Phytochemical And Antimicrobial Properties of Leaf Extracts of Lupinus Arboreus (Yellow Bush) Against Dental Caries Pathogens.

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Abstract: The quests for novel antimicrobial agents from medicinal plants to combat pathogens have become crucial for avoiding the emergence of untreatable microbial infections and reduce the use of synthetic chemicals against these microorganisms. The antimicrobial effect of the crude leaf extract and fractions of (yellow bush) Lupinus arboreus on dental caries pathogens was investigated. The crude water extract of the fresh leaves was obtained by cold maceration, sieved, decanted and evaluated using modified agar-well diffusion method; while the pulverized dried leaves extract was fractionated into ethanol, methanol and Hexane fractions, obtained by 48hours cold maceration and evaluated using modified agar-well diffusion showed that the crude water extract and fractions at varying concentrations, exerted strong antimicrobial activity on some of the test organisms. However, a weak activity was observed on the tested fungi Candida albicans. Methanol and ethanol fractions showed the highest activity on many organisms than the Hexane fraction. The phytochemical studies showed that the extract had the abundance of Saponins, Glycosides, Steroids, Terpenes and Flavonoids. Resins, protein and reducing sugar occurred in moderate amounts, while alkaloids appeared but in trace amount. The pH of the crude extract was determined as 2.90.

Keywords: Phytochemistry, Antimicrobial, Leaf extracts, Lupinus arboreus and Dental Carrier pathogens.

I. Introduction

1.1 Background Information

Plants have been classified as essential sources of medicinal agents for centuries and a huge number of novel drug components have been isolated from natural plant sources (Owolabi *et al.*, 2007). Many of these plants and their extracts were used in traditional medicine. Medicinal plants play a key role in health care with about 80% of the world's populations relying on the use of traditional medicine, predominantly based on plants (Owolabi et al., 2007).

According to World Health Organization (WHO) 2005, medicinal plants would be the best source to obtain a variety of drugs. Plant derived medicines have made large contributions to human health (El-Astal et al., 2005). This is due to the significant healing power of the traditional medicinal systems (Adebolu and Olademeji, 2005). Medicinal plants are distributed worldwide but they are most abundant in tropical countries (Elvin-Lewis, 2001). Natural products from plants may offer new agents of antimicrobial use. A special feature of higher plants is their capacity to produce a large number of organic chemicals of high structural diversity called secondary metabolites (Naovi et al., 1991). Microorganisms have unfavorable effects on the quality and safety of life. Synthetic chemicals are widely used against these microorganisms. Unfortunately, they develop resistance to many antimicrobial agents. The reason for this high resistance to commonly used antimicrobial agents may not be unconnected with their worldwide indiscriminate use in the environment (Anyim et al., 2010). In addition, these antimicrobials sometimes cause allergic reaction and immunity suppression. The use of essential oils from plants improves the human health and environment (Isman, 2000). Plants have provided an arsenal of chemicals to save attack by a microbial invasion (Martins et al., 2004). Literature showed that natural products only and their derivatives represent more than 50% of the drugs in chemical use with one quarter originating from higher plants (Cragg et al., 1997). Lupinus arboreus is easily recognized as a bushy shrub about 1.8m tall with bright vellow sweet smelling leaves and flowers blended with purple and white colours (Pickart and Miller, 1998). Also known as yellow bush, L. arboreus occurs as an invasive species in Northern California coastal dunes (Wear, 1998). But in Nigeria, it is planted widely as ornamental plant (Ohadoma et al., 2011). It is highly nutritive and wholesome hence grown for fodder and it comes close to soy bean in protein content (Rechel, 2006). This study screened the phytochemicals and antimicrobial activity of L. arboreus against dental caries.

Materials And Methods

II.

Collection and Identification of Plant

Leaves of *Lupinus arboreus* were collected in April, 2015 from Trans-Ekulu, Enugu East Local Government Area, Enugu State, Nigeria and official identification was done by Dr. J. I. Nwankwo botanist from Applied Biology and Biotechnology Department, Enugu State University of Science and Technology.

Lupinus arboreus, yellow bush lupine (USA) or tree lupine (UK) is a species of flowering plant in the legume family fabaceae, native to California, where it is widely distributed among coastal shrub and sand dunes. Because it has been widely introduced, there is some uncertainty about its native range; it is thought to be native from point Rayes National sea shore south to San Luis Obispo County (Webb *et al.*, 1996).

It is an evergreen shrub growing 1.8 to 2m in sheltered situations, but more typically 1-1.5m tall. It has green to gray-green palmate leaves, with 5.12 leaflets per leaf. The leaflets are 2-6 centimeters long, often sparsely covered with fine silky hairs (Ohadoma *et al.*, 2010).



Fig: luginus arboreus (Yellow Bush)

Scientific Classification	on	
Kingdom	:	Plantae
(Unranked)	:	Angiosperms
(Unranked)	:	Eudicots
(Unranked)	:	Rosids
Order	:	Fabales
Family	:	Fabaceae
Subfamily	:	Faboideae
Tribe	:	Genisteae
Genus	:	Lupinus
Subgenus	:	Platycarpos
Species	:	L. arboreus
(Webb et al., 1996).		

In spring it bears many racemes, 30cm long, of fragrant, soft yellow, pea-like flowers. Both yellow and lilac to purple flowering forms are known; however, the yellow form is more common. It is capable of tolerating temperatures down to -12°c and living for up to seven years (Webb et al., 1996). The leaves were air-dried at room temperature for 28 days and pulverized into fine powder using a Wahl Blender. The powdered leaves (40g) were extracted each with 200ml of absolute methanol, ethanol and hexane (Sigma Aldrich, Germany) by cold maceration for 48hours respectively. The mixtures were filtered with Watman no 1 filter paper to obtain the methanol, ethanol and hexane extracts, which were evaporated using a rotary evaporator and the concentrated extracts stored in a refrigerator.

Fresh leaves of *L. arboreus* were also collected from the same site above, washed with slow tap running water and macerated using a Wahl blender and squeezed to obtain a crude extract of the fresh leaves. The solution was filtered using Watman filter paper 1 to obtain the crude water extract which was evaporated using (glass condenser) a rotary evaporator and the concentrated crude extract stored in a refrigerator The pH of the extract was determined as 2.90, while an acidic buffer (ethanoate) was used.

The organisms

Pure clinical isolates of *Streptococcus mutans, Staphylococcus aureus, Pseudomonas aeruginosa, Lactobacillus acidophilus, Escherichia coli* and *Candida albicans* were obtained from Medical Microbiology laboratory unit of Enugu State University Teaching Hospital, Park lane, Enugu, Nigeria.

Determination of Sensitivity Test and Inhibitory Zone Diameter (1ZD).

The antimicrobial activity of the test organisms to the four (4) extracts of *L. arboreus* was screened by using the agar-well diffusion method (Perez et al., 1990). An inoculum suspension was streaked uniformly to solidified 20ml miller-Hinton Agar (MHA) for bacteria and Sabouraud Dextrose Agar (SDA) for fungi and the inocula were allowed to dry for 5minutes. Holes of 6mm in diameter were made in the seeded agar using sterile cork borer. Exactly 2 drops of the extract prepared as described earlier were accordingly put into the wells and then allowed to stand for 30minutes for proper diffusion, each plate was then incubated at $37^{\circ}c$ for 24hours for bacteria and 30c for the fungus *C. albicans (5-7days)*. The resulting inhibition zone were measured in millimeters (mm)

Determination of Minimum Inhibitory concentration (MIC)

The Minimum inhibitory concentration was considered the lowest concentration of the sample that prevented visible growth. It was determined using the micro broth dilution technique (Irobi et al., 1993) .The extract and fractions were incorporated at varying concentrating into nutrient broth respectively containing the test organisms in the test tubes. The control experiment containing the growth medium and each of the test organisms, excluding the extract and the fractions were also set. The experiments were incubated at 37°c for 24hours. The lowest concentration of extract and fraction that did not allow microbial growth within the incubation period was taken to be the MIC.

Photochemical Screening of the Crude Extract.

Test for Tannins

About 2ml of the crude extract were stirred with 2ml of distilled water and few drops of $FeCl_3$ solution were added. The formation of a green precipitate was an indication for the presence of tannins (Harbone, 1988).

Test for saponins

Exactly 5ml of crude extract was shaken vigorously with 5ml of distilled water in a test tube and warmed. The formation of obvious stable foam was taken as an indication for the presence of abundant saponins (Harbone, 1988).

Test for flavonoids

To 1ml of crude extract was added 1ml of 10% lead acetate solution. The formation of clear yellow precipitate was taken as a positive test for flavonoids (Harbone, 1988).

Test for terpenes

Exactly 2ml of the organic extract was dissolved in 2ml of chloroform and evaporated to dryness. To dry set up, 2ml of concentrated sulphuric acid was then added and heated for about 2minutes. A grayish colour indicates the presence of terpenes (Harbone, 1988).

Test for steroids

- i. A red colour produced in the lower chloroform layer when 2ml of crude extract was dissolved in 2ml of chloroform and 2ml concentrated sulphuric acid added indicates the presence of steroids.
- ii. The development of greenish colour when 2ml of the organic extract was dissolved in 2ml of chloroform and treated with sulphuric and acetic acids indicates the presence of steroids (Harbone, 1988).

Test for alkaloids

Three millilitres of crude extract were stirred with 3ml of 1% HCL on a steam bath. Mayer's and Wagners reagents were then added to the mixture. Turbidity of the resulting precipitate was taken as evidence for the presence of alkaloids (Harbone, 1988).

Test for reducing sugars.

About 5ml of the extracts were diluted with distilled water. Fehling solutions A and B were added and the mixture warmed. The brick red precipitate at the bottom of the tube indicates reducing sugars (Harbone, 1988).

Test for resins

Exactly ten millilitres of the extract were obtained in a test-tube, the same amount of copper acetate solution was added and the mixture was shaken vigorously and allowed to separate, a green colour indicates the presence of resin (Harbone, 1988).

Test for glycosides

Keller-Kiliani Test: 2ml of extract was dissolved in 2ml of glacial acetic containing a drop of $FeCl_3$ solution. The mixture was then poured into a test tube containing 1ml of concentrated H_2SO_4 . A brown ring at the interphase indicates the presence of a deoxy sugar, characteristic of cardenolides (Harbone, 1988).

Test for proteins and Amino Acids.

Biuret Test: 5ml of extract was mixed with equal volume of 40% NaoH solution and two drops of 1% copper sulphate solution and shaken in a test-tube; the appearance of violet colour indicates the presence of proteins (Harbone, 1988).

Statistical Analysis

Data were entered and analyzed using due procedures and packages of Univariate Analysis of variance (Uni ANOVA) with Duncan Multiple Comparism tests which was employed to test the difference between group means (ref).

III. Results

The effects of different extracts on the tested organisms were determined, and the inhibition zone diameter (IZD) of the isolates measured in millimeters as illustrated in Table 1. The crude water extract showed the highest sensitivity on *S. mutans* (16.5mm) while methanol extraction showed the least activity on *P. aeruginosa* (11mm). However, hexane fraction showed high activity on the *Lactobacillus spp* but had no effect on other tested organisms. Meanwhile, none of the extracts showed sensitivity to *E. coli* and *C. albicans*.

Parameter	S. mutans	L. acidophilus	S. aureus	P. aeruginosa	E. coli IZD(mm)	C. albicans
	IZD(mm)	IZD(mm)	IZD(mm)	IZD(mm)		IZD(mm)
Hexane fraction	-	14.5	-	-	-	-
Methanol fraction	14	15	15	11	-	-
Ethanol fraction	13	16	14.5	13	-	-
Crude water extract	16.5	21	11.5	12.5	-	-

Table 1: The effect of different extracts on the tested organisms and their inhibiting zone diameter.

Key: Where (-) means no inhibition.

The effect of concentrations of hexane fractions on different isolates were carried out is shown in Table 2. The inhibition zone diameter (IZD) and inhibition zone diameter squared (IZD^2) of the various concentrations of the hexane extract were recorded. It was observed that hexane extract showed activity only on the *Lactobacillus spp* and little or no activity on other tested organisms.

Table 2. The effect of concentrations of nexale fractions on different isolates.										
Parameter	IZD	IZD^2	IZD	IZD^2	IZD	IZD^2	IZD	IZD^2	IZD	IZD^2
Conc. (mg/ml)	100		50		25		12.5		6.25	
Log. Conc.	2.00		1.6980		1.3979)	1.0961		0.7958	
S. mutans	-	-	-	-	-	-	-	-	-	-
L. acidophilus	5.0	25.0	4.5	20.2	4.0	16.0	3.5	12.2	3.0	9.0
S. aureus	-	-	-	-	-	-	-	-	-	-
P. aeruginosa	-	-	-	-	-	-	-	-	-	-
E. coli	-	-	-	-	-	-	-	-	-	-
C. albicans	-	-	-	-	-	-	-	-	-	-

 Table 2: The effect of concentrations of hexane fractions on different isolates.

The effect of concentrations of ethanol fraction on different isolates is shown in Table 3. Both the inhibition zone diameter (IZD) and inhibition zone diameter squared (IZD²) were recorded. Ethanol extract showed highest activity on the *Lactobacillus spp at* the concentration of 6.25 mg/ml with inhibition zone diameter of 3.5mm. However, ethanol extract had no effect on *E. coli* and *C. albicans*.

Table 3: The effect of concentrations of ethanol fraction on different isolates

Parameter	IZD	IZD ²	IZD	IZD ²	IZD	IZD ²	IZD	IZD ²	IZD	IZD ²
Conc. (mg/ml)	100		50		25		12.5		6.25	
Log. Conc.	2.00		1.6980		1.3979		1.0961		0.7958	3
S. mutans	9.0	81	6.0	36.0	4.5	20.2	3.0	9	-	-

L. acidophilus	9.0	81	6.0	36.0	4.5	20.2	4.0	16	3.5	12.2
S. aureus	8.0	64	5.0	25.0	4.0	16.0	-	-	-	-
P. aeruginosa	4.5	20.1	4.0	16.0	3.0	9.0	-	-	-	-
E. coli	-	-	-	-	-	-	-	-	-	-
C. albicans	-	-	-	-	-	-	-	-	-	-

The effect of concentrations of methanol extract on different isolates was evaluated, the degree of sensitivity and inhibition zone diameter squared (IZD^2) are illustrated in Table 4. Methanol extract showed the least activity on *P. aeruginosa* at the concentration of 50mg/ml and highest effect on *Lactobacillus acidophilus* at a concentration of 12.5mg/ml, but no sensitivity on *E.* coli and *C. albicans*.

Table 4 . The effect of concentrations of methanol extract on unreferring isolates										
Parameter	IZD	IZD ²	IZD	IZD ²	IZD	IZD ²	IZD	IZD ²	IZD	IZD ²
Conc. (mg/ml)	100		50		25		12.5		6.25	
Log. Conc.	2.00		1.6980)	1.3979)	1.0961		0.7958	
S. mutns	6.0	36.0	5.0	25.0	4.0	16.0	-	-	-	-
L. acidophilus	4.0	16.0	3.5	12.2	3.0	9.0	3.0	9.0	-	-
S. aureus	6.0	36.0	5.0	25.0	4.0	16.0	-	-	-	-
P. aeruginosa	6.0	36.0	3.0	9.0	-	-	-	-	-	-
E. coli	-	-	-	-	-	-	-	-	-	-
C. albicans	-	-	-	-	-	-	-	-	-	-

Table 4: The effect of concentrations of methanol extract on different isolates

The effect of concentrations of crude water extract on different isolates was screened and illustrated in Table 5. The crude water extract showed high level sensitivity to majority of the tested organisms with the highest effect on *P. aeruginosa* with inhibition zone diameter of 3.0mm at the concentration of 6.25 mg/ml but little or no activity on E. coli and *C. albicans*.

Table 5.	Table 5. The effect of concentrations of crude water extract on different isolates.									
Parameter	IZD	IZD ²	IZD	IZD ²	IZD	IZD ²	IZD	IZD ²	IZD	IZD ²
Conc. (mg/ml)	100		50		25		12.5		6.25	
Log. Conc.	2.00		1.6980)	1.3979)	1.0961		0.7958	
S. mutans	10	100	7.5	56.2	5.0	25.0	3.5	12.2	2.0	4.0
L. acidophilus	10	100	7.5	56.2	5.0	25.0	3.5	12.2	2.0	4.0
S. aureus	12	144	8.0	64.0	6.0	36.0	4.4	19.3	2.0	4.0
P. aeruginosa	16	256	11.5	132.2	9.0	81.0	6.0	36.0	3.0	9.0
E. coli	-	-	-	-	-	-	-	-	-	-
C. albicans	-	-	-	-	-	-	-	-	-	-

 Table 5: The effect of concentrations of crude water extract on different isolates.

The effect of the minimal inhibitory concentration (MIC) of the extracts against the isolates was determined and the least concentrations of the various extracts that showed sensitivity to the tested organisms recorded as shown in Table 6. The crude water extract and hexane extract showed the highest activity to P. *aeruginosa* and *Lactobillus spp* 0.625mg/ml for both, while methanol extract showed the least activity on the isolates.

Table 6: The effect of the minimal inhibitory concentration (MIC) of the extracts against the isolates

Isolates	Hexane	Ethanol (mg/ml)	Methanol	CWE
S. mutans	-	>5.0	>10.0	1.25
L. acidophilus	0.625	1.25	>5.0	1.25
S. aureus	-	>10.0	>10.0	1.25
P. aeruginosa	-	>10.0	>10.0	0.625
E. coli	-	-	-	-
C. albicans	-	-	-	-

The Phytochemical constituents of leaf extract of *Lupinus arboreus* were screened through standard procedures using required chemicals and reagents. The phytochemical studies showed that *Lupinus arboreus* extract had the abundance of saponins, glycosides, steroids, terpenes and flavonoids. Resins, proteins and reducing sugar occurred in moderate amounts, while alkaloids appeared but in trace amount as shown in Table 7 below. Both the quantitative and qualitative analyses of the phytochemical constituents were determined; the pH of the extract was determined to be 2.90.

Table 7: Phytochemical constituents of leaf extract of Lupinus arboreus.

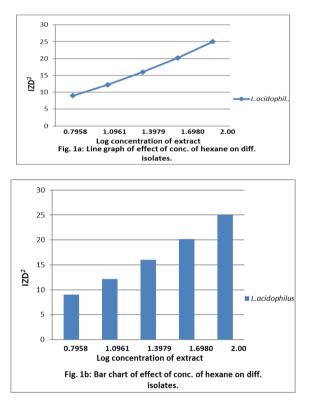
	Phytochemical constituents		yield
1	Saponins	+++	16.8%
2	Glycosides	+++	16.5%

3	Flavonoids	+++	15.9%
4	Steroids	+++	15.8%
5	Terpenes	+++	16.2%
6	Tannins	++	4.2%
7	Resins	++	4.1%
8	Protein	++	4.0%
9	Reducing sugar	++	3.8%
10	Alkaloids	+	1.05%

Key: +++ = Abundantly present, ++ = moderately present, + = present in trace amount.

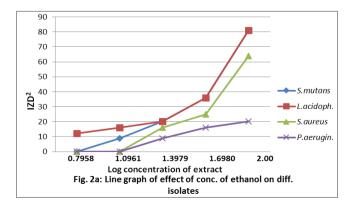
Effect of concentration of hexane extract on different isolates.

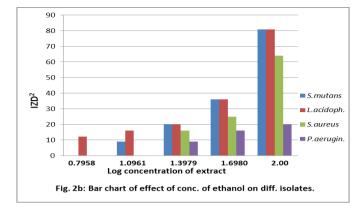
Figures 1a and 1b show the graphical illustrations of the susceptibility of various concentrations of hexane extract on the tested organisms. The graphs show the values of inhibition zone diameter squared against the log concentration of the extracts. The extract showed highest activity on *Lactobacillus spp* at a log concentration of 2.0 with the IZD² value 25mm^2 but no activity on other isolates.



Effect of ethanol extract on tested organisms.

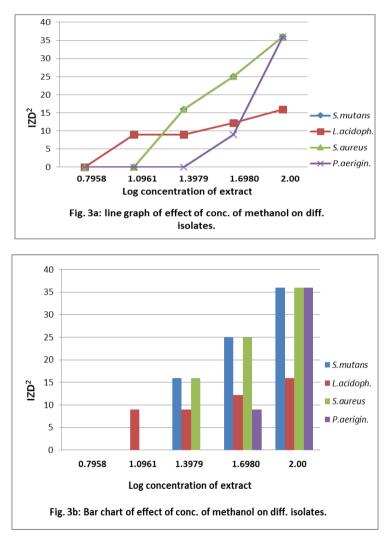
Figures 2a and 2b represents the line graph and bar chart of the result of the effect of concentrations of ethanol extract on the isolates (Table 3) above. The extract showed highest activity on both *S. mutans* and *Lactobacillus spp* at a concentration of 2.00 with inhibition zone diameter squared (IZD²) of 81mm² for both organisms.





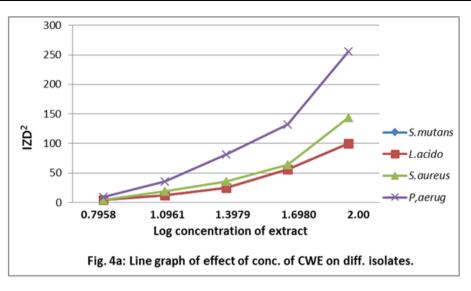
Effect of concentrations of methanol on the isolates.

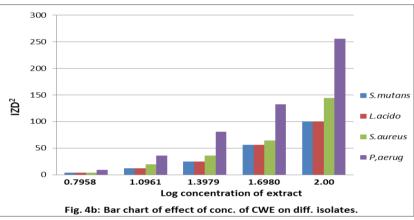
Figure 3a and 3b represent the line graph and bar chart of the result of inhibition zone diameter squared (IZD^2) against the log concentration of the various yields of methanol extract as shown in table 4 above. Methanol showed highest activity at the log concentration of 2.00 on three isolates (*S. mutans, S. aureus, and P. aeruginosa*) with IZD²value of 36mm² for all.



Effect of concentrations of crude water extract on the tested organisms.

Various concentrations of crude water extract showed sensitivity to all the test organisms except *E. coli* and *C. albicans* as shown in table 5 above. Figure 4a and 4b below show graphical representation of the inhibition zone diameter squared (IZD^2) against log concentration of the extract yields, with highest activity on *P. aeruginosa* with IZD^2 value of 256mm² at a log concentration of 2.00.





IV. Discussion

The results in the study indicated that crude and solvent guided extractions were prepared from the leaves of *Lupinus arboreus*. The direct extract from the fresh leaves yielded the crude water extract (CWE), while the solvent extraction of the dried leaves yielded the hexane, ethanol and methanolic fractions. The analysis of the plant extracts revealed the presence of phytochemicals which are known to exhibit medical and physiological activities (Bipul *et al*; 2013). For example tannins are polyphenolic compounds that bind to proline rich protein that interferes with protein synthesis and have shown to have antibacterial activity (Biwas *et al*; 2013).

Flavonoids are hydroxylated polyphenolic compounds known to be produced by plants in response to microbial infections to which this aspect has been extensively studied and found to have antimicrobial activity against an array of microorganisms invitro (Ohadoma *et al.*, 2010). Terpenes, saponins, glycosides have all been found to have inhibitory effects on gram-positive organisms due to their inability to form complexes with extracellular and soluble proteins and bacterial cell walls (Biwas *et al.*, 2013). Therefore the phytochemical analysis revealed that the crude water, hexane, ethanol and methanol extracts have chemical compounds that have been found to possess antibacterial activities which could contribute to the result obtained from antimicrobial analysis. Table 7 shows the qualitative representation of the phytochemical constituent of *Lupinus arboreus*.

Several studies had revealed the antimicrobial efficacy of *Lupinus arboreus* crude water extract, hexane fraction, ethanol and methanolic fractions against clinical isolates of gram-negative and gram-positive bacteria responsible for majority of the multidrug resistant infections in Nigeria (Kesah et al., 2003) and Salmonella (Akinyemi et al., 2000), urinary tract and asymptomatic genital infections, otitis media and wound infections by *P. aeruginosa* (Oyeka et al., 1995) and *S. aureus* (Akerele et al., 2002), upper respiratory tract infections, periodontal disease, dental caries and osteomyelitis in children by *Streptococcus species* and *Bacillus species* (Onuba, 1992). *Lupinus arboreus* showed appreciable activity against these bacteria using the method of agarwell diffusion on *S. mutans, L. acidophilus, S. aureus* and *P. aeruginosa*, but had weak activity on the tested fungi *C. albicans*, hence it is a broad-spectrum antimicrobial (Ohadoma et al., 2012).

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Extrapolations from the results of susceptibility of concentrations of extract and fractions on the tested isolates showed their MIC values. From the result of the minimum inhibitory concentration (MIC), it was observed that the greater the IZD produced, the lower the MIC and more potent the agent. However, the fractions and extract had varying MICs on individual organisms. Although, it showed no activity on other organisms used in the study, the hexane fraction when compared with the other fraction showed the highest activity only on *Lactobacillus acidophilus* (MIC 0.625mg/ml), followed by ethanol extract (MIC 1.25mg/ml), crude water extract (1.25mg/ml) and methanolic fraction (>5.0mg/ml). The ethanol fraction showed activity on other organisms as follow: *S. mutans* (>5.0mg/ml), *S. aureus* (MIC >5.0mg/ml) and *P. aeruginosa* (MIC >10.0mg/ml) but highest activity on *Lacidophilus* (MIC <10.0mg/ml) when compared with crude water extract on *L. acidophilus* (MIC 1.25mg/ml), *S. aureus* (MIC 1.25mg/ml) and *P. aeruginosa* (MIC 1.25mg/ml). This proves the potency of the extracts and reveals their high affinity to inhibit the growth or cause the death of microorganisms (bacteristatic and bactericidal characteristics) especially the dental caries pathogens (Ohadoma *et al.*, 2010). When ANOVA was employed with the inhibition zone diameter stratification, there was a significant difference

The study is a preliminary assessment of the easily available medications of plant origin which can be effective in the treatment of dental caries. To identify the active compound involved in the anticariogenic activity of the efficient *L. arboreus* extract revealed in the present study, the phytochemical screening was painstakingly conducted. Ten compounds were identified, majority of which are known for their curative efficiency as anti-inflamatory and remarkable antibacterial activity. This natural therapeutant can meet out challenges faced in dental caries management and can be incorporated into an efficient community-based health care system.

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