^{c}	14.00±1.73^{b}	24.00±1.00^{d}	0.00±0.00^{a}	0.00±0.00^{a}
Lactobacillus acidophilus	0.00±0.00^{a}	20.00±2.00^{a}	11.00±1.00^{a}	20.00±1.00^{c}	23.00±1.00^{e}	0.00±0.00^{a}

Values are mean zone of inhibition (mm) ± Standard deviation of three replicate

$^{a-d}$Means in the same column not sharing a common letter are significantly different (P ≤ 0.05) by Duncan's multiple range test

Table 2. Minimum inhibitory concentration (MIC) of the mouthwashes on the test isolates

Organisms	Brett			Colgate Plax			Listerine			Dentiplus		
	70%	50%	30%	70%	50%	30%	70%	50%	30%	70%	50%	30%
Staphylococcus aureus	10.00±0.00^{a}	8.00±0.00^{a}	0.00±0.00	20.00±2.64^{a}	15.00±1.73^{a}	12.00±1.00^{a}	18.0±1.00^{d}	16.00±1.73^{c}	12.00±1.00^{b}	10.00±1.73^{ab}	6.00±1.00^{b*}	0.00±0.00^{a}
Staphylococcus epidermidis	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00	20.00±1.73^{a}	15.00±1.00^{a}	10.00±1.73^{a}	6.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	15.00±1.73^{c}	11.00±0.00^{c}	0.00±0.00^{a}
Streptococcus mutans	5.00±1.00^{b}	0.00±0.00^{a}	0.00±0.00	18.00±1.73^{a}	14.00±1.00^{a}	11.00±0.00^{a}	18.00±1.73^{d}	12.00±2.64^{c}	8.00±1.73^{b}	7.00±2.64^{a}	0.00±0.00^{a}	0.00±0.00^{a}
Streptococcus pneumoniae	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00	18.00±1.00^{a}	16.00±2.00^{a}	10.00±1.00^{a}	8.00±1.00^{bc}	6.00±2.00^{b}	0.00±0.00^{a}	13.00±1.73^{bc}	10.00±1.00^{c}	6.00±1.00^{b*}
Streptococcus pyogenes	8.00±1.73^{c}	5.00±1.73^{b}	0.00±0.00	22.00±1.00^{b}	16.00±1.73^{a}	11.00±1.73^{a}	12.00±1.73^{c}	0.00±0.00^{a}	0.00±0.00^{a}	20.00±1.00^{d}	14.00±2.64^{cd}	12.00±2.00^{c}
Lactobacillus acidophilus	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00	18.00±2.64^{a}	15.00±2.00^{a}	12.00±1.00^{a}	8.00±1.73^{b}	6.00±0.00^{a}	0.00±0.00^{a}	16.00±1.73^{c}	12.00±2.64^{d}	8.00±2.64^{c}

Values are mean zone of inhibition (mm) ± Standard deviation of three replicate

$^{a-d}$Means in the same column not sharing a common letter are significantly different (P = .05) by Duncan's multiple range test

Table 3. Antibacterial activities of ethanolic extract of *Vernonia amygdalina* as compared with ciprofloxacin on the test isolates

Isolates	Zones of inhibition (mm)		
	50mg/ml Concentration	Positive control (ciprofloxacin 30µg)	Negative control (30% DMSO)
Staphylococcus aureus	26.00±2.00[c]	13.00±1.73[d]	0.00±0.00[a]
Staphylococcus epidermidis	22.00±2.00[b]	10.00±1.00[c]	0.00±0.00[a]
Streptococcus mutans	14.00±2.00[a]	3.00±1.00[b]	0.00±0.00[a]
Streptococcus pneumoniae	15.00±2.64[a]	21.00±2.00[e]	0.00±0.00[a]
Streptococcus pyogenes	16.00±2.00[a]	0.00±0.00[a]	0.00±0.00[a]
Lactobacillus acidophilus	17.00±2.64[a]	23.00±1.00[e]	0.00±0.00[a]

Values are mean zone of inhibition (mm) ± Standard deviation of three replicate
[a-d] Means in the same column not sharing a common letter are significantly different (P ≤ 0.05) by Duncan's multiple range test

The minimum inhibitory concentration (MIC) of the stem extract of *V. amygdalina* was observed to be 12.5 mg/ml, with the least value observed on *Streptococcus mutans* and *lactobacillus acidophilus* as shown in Table 4.

This is in line with the findings of Anibijuwon et al. [30] in their research on the Antimicrobial activities of *Vernonia amygdalina* against oral microbes, where the ethanolic extract of *V. amygdalina* had a least minimum inhibitory concentration on *S. mutans*. It is known to be the chief pathogen responsible for the formation of dental plaque which normally results in caries [31]. Its susceptibility to *V. amygdalina* in this study confirms the use of this plant as a chewing stick locally in removing dental plaque.

Table 4. Minimum Inhibitory concentration of the ethanolic extract of *Vernonia amygdalina* on the test isolates

Organisms	Zones of inhibition(mm)		
	25mg/ml	12.5mg/ml	6.25mg/ml
Staphylococcus aureus	20.00±1.73[c]	12.00±2.00[b]	0.00±0.00
Staphylococcus epidermidis	15.00±2.00[b]	11.00±2.00[b]	0.00±0.00
Streptococcus mutans	11.00±1.73[a]	6.00±1.73[a]	0.00±0.00
Streptococcus pneumoniae	10.00±1.00[a]	7.00±2.64[a]	0.00±0.00
Streptococcus pyogenes	11.00±1.00[a]	9.00±1.00[ab]	0.00±0.00
Lactobacillus acidophilius	15.00±1.00[b]	6.00±1.00[a]	0.00±0.00

Values are mean zone of inhibition (mm) ± Standard deviation of three replicate
[a-c] Means in the same column not sharing a common letter are significantly different (P ≤ 0.05) by Duncan's multiple range test.

Phytochemical test of the plant extracts revealed the presence of some bioactive components like saponins, and steroids which might be responsible for the antibacterial activity of the extract [32] as shown in Table 5.

Table 5. Phytochemical screening of ethanol extract of *Vernonia amygdalina*

Phytochemical tests	Presence/absence
Saponins	+ve
Flavonoids	-ve
Phlobatannin	-ve
Alkaloids	+ve
Anthraquinone	+ve
Steroids	+ve
Cardiac glucoside	
Legals Test	+ve
Salkowski Test	+ve
Keller Killian Test	+ve
Liebermans	+ve

Keys: + = presence, - = absence

The presence of cardiac glycosides and steroids have been documented to inhibit many bacteria and found to possess antioxidant potentials. Also, alkaloids have been found to interfere with cell division in microorganisms, these phytochemicals inhibit life processes in microbes [23].

Studies have revealed some other medicinal plants also possess antimicrobial activity against these test isolates. The ethanolic extract of *Polish propolis* has been shown to inhibit *Streptococcus mutans* and *Lactobacillus acidophilus* [33]. Remarkable results were obtained using leaf, phloem and latex of *Croton urucurana* against the bacteria *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Streptococcus pyogenes* [34]. Ethanolic extracts of *Fagara xanthoxyloides* (Pako Ata), Garcinia kola (Pako Orogbo) and *Anogeissus leiocarpus* (Pako Ayin) exerted great antibacterial effects on *Lactobacillus acidophilus* [35].

4. CONCLUSION

This study has been able to establish the antibacterial activity of *V. amygdalina* against bacteria causing tooth infection as compared to various brands of mouthwashes and commercial antibiotics. Quaternary compounds, like Cetylpyridinium chloride (CPC) should be among the active ingredients being used in the production of mouthwashes as it has a broad spectrum antimicrobial activity. Since the extract contains natural antimicrobial ingredients, the plant's stem in the form chewing stick is therefore recommended for usage by the local populace. The bioactive components can also be incorporated as ingredients in manufacturing mouthwashes. Further purification of the extract and identification of the active component is necessary to enhance greater antibacterial potency.

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COMPETING INTEREST

Authors have declared that no competing interests exist.

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