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ASSESSMENT OF MICROBIAL AND PHYTOCHEMICAL PARAMETERS IN THE EFFLUENTS OF LOCUST BEANS (*Parkia biglobosa*).

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ABSTRACT

African locust bean effluent contains a range of microbes and has been found to be of health benefit to human. The present study aims to identify various microorganisms associated with African locust beans effluents and also determine its phytochemical parameters. Cooked locust beans under hygienic condition in the laboratory containing the effluent with chaffs (EFWS) and the effluent without the chaffs (EFWOS) were used in this study. The conventional and molecular methods of identification of microorganisms were employed in the identification of microbial isolates of the effluent. Quantitative and qualitative analyses were used to determine phytochemical components in the effluents using standard methods. *Bacillus subtilis*, *Enterobater faecalis*, *Enterobacter aerogenes*, *Acaligenes faecalis*, *Lactobacillus* sp., *Aspergillus niger*, *Aspergillus flavus*, *Penicillium*, *Rhizopus* and *Candida* species were identified using conventional method. The molecular techniques revealed that the bacteria had (98-100%) homology with *Bacillus subtilis* 8700:2, *Lactobacillus paracasei* D12-5 and *Enterobacter ludwigii* TB-136 while the fungi had remote identity (88-98%) with *Aspergillus flavus* Z5, *Geotrichum candidum* OUCMBI101144 and *Trichomonacaea* sp. LMI. Predominant microorganisms isolated were *Bacillus subtilis* and *Aspergillus flavus*. The phytochemical components of the effluents were saponin, tannin, flavonoid, terpernoid, and alkaloid. Saponin had the highest concentration with 39.82 mg/g and 39.27 mg/g for EFWS and EFWOS respectively while tannin had the lowest concentration with 4.17 mg/g for the effluents. This study has provided additional and useful information on the diversity of microorganisms and the phytochemical constituents associated with the effluents of *Parkia biglobosa* for those interested in consuming the effluents for therapeutic purpose.

Keywords: Locust beans, Polymerase Chain Reaction, Microorganisms, Phytochemical,

INTRODUCTION

African locust beans, (*Parkia biglobosa*) is from a perennial tropical legume tree which belong to the sub-family mimosoideae and the family leguminosae (now family fabaceae) (Akande *et al*., 2010). *Parkia biglobosa* seed has been said to contain some phytochemicals that are useful for health (Alabi *et al*., 2015). Phytochemical are non-nutritive plant chemicals that have protective or disease preventive properties. Phytochemicals exert antimicrobial activities through different mechanisms, for example, flavonoids exhibit a wide range of biological activities which include antimicrobial, anti-inflammatory, analgesic, anti-allergic effects and cytostatic and antioxidant properties (Maikai *et al*., 2009). Fermented African locust bean (*Parkia biglobosa*) effluent has been

found to be of health benefit to human, due to its high nutritional and medicinal values (Abdulkarim *et al*., 2005). In folklore medicine, *Parkia biglobosa* is used by traditional healers in Southwest Nigeria and Senegal for the treatment of Diabetes Mellitus (Abo *et al*., 2008 and Die yea *et al*., 2008) and in Northern Nigeria, it is used in the treatment of diarrhea (Abdulkarim *et al*., 2005). However, the locust bean effluent, which is the waste product from fermentation process has foul smell and can be viewed as nuisance to the environment where it is being processed.

African locust beans effluents have diverse microorganisms. In our previous studies, *Rhizopus stolonifer*, *Saccharomyces cerevisiae*, *Bacillus subtilis*, *Staphylococcus aureus* and *Micrococcus luteus* were isolated from the effluent of fermented African locust beans using conventional techniques, however, with its limitations (Olukunle and Buari, 2018). Molecular identification on the other hand, provides accurate and reliable identification tool (Dolci *et al*., 2015). Molecular identification of microorganisms also deals with the naming at gene levels hence, it is more accurate and reliable (Surajit *et al*., 2014). There is little information currently available on molecular characterization of microorganisms and phytochemical constituents present in the effluents of fermented *Parkia biglobosa* obtained from Southwest, Nigeria. This work therefore, is designed to isolate microorganisms associated with the effluents of *Parkia biglobosa* and also to identify its phytochemical constituents. This work will provide additional scientific information on microorganisms and the phytochemical constituents of African locust beans effluents for those interested in consuming the effluent for therapeutic purpose.

MATERIALS AND METHODS

Sample Source

The locust bean seeds were purchased at "Oja Oba" in Ikare, Ondo State, Nigeria and washed thoroughly, after which, they were cooked for 6 h until the coats were soft enough to be removed. The effluent (effluent from the boiled locust beans) was decanted and kept in a sterile air tight container, while the coats (chaffs) of the cooked seeds were removed; re-washed and cooked until it was very soft. Then the effluent was also decanted and kept in another air tight container. The samples were transported to Microbiology Laboratory of the Federal University of Technology, Akure, for further analyses.

Sample preparation and isolation method

One milliliter (1mL) of the *Parkia biglobosa* effluent was collected and homogenized with 9 mL of sterile distilled water. The resulting homogenates were diluted appropriately by the method of Uzuegbu and Eke (2001). Ten-(10) fold serial dilution was performed and the desired diluent of each sample was placed on various agars using the pour plate method.

Isolation and Identification of Bacteria

Each bacterial isolate from the effluents (with chaff and without chaff) was purified and identified using colonial characteristics (edges, texture, elevation, colour, pigmentation, and size etc) and cell morphology (shape, arrangement and Gram reaction). Bacterial isolates from each plated Petri dishes were sub cultured. Identification of bacteria for motile, gas production, catalase, citrate utilization, oxidase, indole production, H_2S production, starch hydrolysis, methyl red, Vogues-Proskauer and sugar fermentation tests were carried out on the isolates according to the methods described by Olutiola *et al.* (2000).

Purification and Identification of Fungal Isolates

Isolates of different fungi were sub-cultured repeatedly on Potato dextrose agar (PDA) medium using the inoculating needle and incubated at room temperature for 72 hours, until pure cultures were isolated and identified based on the microscopic and macroscopic characteristics of the hyphal mass, nature of the fruiting bodies and the morphology of the cells and spores. Pure fungal isolates were maintained on Potato dextrose agar slants in the refrigerator at 4ºC until further investigative procedure (Barnett and Hunter, 1998).

Extraction of bacterial DNA

One (1) mL of 24 hours bacterial broth culture was added to 750 µL of lysis solution to a ZR Bashing Breadth lysis tube. The tube was placed on a vortex mixer for about 5 min; after which it was spun in a microcentrifuge ≥ 10,000 rpm for 1min. About 400µL of the supernatant was transferred to a Zymo-spinTH IV spin filter in a collection tube, spun at 7,000 rpm for 1min and 1200 µL of binding buffer was added to the filtrate in collection tube. Eight hundred (800) µL of this mixture was transferred to a Zymo-spin IIC column in a collection tube and spun at 1000 rpm for 1min and the flow through was discarded from the collection tube. Another 800µL of the mixture was transferred into the same Zymo-spin IIC column and spun at 10,000 rpm for 1min. Two hundred (200) µL of DNA was pre-washed and buffer was added to the Zymo-spin IIC column in a new collection tube and was spun at 10,000 rpm for 1min. The Zymo-spin IIC column was transferred to a clean 1.5 mL microcentrifuge tube, 100 µL of the Elution buffer was added directly to the column matrix and was spun at 10,000 rpm for 30 sec to elude the DNA.

Agarose gel- electrophoresis

The eluted DNA was run on agarose gel electrophoresis in order to determine the quality and integrity of the DNA. Agarose powder was weighed (0.8g) and a 100 mL of I X TBE (Tris Borate EDTA) buffer was added to it, dissolved by boiling using a heater magnetic stirrer, cooled to about 60°C and 10 µL of ethidium bromide was added, this was mixed by swirling gently and poured into an electrophoretic tray with the comb in place to obtain a gel thickness of about 4-5 mm. The gel was allowed to solidify for about 20 min, the comb was carefully removed and the tray placed in the electrophoretic tank. The buffer 1 X TBE (Tris Borate EDTA) was poured into the tank, 15µL of the sample was mixed with 2µL of the loading dye. Samples were carefully loaded into the wells created by the combs, then the electrode was connected to the power pack and electrophoresis was at 60-100 V until the loading dye had migrated about 3-quarter of the gel. The electrode was then disconnected and the gel visualized with ultraviolet light illumination and photographed with a digital imaging system (Alpha image 2000, Alpha innotech, S and lendra A).

Polymerase Chain Reaction Analysis for 16S rRNA and 18S rRNA DNA Amplification

The universal primer set that was used to amplify 16S rRNA gene in bacteria was 27F and 1492R. The nucleotide sequence of the forward primer was 5'AGAGTTTGATCTGGCTCAG-3' while the sequence for the reverse primer was 5'-GGTTACCTTGTTACGACTT-3'. A portion of the 16S rRNA bacterial gene was amplified by PCR from the total extracted DNA. The polymerase chain reaction (PCR) programme used was initial denaturation temperature at 94°C for 3 min followed by 30 cycles of 94°C at 1 min, 55°C for 1 min for annealing, 72°C for 1 min and final extension 72°C for 10 min (Joshi and Deshpande, 2011). For 18S rRNA gene in fungi, nucleotide sequences of the primer were NS1-5'-GTAGTCATATGCTTGTCTC-3' and NS2-5'-GGCTGCTGGCACCAGACTTGC-3' with cycling parameter of initial denaturation temperature at 95°C for 2 min followed by 35

cycles of denaturation at 95°C for 30 seconds annealing at 56°C for 30 seconds, extension at 72°C for 1 min, and final extension at 72°C for 10 min.

Purification and Sequencing of PCR Products

Clean-up of PCR products was done by the use of Exo-SAP mix (Exonuclease-Shrimp Alkaline Phosphatase) reagent. Seven (7) µL of PCR product from each of three was added in 3µL Exo-SAP mixture in tube. Purified PCR products were further amplified in one direction with the 16S primers using Big Dye Terminator Ready Reaction Mix (ABI) and sequencing of amplified product was done. The mixture was a combination of 9 µL, Hi Di formamide with 1µL of purified sequencing making a total of 10 µL. The samples were loaded into the machine and the results was produced in form of A, C, T and G (Adenine, Cytocine, Thymine and Guanine respectively). The nucleotides were compared with other nucleotides using BLASTn tools from the National Centre for Biotechnology Information (NCBI).

Phytochemical Analysis

Qualitative and quantitative determination of phytochemical components of *Parkia biglobosa* effluents were determined using standard methods, as described by Sofowora, (1993); Ayoola, et al., (2008) and Savithramma et al., (2011). They include alkaloid, saponin, tannin, phlobatannin, anthraquinone, flavonoid, steroid and terpenoid while cardiac glycosides include Legal's Test, Lieberman's Test, Salkowski's Test and Keller-Killiani's Test.

Statistical analysis of data obtained

Data obtained were subjected to one-way analysis of variance while the mean were compared by Duncan's New Multiple Range Test at 95% confidence interval using Statistical Package for Social Sciences version 16.0.

RESULTS

Characteristics and Occurrence of Isolated Bacteria

Morphological and biochemical characteristics of the bacteria isolated from the locust beans effluents are shown in Table 1. The following bacteria were isolated from the locust beans effluent; *Bacillus subtilis*, *Enterobater faecalis*, *Enterobacter aerogenes*, *Acaligenes faecalis* and *Lactobacillus* sp. The genera of *Bacillus*, *Enterobacter* and *Lactobacillus* are Gram positive while *Acaligenes* is Gram negative.

Table 1: Morphological and Biochemical Characteristics of isolated Bacteria from Effluent of *Parkia biglobosa*

Characteristics	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5
Gram staining/ shape	+R	+C	-R	-R	+R
Cellular and morphological characteristics	Opaque, white waxy growth	Clear, smooth, small, round	Abundant, thick, glistering growth	Thin, white, spreading viscous growth	Moderate, regular, creamy, opaque, raised, dry, entire and very large
Catalase	+	-	+	+	-
Oxidase	-	-	-	+	ND
Citrate	-	-	+	+	-
Motility	-	-	+	+	-
Indole production	-	-	-	-	-
H_2S production	-	-	-	-	-
Starch hydrolysis	+	-	-	-	-
Mannitol	A	+	-	-	-
Glucose	A	-	A	-	AG
Sucrose	A	A	A	-	+
Lactose	-	A	A	-	+
M.R	-	+	-	-	ND
V.P	+	-	+	-	ND
Probable microorganism	*Bacillus subtilis*	*Enterobacter faecalis*	*Enterobacter aerogenes*	*Alcaligenes faecalis*	*Lactobacillus* sp.

Legend: + = Positive; - = Negative; A = Acid production; G = Gas production; MRP = Methyl Red Test
V.P = Voges Proskauer test

Characteristics and Occurrence of Fungal Isolates

Microscopic and macroscopic characteristics of the fungi isolated are shown in Table 2. The fungi isolated from the effluent were *Aspergillus flavus*, *Aspergillus niger*, *Penicillium* sp., *Saccharomyces* sp. and *Geotrichum candidum*.

Table 2: Colonial and Morphological Characteristics of the fungal isolates

Cultural characteristics	Spores/ conidia arrangement under the microscope	Identity of isolates
White base with yellowish-green colour appearance	Conidial heads radiate and typically vesicles globose, surface contain many flask shaped phiades and chains of conidia. Septate and no collumella	*Aspergillus flavus*
Colonies are thin, spreading, soft, creamy and white in the anamorph state.	Characterized by hyphae that appear creeping, mostly submerged and septate. The hyphae colour appears to be hyaline or lightly pigmented. Chlamydospores are sub globose, solitary, borne on undifferentiated hyphae.	*Geotrichum candidum*
Flat, soft, smooth, moist, glistering surface and cream in colour	Nitrate positive, and fermented carbohydrate, produces ascospores, ascospores are globose, hyphae are absent, but has pseudo hyphae.	*Saccharomyces* sp.
Yellowish green	Highly branched network of multinucleate septate, usually colourless hyphae, branched conidiophores sprout on the mycelia, bearing individual constricted conidiophores, conidiophores are usually green in colour.	*Penicillium* sp.
White base with brown colour	Conidial heads radiate and typically vesicles globose, surface contain many flask shaped phiades and chains of conidia. Septate and no columella.	*Aspergillus niger*

Amplification of 16S rRNA of the bacteria

The PCR amplification of genomic DNA targeted to amplify the 16S rRNA of the bacteria and 18S rRNA of the fungal isolates are shown in Plates 1 and 2 respectively.

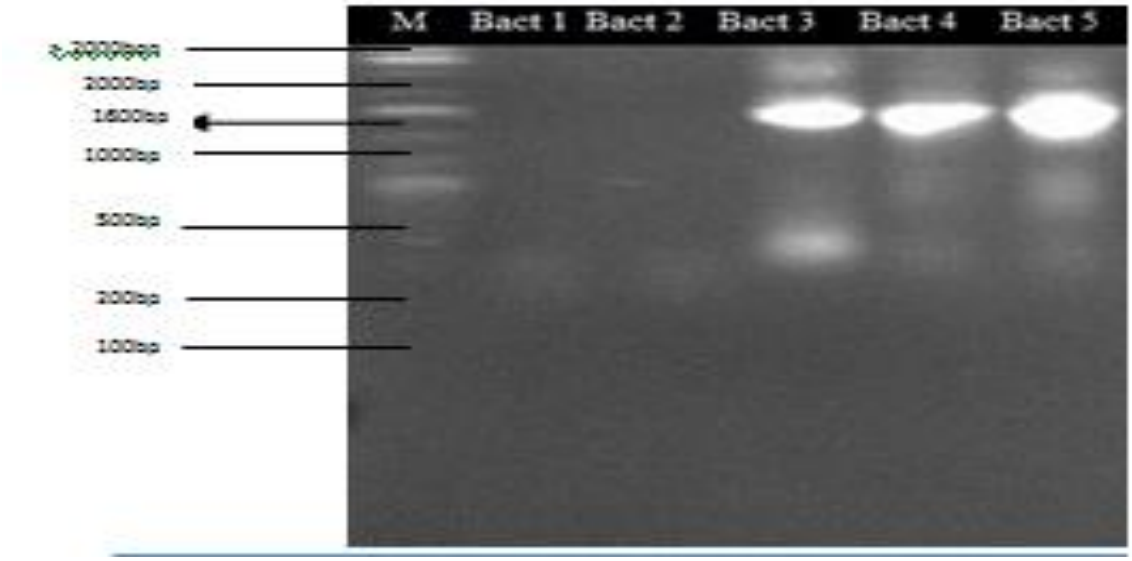

Plate 1: PCR amplification of genomic DNA targeted to amplify the 16S rRNA of the bacterial isolates.

Legends: M: Molecular marker; Bact 3: BOOS1; Bact 4: BOOS2; Bact 5: BOOS3

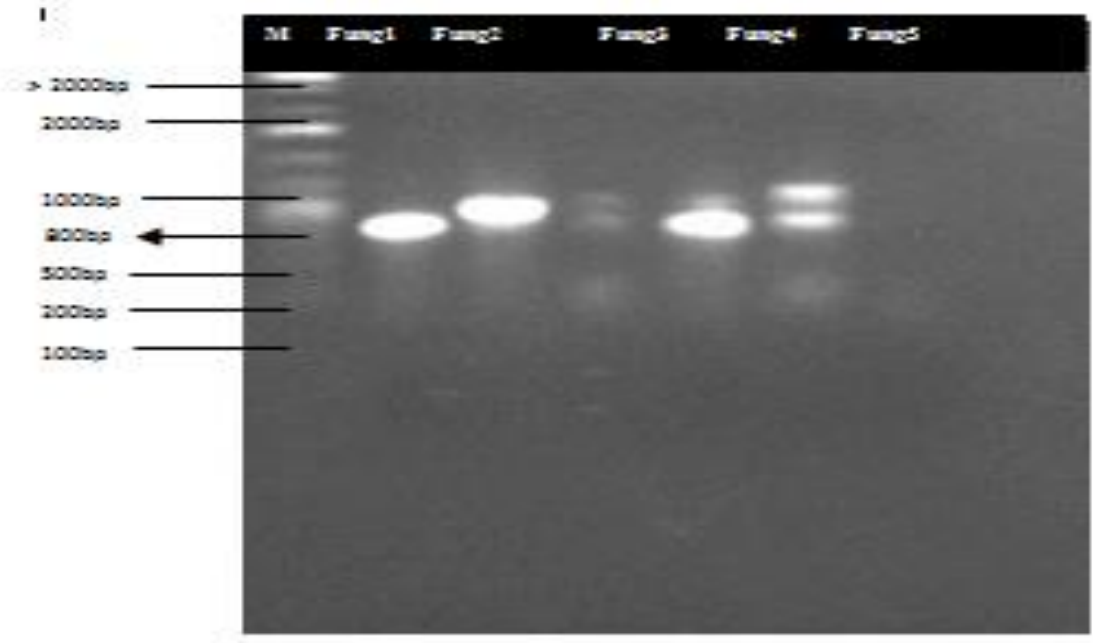

Plate 2: PCR amplification of genomic DNA targeted to amplify the 18S rRNA of the fungal isolates.

Legends: M: Molecular marker; Fungi 1: FOOS1; Fungi 2: FOOS2; Fungi 4: FOOS3

Molecular Identities of the Bacterial and Fungal Isolates

The bacteria isolated in this study using molecular techniques, had 100%, 99% and 98% of similarity identity with: *Lactobacillus paracasei* 8700:2, *Bacillus subtilis* D12-5 and *Enterobacter ludwigii* TB-136 respectively when compared with the query in NCBI database. The results of the bacterial isolates are shown in Table 3.

The fungi identified by molecular techniques are FOOS1, FOOS2 and FOOS3, having 89%, 99% and 89% similarity with *Aspergillus flavus* Z5, *Geotrichum candidum* OUCMBI101144 and *Trichocomaceae* sp.LM1 respectively when compared with the query in NCBI database. The results of fungal isolates are presented in Table 4.

Table 3: Molecular Characteristics of the Bacterial Isolates

Bacterial Isolates	Homologous Genes	% of Identity	Accession No	Strains	Bacteria
BOOS1	16S ribosomal RNA partial sequence	100%	CP002393.1	8700:2	*Lactobacillus paracasei*
BOOS2	16S ribosomal RNA partial sequence	99%	CP014858.1	D12-5	*Bacillus subtilis*
BOOS3	16S ribosomal RNA partial sequence	98%	KF817747.1	TB-136	*Enterobacter ludwigii*

Table 4: Molecular Characterization of Fungal Isolates

Fungal Isolates	Homologous Genes	% of Identity	Accession Number	Strain	Microorganisms Description
FOOS1	18S rRNA partial sequence	98%	KP784374.1	Z5 18S	*Aspergillus flavus*
FOOS2	18S rRNA partial sequence	89%	706920.1	OUCMBI101144	*Geotrichum candidum*
FOOS3	18S rRNA partial sequence	88%	EF060395.1	LM1 18S	*Trichocomacaea* sp.

Qualitative Phytochemical Composition of Locust Beans Effluents

The composition of locust beans effluent based on the qualitative phytochemical screening is presented in Table 5. The phytochemical composition was present in the effluent with chaffs and effluent without chaffs with the exception of phlobatannin, alkaloid and anthraquinome.

Table 5: Qualitative Phytochemical Composition of Locust Beans Effluent

	EFWS	EFWOS
Saponin	+	+
Tannin	+	+
Phlobatannin	-	-
Flavonoid	+	+
Steroid	+	+
Terpenoid	+	+
Alkaloid	-	-
Anthraquinone	-	-
CARDIAC GLYCOSIDE		
Legal Test	+	+
Keller kiliani Test	+	+
Salkwoski Test	+	+
Lieberman Test	+	+

Legend: EFWS: Effluent with chaffs; EFWOS: Effluent without chaffs; +: Positive; -: Negative

Quantitative Phytochemical Composition of the Locust Beans Effluents

Figure 1 illustrates the qualitative phytochemical composition of the locust beans effluent used in this study. The phytochemical components include saponin, tannin, alkaloid terpenoid, cardiac glycoside, and flavonoid. Saponin had the highest value of 39.82 mg/g and 39.27mg/g with effluent containing chaffs and effluent without chaffs respectively whereas, flavonoid had the lowest value of 4.17 mg/g and 7.00 mg/g in effluent with chaffs and effluent without chaffs respectively.

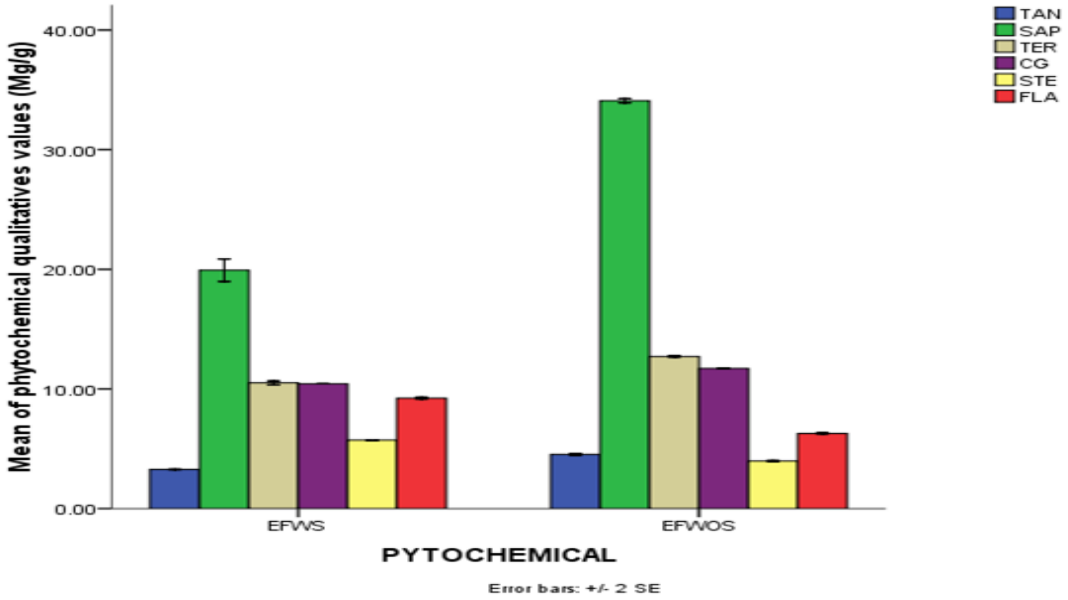

Figure 1: Quantitative Phytochemical Composition of the Locust Beans Effluents

Legend: EFWS: Effluent with chaffs; EFWOS: Effluent without chaffs; TAN: Tannin, SAP: Saponin, TER: Terpenoid; CG: Cardiac glycoside, ALK: Alkaloid, FLA: Flavonoid

DISCUSSION

In this study, conventional and molecular methods of identification of microorganisms were used. The conventional method reveals the morphological and biochemical activities exhibited by the microorganisms while the molecular characterization on the other hand, helps to discover new microbes and to identify to the species and strain levels based on their genetic properties (Ricardo *et al*., 1993). The predominant bacterium isolated from the effluent was *Bacillus subtilis*. Succession of microorganisms involved during the fermentation of African locust beans seeds have been studied by many researchers (Odunfa and Oyewole, 1986; Ogbadu *et al*., 1988; Oguntoyinbo *et al*., 2007) and also reported that one of the predominant microorganisms present throughout the fermentation was *Bacillus* species with characteristics similar to *Bacillus subtilis*.

This result also correlates with that of Oladunmoye (2007) who reported the identities of the bacteria involved in the fermentation of locust beans as *Staphylococcus aureus* and *Bacillus* sp. while the identities of fungi were *Fusarium*, *Aspergillus* and *Penicillium* species. The most predominant fungi isolated from the effluents was *Aspergillus flavus*. This is in accordance with the report of Nwadiaro *et al*. (2015) who reported that out of seventeen fungal species that were isolated, *Aspergillus* species were the most dominant fungi.

The presence of *Bacillus subtilis* and the lactic acid bacteria; *Lactobacillus* sp. are capable of increasing the protein and fat contents in the effluent. In addition, these microorganisms are responsible for the fermentation of African locust beans (Ogbadu and Okagbue, 1988). Both microorganisms (*Bacillus subtilis* and *Lactobacillus* sp.) are attributed with giving the characteristic flavour of fermented locust beans (Campbell, 1980; Odunfa, 1985). The presence of such bioactive compounds has been linked to the antibacterial activity such as inhibition of growth (De and Ifeoma 2002) and providing some

protection to the plant against microbial infections (Fransworth, 1982).

The phytochemical screening of the effluent with chaffs and effluent without chaffs revealed the presence of saponin, tannin, flavonoid, terpenoid, and steroid. This finding also correlates with the report of Obajuluwa *et al*. (2010) who reported that *Parkia biglobosa* has been reported to be rich in tannins, flavonoids and saponins among others which are secondary metabolites, known to have antibacterial activities. Alkaloids, phlobatannin and anthraquinone were absent. This may be responsible for the inability of the locust beans effluent to inhibit the growth of some microorganisms when subjected to antimicrobial test as suggested by Sherah *et al*., (2014).

In this study, the values of phytochemical properties revealed the exposition of broad range of biological activities, comprising of antimicrobial, anti-angiogenic, anti-inflammatory, anti-allergic effects, cytostatic, analgesic, and antioxidant properties (Maikai *et al*., 2009). Several health promoting functions of flavonoids in microorganisms have been confirmed by the ability to scavenge hydroxyl radicals, lipid peroxyl radicals and superoxide anion radicals. These flavonoids are essential for prevention of diseases associated with oxidative damage of membrane, DNA and protein (Scabelt, 1991). The antibacterial activity of this metabolite had been reported to be as a result of their ability to form complexes with bacterial cell walls, extracellular and soluble proteins (Scabelt, 1991). Tannin acts by iron deprivation, hydrogen bonding, or specific interaction with protein such as enzymes, cell envelopes and complex formation with polysaccharides Hisanori *et al*., (2001). Herbs that contain tannin are used in the treatment of intestinal disorders such as diarrhoea and dysentery, thus exhibiting antimicrobial activity (Dharmananda, 2003). Saponins are known to produce inhibitory effect on inflammation (Just *et al*., 1998). Saponins have also been reported to possess antibacterial property with their mode of action attributed to their ability to cause leakage of protein and certain enzymes from bacterial cells (Tamail *et al*., 2011). Cardiac glycosides are an important class of naturally occurring drugs whose actions help in the treatment of congestive heart failure (Ikedia *et al*., 1995). This group of phytochemical compound was detected in the effluent, thereby, making this plant useful for the treatment of cardiac infections along with other ailments such as dental caries and cough among Yoruba people of Nigeria. Steroid compounds also present in *P. biglobosa* effluent have been reported to have immune-enhancing benefits (Donald *et al*., 1997; Berges *et al*., 1995). Taking together all these facts, the use of *Parkia biglobosa* effluent as part of local and conventional medications for treatment of diseases should be encouraged.

CONCLUSIONS

This study has established that African locust bean seed effluent contains arrays of microorganisms. *Bacillus subtilis* and *Aspergillus flavus* are established to be the most predominant microorganisms present in the effluent. This study also confirms some secondary metabolites such as tannins, saponins, terpenoids, steroid and flavonoids in reasonable amount, hence the effluents are confirmed to contain active secondary metabolites with antimicrobial and other medicinal properties that can be explored for therapeutic use instead of discarding it.

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