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PHYTOCHEMICAL AND ANTIMICROBIAL ANALYSIS OF *SPONDIAS MOMBIN LINN.* EXTRACTS ON CARIOGENIC ORGANISMS

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ABSTRACT
Caries, oral-mucosal and periodontal diseases are the most prevalent oral health problems globally. They are prevalent in all ages, demographic and socioeconomic groups. Due to the high prevalence of oral diseases and increased microbial resistance to antibiotics, there is need for alternative methods of oral diseases management. Phytochemicals isolated from plants, which are used in traditional medicines, are considered to be safe and effective alternatives compared to synthetic chemicals. This study was aimed at analyzing the phytochemicals and antimicrobial potentials of *Spondias mombin linn.* extracts on cariogenic organisms. The cariogenic organisms were isolated from dental lesions of carious patients attending Enugu dental clinic, Enugu State, Southeastern Nigeria using standard microbiological and molecular diagnostic methods. Methanolic, ethanolic and aqueous extracts of the leaf and stem-bark of *Spondias mombin linn.* were used for the analysis. Agar-well and disc diffusion methods were used to determine the antimicrobial sensitivities of these agents on the isolates. Phytochemical analysis of leaf and stem-bark of *Spondias mombin linn.* shows the presence of dihydrocytisine, ammodendrine, spartein, sapogenin, naringenin, cardiac glycoside, tannin, epihendrine, kaempferol, ribalinidine, anthocyanin, flavones, catechin, flavonones, aphylidine, oxalate, proathocyanidin, steroid, phytate and cynogenic glycoside. The isolates were *Klebsiella pneumoniae strain kp1804, Enterobacter cloacae strain D4skcTm4, Bacillus subtilis strain A7, Staphylococcus sciuri strain Ty-42, Escherichia coli strain m-196-3* and *Candida albicans AMA12*. The MIC and MBC ranged from 25mg/ml and 12.5mg/ml to 12.5mg/ml and 6.25 mg/ml. The study revealed that *Spondias mombin linn.* leaf and stem-bark extracts could serve as good alternative to conventional antimicrobial agents in the treatment of oral diseases especially dental caries.

KEYWORDS: Oral diseases, Cariogenic, Phytochemicals, Antimicrobials.

INTRODUCTION
Dental caries is a dynamic dieto-microbial disease involving cycles of demineralization. It is a chronic multifactoral disease, which causes destruction and demineralization of hard tissues of the teeth by acid production occurring from bacterial fermentation (ADA, 2012). Although a significant body of knowledge is now available on how to maintain healthy teeth such as use of lozenges, mouth washes, oral diseases continue to be a major health problem worldwide. Dental caries and periodontal disease are among the most important global oral health problems (Amadi *et al*., 2007).

According to the world oral health report (2003), dental caries remains a major public health problem in most industrialized countries, affecting 60-90% of school children and vast majority of adults. Irrespective of geographical location in the world, both male and females are affected by the condition. Various factors including plaques formation, microorganisms, suitable carbohydrate (mainly sucrose), susceptible tooth surface and time are amongst factors necessary to produce dental caries (Frei, 2012).

In spite of the overwhelming influences and dependency on modern medicine and tremendous advances in synthetic drugs, large segments of the world population depend on drugs from plants (Joy *et al*., 2001). Poverty, inadequacy of health services, shortage of health workers, rampant shortage of drugs and equipment in existing health facilities make traditional drug an important component of healthcare in Africa. *Spondias mombin linn.* is a fructiferous tree that belongs to the family *Anacardiaceae.* It grows in the coastal areas and rainforest into big tree of up to 15-22m in height. The tree is common in Nigeria, Brazil and several other tropical rainforests of the world with high genetic variability among populations (Ayoka *et al*., 2008).

Spondias mombin linn is called Hog plum in English, *akika* in Yoruba, *ijikara* or *isikala* in Igbo, *tsaadar lamarudu* in Hausa, *chabbuli* in Fulani and *nsukakara* in Effik. Both bark and flowers are used in folk medicine for treatment of digestive tract aliments, lower back pain, rheumatism, angina, sore throat, toothache, malarial fever, congestion, diarrhea, urethritis, metrorrhagia and as contraceptive. The plant extracts exhibit antibacterial properties and a decoction of the bark or root bark is considered antiseptic. The roots are regarded as antifungal and the leaf decoction are used in treating dental caries. The decoction of the bark can be taken for severe cough with inflammatory symptoms, while the leaves can be combined with uraric picta and used as gargle to treat dental caries and sore throat. A wide variety of secondary metabolites in medicinal plants having *in vitro* antimicrobial activities provide a hope for novel drug compounds (Aladesanmi *et al.*, 2007).

This study was aimed at analyzing the phytochemicals and antimicrobial potentials of *Spondias mombin Linn* leaf and stem-bark extracts on cariogenic organisms.

MATERIALS AND METHODS

Sample collection and identification

Fresh leaves and barks of *Spondias mombin linn* were collected at Ezu forest in Amansea, Awka –North Local Government Area, Anambra State, Nigeria. The samples were properly identified and confirmed by Prof. A. I. Izundu of Botany Department, Nnamdi Azikiwe University Awka, Anambra State, Nigeria.

Study design

The study was designed in such a way that clinical specimens were collected only from patients suffering from tooth decay or tooth cavity. The support of dental surgeons in the clinic was solicited. An ethical clearance was obtained from the hospital management board. Clinical examinations of oral cavities of the patients were done to ascertain their dental health. The specimens were transferred to the Microbiology laboratory in an aseptic ice cooler for further analysis.

Specimen Collection

Thirty clinical specimens obtained from patients with carious lesions were used for this study. Brain heart infusion broth was used as the transport media. The tooth of patients that present with carious lesions was extracted by the dental surgeon under strict aseptic conditions. Patients were made to gaggle their mouth with sterile water. The tooth and the surrounding field were cleaned with 3% hydrogen peroxide and then decontaminated with 2.5% sodium hypochlorite solution. The food debris was removed using a dental excavating instrument. The extracted teeth were introduced into Bijou bottles containing sterile brain heart infusion broth and labeled accordingly. Sterile swab sticks and dental probes were used by the dentist in collection of specimen from second group of patients, whose carious lesion did not require tooth removal. The collected specimens were stored in an ice cooler and transported to the Microbiology laboratory for further analysis.

Isolation and identification of Cariogenic Organisms

The specimens were inoculated on Azide Blood Agar plates, chocolate agar and potatoes dextrose agar using the spread plate method. The plates were incubated aerobically at 37^{o}C for 24 hours and anaerobically at 37^{o}C for 72hours. Pure culture colonies were identified by macroscopic examination of colony growth on agar plates, colony morphological characteristic, hemolytic activity, gram staining reaction and motility test. Other identification tests include catalase, growth in 65% NaCl, Arginine hydrolysis and sugar fermentation tests (Cheesbrough, 2010). The yeast isolates were identified using Lactophenol mount, Sugar fermentation and germ tube tests (Udemezue and Oyeka, 2021). Chromosomal DNA extraction, PCR and nucleic acid sequencing procedures were used to confirm the isolates (Morey *et al*., 2013).

Preparation of plant extracts

Extraction of leaf and Stem-bark of *Spondias mombin Linn.*

The collected leaves and bark were rinsed under a running tap and air-dried for three weeks at room temperature, 25^{o}C. The dried leaves and bark were pulverized using electrical blender into fine powder. Exactly 50g of the powdered plant materials were measured out separately using weighing beam balance and each introduced into 250ml of 95% Methanol, ethanol and distilled cold water respectively. Each mixture was kept in a rotator shaker at room temperature, (28±2^{o}C), for 24 hours. It was then filtered using sterile Whatman no. 1 filter paper. The filtrate were then subjected to a slow but complete solvent evaporation using a regulatory hot plate at a temperature of 40-60^{o}C to obtain the extracts.

Sterility Test

All extracts were cultured on nutrient agar and Sabouraud's dextrose agar plates and incubated for 24-48 hours at 37^{o}C. This was done to ensure that the extract were sterile before use.

Phytochemical analysis of *Spondias mombin linn* extracts

The phytochemical constituents of *Spondias mombin linn.* extracts (the leaves and bark) were analyzed using the methods described by Ogundiya *et al.* (2002). The methods include Hager's test (alkaloids), Wagner's test (alkaloids), Ferric chloride test (tannins), Salkowski test (terpenoids), Sodium hydroxide test (flavonoids), Frothing test (saponins) and steroids tests.

Extracts Quantification using GC-MS

Quantitative analysis of the phytochemicals was performed on a BUCK M910 Gas chromatography equipped with HP-5MS column (30 m in length × 250 μm in diameter × 0.25 μm in thickness of film). Spectroscopic detection by GC–MS involved an electron ionization system which utilized high energy electrons (70 eV). Pure helium gas (99.995%) was used as the carrier gas with flow rate of 1 mL/min. The initial temperature was set at 50 –150 °C with increasing rate of 3 °C/min and holding time of about 10 min. Finally, the temperature was increased to 300 °C at 10 °C/min. One microliter of the prepared 1% of the extracts diluted with respective solvents was injected in ansplitless mode. Relative quantity of the chemical compounds present in each of the extracts was expressed as percentage based on peak area produced in the chromatogram (Buss *et al.*, 2010).

Identification of chemical constituents

Bioactive compounds extracted from different extracts were identified based on GC retention time on HP-5MS column and matching of the spectra with computer software data of standards (Replib and Mainlab data of GC–MS systems).

Antimicrobial susceptibility screening of the plant extracts

Antibacterial activities of the methanolic, ethanolic and aqueous extracts were evaluated using the agar-well diffusion method. Zones of inhibition against the test organisms were examined. Mueller Hinton agar plates were prepared and allowed overnight to check sterility. A sterile 6mm cork borer was used to bore wells on the agar plates. Using a sterile inoculation loop, colonies of 24hours plate culture of each isolate was transferred aseptically into a sterile test tube containing 10mls normal saline and was standardize to 0.5 McFarland turbidity. A sterile swab stick was used to spread the resulting solution uniformly on the surface of Mueller Hinton agar. This was allowed to stand for 30min and 0.5ml of the plant extracts were aseptically seeded into the wells. Conventional antibiotics were used as positive control while sterile distilled water was used as negative control. The plates were incubated at 37^{o}C for 24 hours. The inhibition zones diameter were measured and recorded accordingly (Udemezue and Oyeka, 2021).

Determination of MIC and MFC of the plant extracts using broth dilution method

Various concentrations of the test agents, from the stock solution of 100mg/ml, were made in nutrient broth by double fold serial dilution to obtain, 100mg/ml, 50 mg/ml, 25 mg/ml, 12.25mg/ml and 6.325 mg/ml. Each dilution in a test-tube was inoculated with 0.2 ml of the broth culture of test isolates diluted to 0.5 McFarland standard. All the tubes were incubated at 37°C for 24 hrs. The lowest concentration showing no visible growth (as compared with a negative control) was recorded as the minimum inhibitory concentration (MIC) for each organism. The MFC was determined by transferring 1ml from each negative tube in MIC assay, onto the surface of freshly prepared nutrient agar plates (without the test agents) using the spread plate method and incubated at 37°C for 48 hrs. The lowest concentration showing no visible growth was recorded as minimum bactericidal concentration (MBC) for each organism (Udemezue and Oyeka, 2021).

Statistical Data Analysis

The results of these tests were analyze statistically using one way (ANOVA) followed by Duncan's posthoc multiple comparison test using SPSS package version 25.0 for windows, to determine the significant different. The results was expressed as mean $\pm S.D.$ P values < 0.05 was considered as statistical significant.

RESULTS

Table 1: Phytochemical analysis of *Spondias mombin linn.* leaves and bark extracts.

Component	Leaves extract (µg/ml)	Bark extract (µg/ml)
Dihydrocytisine	10.9321	7.3151
Ammodendrine	10.9745	3.6162
Spartein	9.2474	8.9813
Sapogenin	24.5141	6.5320
Naringenin	10.2164	5.3615
Cardiac glycoside	26.3705	6.2916
Tannin	4.6423	4.8170
Epihendrine	8.9219	8.9789
Kaempferol	13.1795	3.9056
Ribalinidine	17.1435	6.1510
Anthocyanin	8.6526	7.4351
Flavones	9.3922	7.3862
Catechin	15.5078	3.9744
Flavonones	6.5616	6.6402
Aphylidine	9.7040	3.0007
Oxalate	1,4314	0.9699
Proathocyanidin	6.2794	6.7076
Steroid	7.0857	6.0613
Phytate	14.1787	7.9020
Cynogenic glycoside	-	3.0029

Table 2: Inhibition zone (mm) produced by the crude ethanolic extracts of *Spondias mombin linn.* against test organisms.

Concentration of extract in mg/ml.

Isolates	6.25	12.5	25	50	100	Cpr	Am	Fvalue
Staphylococcus sciuri	0 ± 0.0^{a}	0 ± 0.0^{a}	9 ± 0.19^{d}	11 ± 0.20^{d}	13 ± 0.11^{c}	15 ± 0.20^{c}	21 ± 0.21^{g}	61.0^{***}
Enterobacter cloacae	0 ± 0.0^{a}	0 ± 0.0^{a}	6 ± 0.17^{c}	9 ± 0.14^{d}	10 ± 0.23^{d}	23 ± 0.45^{g}	18 ± 0.27^{f}	87.0^{***}
Bacillus subtilis	0 ± 0.0^{a}	10 ± 0.16^{d}	10 ± 0.20^{d}	11 ± 0.26^{d}	12 ± 0.12^{e}	21 ± 0.34^{g}	15 ± 0.45^{e}	431.9^{***}
Escherichia coli	3 ± 0.0^{a}	6 ± 0.14^{b}	8 ± 0.18^{c}	12 ± 0.14^{c}	15 ± 0.23^{e}	12 ± 0.23^{d}	$15\pm0,29^{c}$	129.8^{***}
Klebsiella pneumonia	0 ± 0.0^{a}	0 ± 0.0^{a}	0 ± 0.0^{a}	3 ± 0.18^{b}	6 ± 0.23^{c}	27 ± 0.33^{h}	27 ± 0.29^{h}	420.2^{***}

*P< 0.05, **P<0.01, ***P<0.001, Cpr= ciprofloxacin, Am= amoxil NB : Different superscript letters indicates statistically significant differences.

Table 3: Inhibition zones (mm) produced by the crude methanolic extract of *Spondias mombin linn.* against test organisms.

Concentration of extract in mg/ml.

Isolates	6.25	12.5	25	50	100	Cpr	Am	Fvalue
Staphylococcus sciuri	0±0.0[a]	5±0.21[b]	7±0.19[b]	10±0.20[c]	14±0.11[e]	15±0.20[e]	21±0.21[g]	203.6***
Enterobacter cloacae	0±0.0[a]	6±0.22[b]	5±0.17[b]	11±0.14[c]	14±0.23[e]	23±0.44[g]	18±0.27[f]	164.9***
Bacillus subtilis	0±0.0[a]	0±0.0[a]	1±0.20[a]	4±0.26[b]	5±0.12[b]	21±0.34[g]	15±0.45[e]	733.0***
Escherichia coli	0±0.0[a]	3±0.18[b]	9±0.14[c]	13±0.18[d]	16±0.23[e]	12±0.23[d]	15±0.29[e]	82.8***
Klesilla pneumonia	0±0.0[a]	0±0.0[a]	7±0.22[b]	7±0.18[b]	10±0.23[c]	27±0.33[h]	27±0.29[h]	87.0***

*P< 0.05, **P<0.01, ***P<0.001, Cpr= ciprofloxacin, Am= amoxil NB: Different superscript letters indicates statistically significant differences

Table 4: Inhibition Zones (mm) produced by the crude aqueous extracts of *Spondias mombin linn.* against test organisms.

Concentration of extract in mg/ml

Isolates	6.25	12.5	25	50	100	Cpr	Am	Fvalue
Staphylococcus sciuri	0±0.0[a]	0±0.0[a]	0±0.0[a]	5±0.20[b]	5±0.11[b]	15±0.20[e]	21±0.21[g]	453.1***
Enterobacter cloacae	0±0.0[a]	0±0.0[a]	0±0.17[a]	1±0.14[a]	5±0.23[a]	23±0.45[g]	18±0.27[f]	319.0***
Bacillus subtilis	0±0.0[a]	0±0.0[a]	0±0.0[a]	0±0.0[a]	1±0.12[a]	21±0.34[g]	13±0.45[e]	114.60***
Escherichia coli	0±0.0[a]	0±0.0[a]	0±0.0[a]	3±0.18[b]	1±0.23[a]	12±0.23[d]	15±0.29[e]	444.2***
Klebsiella pnumoniae	0±0.0[a]	0±0.0[a]	0±0.0[a]	0±0.0[a]	1±0.23[a]	27±0.33[h]	27±0.29[h]	554.7***

*P< 0.05, **P<0.01, ***P<0.001, Cpr= ciprofloxacin, Am= amoxil NB: Different superscript letters indicates statistically significant differences

Table 5: Minimum Inhibition Concentration (MIC) in mg/ml of the methanolic extracts of *Spondias mombin linn.* leaves and bark against test isolates.

Isolates	Leaves	Bark
Staphylococcus sciuri	25	25
Enterobacter cloacae	50	50
Bacillus subtilis	25	25
Escherichia coli	50	50
Klebsiella pneumonia	50	50

Table 6: Minimum Inhibition Concentration (MIC) in mg/ml of the ethanolic extracts of *Spondias mombin linn.* leaves and bark against test isolates.

Isolates	Leaves	Bark
Staphylococcus sciuri	50	25
Enterobacter cloacae	25	25
Bacillus subtilis	25	50
Escherichia coli	25	25
Klebsiella pneumonia	25	25

Table 7: Minimum Inhibition Concentration (MIC) in mg/ml of aqueous extracts of *Spondias mombin linn.* leaves and bark extracts.

Isolates	Leaves	Bark
Staphylococcus sciuri	12.5	12.5
Enterobacter cloacae	12.5	12;5
Bacillus subtilis	12.5	12.5
Escherichia coli	25	25
Klebsiella pneumonia	12.5	12.5

Table 8: Minimum Bactericidal Concentration (MBC) in mg/ml of the methanolic extract of *Spondias mombin linn.* leaves and bark against test isolates.

Isolates	Leaves	Bark
Staphylococcus sciuri	12.5	12.5
Enterobacter cloacae	25	25
Bacillus subtilis	12.25	12.25
Escherichia coli	25	25
Klebsiella pneumonia	25	25

Table 9: Minimum Bactericidal Concentration (MBC) in mg/ml of the ethanol extracts of *Spondias mombin linn.* leaves and bark against test isolates.

Isolates	Leaves	Bark
Staphylococcus sciuri	25	12.5
Enterobacter cloacae	12.5	12.5
Bacillus subtilis	12.5	25
Escherichia coli	12.5	12.5
Klebsiella pneumonia	12.5	12.5

Table 11: Minimum bactericidal concentration (MBC) in mg/ml of aqueous extracts of *Spondias mombin linn.* leaves and bark against test isolates.

Isolates	Leaves	Bark
Staphylococcus sciuri	6.25	6.25
Enterobacter cloacae	6.25	6.25
Bacillus subtilis	6.25	6.25
Escherichia coli	12.5	12’5
Klebsiella pnumoniae	6.25	6.25

DISCUSSION

The study was designed to explore the phytochemicals of *Spondias mombin Linn* and its antimicrobial activities on carious organisms in order to ascertain it usage in dental caries treatment as well as carious prevention. Despite the wide range of available conventional antimicrobials, the microbes render them ineffective through resistance development. An important potential strategy to help combat the resistance problem involves the discovery and development of new active agents capable of partly or completely suppressing bacterial resistance mechanisms. The extracts quantification of *Spondias mombin linn.* leaf and bark was conducted using the GC-MS; and shows the presence of dihydrocytisine, ammodendrine, spartein, sapogenin, naringenin, cardiac glycoside, tannin, epihendrine, kaempferol, ribalinidine, anthocyanin, flavones, catechin, flavonones, aphylidine , oxalate, proathocyanidin, steroid, phytate and cynogenic glycoside.

In a study by Carbral *et al.* (2016), phytochemical prospecting and thin layer chromatography analysis revealed that *Spondias mombin linn.* extract has, in its constitution, phenolic compounds, flavonoids, tannins and sponine and these constituents exhibited antimicrobial activity. This findings is in line with the result of phytochemical analysis obtained in this study. Thus, it may be suggested that the effectiveness of *Spondias mombin linn.* extract as an antimicrobial agent, could be related to the presence of these metabolites. It was observed that cold water extracts generated the lowest inhibitory concentration. This may be due to its inability to fully extract all the active antimicrobial components of the plant. This agrees with similar study done by Ogundiya *et al. (*2008). The influence of solvent used for extraction on the inhibitory capacity of the extract on the test organism has been reported by Al-Bayati and Sulaiman (2008). From the result of this study, methanolic extracts exhibited more pronounce inhibition than the ethanolic and cold water extracts.

The isolates obtained were *Enterobacter cloacae strain D4skcTm$_4$, Bacillus subtilis strain A7, Staphylococcus sciuri strain Ty42, Escherichia coli strain M-196-3, Klebsiella pneumonia strainKP1804, and Candida albicans strain CBS 5138. Escherichia cloacae, E. coli, E. aerogenes* and most strains of *E. sakazakii* are intrinsically resistant to ampicilin, as well as first and second generation cephalosporins as a result of an inducible ampc chromosomal β-lactamase that is controlled by both positive and negative regulators (Chow *et al.*, 2012). Thus, the strong need for a better alternative. This study revealed that *Spondias mombin linn.* extracts will serve as better alternative.

Moreover it was observed that the zones of inhibition varied from one organism to another at different concentration. Similar results have been reported by several researchers. According to Willey *et al*. (2015), the activity of antimicrobial agents is concentration dependent. Cowman (2009) also indicated that the position of zone edge (inhibition diameter) is determined by the initial population density of organism, their growth rate and rate of diffusion of the antimicrobial agent. The study also showed that bark extract of *Spondias mombin linn* showed more inhibitory potential than the leave extract. This can be due to slight difference in their phytochemical composition as it was observed that cynogenic glycoside is only present in the bark extracts of *Spondias mombin linn*.

Candida albicans was the major fungi encountered in this study and this agrees with Todur 2012, who reported *C. albicans* as the most common yeast isolated from the oral cavity.

CONCLUSION

Prevention of dental caries is challenging, as the incidence of the disease is very high in general population and it occurs in economically deprived people who cannot afford the commercially available oral hygiene products. Hence, in order to prevent dental caries, it is time to focus our attention toward natural resources which have vast abilities to inhibit the growth of microbes that are responsible for caries. For this, we need to isolate the bioactive compounds from plants with little or no harmful effects and *Spondias mombin linn.*, from this study, has proven effective.

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