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Table of Content

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Full Length Research Paper

Isolation and characterization of a novel keratinolytic bacterial strain from soil samples of chicken feather keratinaceous-waste dumping sites of Ngaoundere (Cameroon)

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This study aims at characterizing new chicken feather-degrading bacteria isolated from soils of keratinic waste collected from four dumping sites of Ngaoundere. Fifteen proteolytic bacteria were isolated with three (D1, D2 and F1) showing the capacity to degrade feather in Basal Salt Media. Highest degradation rate (71.11 ± 3.05%) of feather was obtained from isolate D2 from Municipal slaughterhouse. The partial characterization of the strain D2 based on morphological and biochemical tests revealed that it is a *Bacillus* sp. The keratinolytic activity was positively correlated to the bacterial growth with the highest value (13.76 U.ml^{-1}) obtained at the stationary phase after 120 h of incubation. The optimal conditions for the keratinolytic activity of this isolate were determined to be pH 8.0 and temperature of 45°C. Thus, the Bacillus strain (D2) isolated is a promising strain for the management and valorization of feather wastes.

Key words: Chicken feather-degrading bacteria, keratinolytic enzyme activity, characterization, Ngaoundere, Cameroon.

INTRODUCTION

Proteases are enzymes consisting of one or more polypeptide chains that catalyze the hydrolysis of proteins by cleaving the peptide bond that binds two amino acids and belong to the class of peptidases (Drouin, 2005). They are the most produced and used industrial enzymes in the world, accounting for about 65% of the global

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industrial enzymes market (Sahoo et al., 2012). Proteases are divided into several groups among which keratinases. Most keratinases described are of microbial origin and are described to be produced only in the presence of a keratinous substrate (Bach et al., 2015). They possess a unique feature compared to other proteases, to effectively degrade keratinaceous materials found mainly in animal slaughter waste such as horns, hairs, hooves, nails and feathers.

One of the main sources of the keratins is poultry industry. Indeed, in the poultry industry, about 400 million chickens are processed every week in the world; which generates a total of about five million tons of feather (Godheja and Shekhar, 2014). Concerning Cameroon, there is about 50 million of chickens produced per year, which generates about 6000 tons of feather waste annually. With the improvement of local chicken breeding technics and growth production volume, there is a subsequent increase in production of feather waste (FAO, 2013; IPAVIC, 2016). This leads to the problem of waste disposal, and high risk of environmental pollution due to the recalcitrant nature of keratinaceous materials. Indeed, keratin contains in its structure disulfide bonds from a high level of cysteine, responsible for its three-dimensional stability, making it a non-degradable by common enzymes such as trypsin, pepsin and papain (Vidmar and Vodovnik, 2018). To solve the environmental pollution problem, chicken feathers have been valued for several years in the production of animal food after physical or chemical hydrolysis (Papadopoulos et al., 1986). But the high temperatures and pressures used in these treatments require large amounts of energy and the drastic conditions used also lead to the degradation of many essential amino acids (lysine, methionine, tryptophan), giving a product with poor and variable nutritional quality (Cai et al., 2008). This has prompted researchers to consider new possibilities, especially biological treatments in mild conditions through the use of microorganisms producing keratinases which will facilitate the disruption of disulfide bridges of keratins (Papadopoulos et al., 1986). It has also been shown that keratinases produced can have a variety of applications, particularly in production of animal feed (Suntornsuk and Suntornsuk, 2003), in the detergent (Gessesse et al., 2003), leather, textiles and wool industries (Alexandre et al., 2005).

The investigations carried out (including isolation, identification and characterization of keratinase-producing microorganisms) have shown that the soils of keratin waste dumpings constitute a reservoir of microbial strains with potential keratinolytic activities of which the most efficient are the species of the genus Bacillus; followed only by species of fungi (Mehta et al., 2013; Vidmar and Vodovnik, 2018). However, although several keratinolytic microbial strains have already been isolated worldwide, their effectiveness varies, according to the ecosystems in which they are isolated from and strain specific keratinolytic capacity. On this basis, the search of new keratinolytic microbial strains remains a challenge. In the Cameroonian ecosystem, such studies have not been done and the potential for finding new and even better bacterial strains for chicken feather degradation needs to be investigated. The aim of this study was to screen Cameroonian ecosystem where keratinous waste are dumped, to find, isolate and characterize bacteria having high ability to degrade the keratinous wastes, especially chicken feathers.

MATERIALS AND METHODS

Chicken feathers samples were collected at a poultry processing plant in the Marza city (Ngaoundere, Cameroon). Soil samples were separately taken with sterile spatula at 5 cm depths from the surface of the soil of five different sites: the small market of Ngaoundere town (01 sample) and from municipal (04 samples), Gada-bini (02 samples) and Manwi (01 soils sample) slaughterhouses, Marza city (poultry processing plant)) and transported in sterile glass jars to the laboratory (Table 1). The selected sites are the main slaughtering points of Ngaoundere where the huge quantities of keratin-rich waste (wool, horns, hooves, feathers) are generated and dumped onto the soils.

Chicken feather processing and feather meal preparation

Chicken feathers were washed thoroughly with distilled water, oven-dried (45°C) until constant mass and characterized for their chemical composition using standard methods: proteins (Devani et al., 1982), fat (Bourely, 1982) and ash (A.O.A.C method, 1975). One part of the dried feathers was cut into short fragments of 5 cm to be used in the basal cultivation medium. Another part was grinded in a blender to fine powder, sifted (Φ = 1 mm) and used as a the source of carbon and nitrogen in the preparation of Feathers Meal Agar (FMA) (composition ($g.L^{-1}$): 0.5g of NH_4Cl, 0.5 g of NaCl, 0.3 g of K_2HPO_4, 0.4 g of KH_2PO_4, 0.1 g of MgCl-$6H_2O$; 0.1 g of yeast extract, 15 g of agar and 15 g of feather meal).

Isolation and screening of keratinolytic bacterial strains

For the isolation of feather-degrading bacteria, 10 g of different soil samples from each location were suspended in sterile saline water and were shaken for 30 min (150 rpm). Serial dilutions were made up to 10^{-6}. A volume of 0.1 ml of the different dilutions was spread on the surface of sterile FMA and incubated at 37°C for 48 h. Different bacterial colonies isolated were purified and aseptically inoculated for primary screening of proteolytic activity onto sterile Skim Milk Agar (SMA) (containing: peptone 1%, NaCl 0.5%, yeast extract 0.3%, agar 10%, sterile skim milk powder 10%). After incubation at 37°C for 24 h, colonies were examined for the formation of a clear halo (hydrolysis zone of casein) around their growth and the proteolysis ratio (PR) which is proportional to the amount of proteases released by the microbial cells into the medium during the incubation was calculated (Habbeche et al., 2014) as follows:

$$PR = \frac{\text{diameter of the halo}}{\text{diameter of the colony}}$$

The strains showing a clear halo onto SMA medium were selected

Table 1. Soil samples description.

Sampling sites	Soils sample nature		Sample code
Municipal slaughterhouse	Soil	Contains residues of wool and flesh	A
		Deposit of beef hooves	B
		Deposit n°1 of beef horns	C
		Deposit n°2 of beef horns	D
Small market	Soil	Deposit of chicken feather	E
Manwi slaughterhouse	Soil	Deposit of wool, flesh and animal excrement	F
Gada-Bini slaughterhouse	Soil	Deposit of beef horns	G
		Deposit of beef hooves	H

to test their ability to hydrolyze also the keratinaceous keratin feather in FMA. Once FMA was prepared, it was poured into the sterile Petri dishes and inoculated by central stitching with the previously selected proteolytic strains. After incubation at 37°C for 4 days, the formation of a clear halo around the colonies was examined. The formation of a clear halo is an indicator of keratinolytic activity and was confirmed by evaluation the degradation rate after culture in Basal Salt Medium (BSM) (composition $g.L^{-1}$: 0.5 g of NaCl, 0.2 g of $MgSO_4$, 0.22 g of $CaCl_2$, 0.3 g of K_2HPO_4, 0.4 g of KH_2PO_4, 0.5 g of yeast extract). Residual feathers remaining in the culture broth were collected by filtration through filter paper (Whatman no. 1) after 8 days of incubation and drying (45°C) until constant weight. The degradation rate was estimated as the ratio of residual weight to that of the initial weight (Haggran, 2014).

$$Degradation\ rate\ (\%) = \frac{(\mathrm{TF} - \mathrm{RF}) \times 100}{\mathrm{TF}}$$

Where TF is the feather weight before bacterial activity and RF is the residual feather weight.

Identification of the high feather-degrading isolate

Bacteria were partially identified based on morphological and biochemical tests described in Bergey's manual of systematic bacteriology (Bergey's et al., 1974).

Keratinase production

For enzyme production, the mixture of 2.5 g of whole feather and 250 ml of BSM in Erlenmeyer was inoculated with 5% (v/v) of the inoculum 10^6 $CFU.mL^{-1}$ of the selected bacteria (with the high feather-degrading potential), incubated at 37°C under stirring (150 rpm) up to 08 days. At 24 h intervals, a volume of 15 ml of culture broth was withdrawn, centrifuged at 4°C (5000 g for 20 min) and the supernatant containing crude extracellular keratinase blend was collected. The pH values of culture broths, the keratinolytic activities and the concentration of soluble proteins in the supernatants were investigated.

Effect of pH and temperature on bacterial keratinase activity

The effect of pH on keratinase activity was studied by adjusting the initial pH of the culture broth (BSM: Basal Salt Medium) with a solution of 1 N NaOH at different pH (pH 6, 7, 8, 9 and 10), followed by the inoculation with 5% (v/v) of 10^6 $CFU.mL^{-1}$ and incubated at 37°C in an incubator under stirring (150 rpm) for 8 days. The optimum temperature for keratinolytic enzyme activity was determined by evaluating the enzyme activity at the optimum pH at different temperatures (25, 37 and 45°C) using the same culture broth and incubated up to 8 days.

Assay of Keratinase activity

Soluble keratin was prepared using the modified protocol of Wawrzkiewicz et al. (1987). The powder of feather (10 g) was mixed with 500 ml of Dimethyl Sulfoxyd (DMSO). The mixture was heated at 100°C for 120 min under reflux. Soluble keratin was then cold precipitated at 4°C for 2 h by adding 1000 ml of cold (-20°C) acetone, centrifuged at 4°C (5000 g for 20 min). The precipitate obtained was washed thoroughly, thrice, with distilled water, and dried at 40°C in a vacuum dryer. The dried precipitate (1 g) was dissolved in 20 ml of 0.05 N NaOH and the pH was adjusted to 8 using 0.1 N HCl. The resulting solution was finally diluted to 200 ml with 0.05 M Tris-HCl buffer pH 8 and used as keratin solution.

The keratinolytic activity was evaluated by mixing 1.0 ml of crude enzyme solution with 1 ml of keratin solution at 50°C in a water bath for 10 min. The reaction was stopped by adding 2.0 ml 0.4 $mol.L^{-1}$ trichloroacetic acid (TCA) and the mixture centrifuged at 4°C (5000 g for 20 min). The absorbance of the supernatant was measured at 280 nm against a control prepared by incubating the enzyme solution with 2.0 ml TCA without the addition of keratin solution. One unit of keratinolytic activity was defined as an increase of corrected absorbance of 280 nm (A280) (Gradisar et al., 2005) with the control for 0.01 under the conditions described above. The enzyme activity was calculated as follows (Cai et al., 2008):

$$U = \frac{4 \times \mathrm{n} \times \mathrm{A280}}{0.01 \times 10}$$

Where n is the dilution rate; 4 is the final reaction volume (ml) and 10 is the incubation time (min).

Protein measurement

Soluble proteins were measured by the method of Lowry et al. (1956) with bovine serum albumin (BSA) as a standard.

Statistical analysis

The results obtained were expressed as mean ± standard deviation. The DUNCAN test was performed using STATGRAPHICS Centurion XVI to compare the means with significant differences

Table 2. Chemical composition of feather.

Component	Content (%)
Proteins	76.44 ± 3.42
Fat	0.46 ± 0.01
Ash	1.58 ± 0.14
Dry matter	86.51 ± 0.21

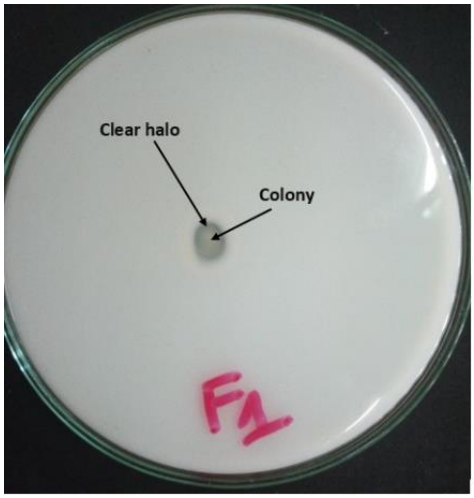

Figure 1. Petri dish with colony (F1) presenting zone of clearance on SMA.

and the correlation tests to measure the links between the different variables.

RESULTS

The chemical composition of chicken feathers waste used in this study (proteins, fat and ash content) are presented in Table 1. Proteins appear as a major compound of feather with a value of 76.44%. Ash and fat content respectively represented 0.46 and 1.58%. The chemical composition of chicken feather indicated that it is a proteinaceous substrate, which can constitute a principal source of carbon and energy for many microorganisms. Therefore, these microorganisms do not require other C- and N- nutrients for growth in laboratory culture (Table 2).

Bacterial isolates with feather-degrading potential

A total of fifteen bacterial colonies (A1, B3, B5, C2, C3, D1, D2, E2, E4, F1, F2, F3, F4, H1, H2) were found to have a clear halo on SMA medium (Figure 1) and were considered as proteolytic strain. Their proteolysis ratio was ranged between 1.05 and 3.0 with four strains (D1, D2, E2 and F1) showing higher proteolysis ratio (total weight as compared to degraded weight) which are presented in Table 3.

The isolates that showed higher proteolysis ratio were screened for their capacity to degrade feather. Three isolates namely D1, D2 and F1 were able to use feather as carbon and nitrogen sources and were thus able to grow on FMA and degrade feather in submerged fermentation (in BSM) (Figure 2). Out of the three isolates, D2 have presented the highest degradation rate (Table 3) and was selected for further analysis (Table 4).

Characteristics of the feather degrading isolate D2

According to biochemical parameters, isolate D2 belongs to the genus Bacillus (Table 5).

Keratinolytic activity

Keratinolytic activity and growth of the isolate D2 was investigated during cultivation in BSM containing feather as unique source of carbon and nitrogen (Figure 3). The

Table 3. Proteases producing strains and their proteolysis ratio on SMA.

Location	Isolate	Proteolysis ratio
Municipal slaughterhouse	D1	2.67
	D2	2.67
Small market	E2	2.0
Manwi slaughterhouse	F1	3.0

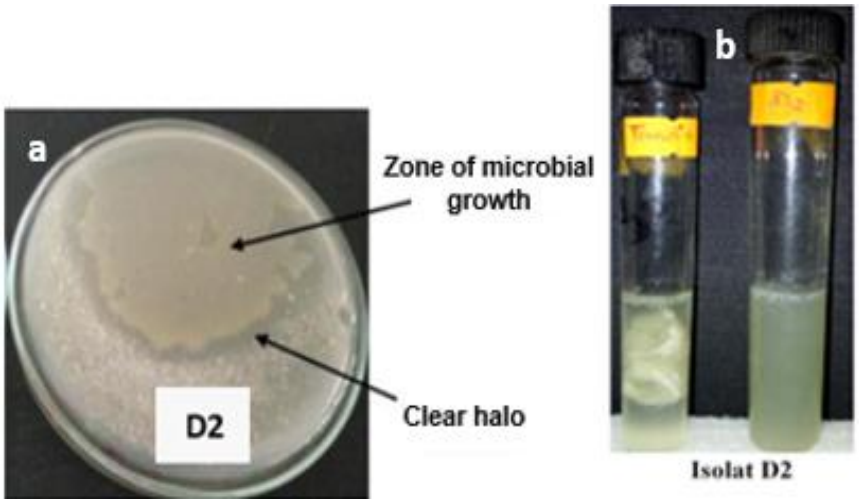

Figure 2. Petri dish with colony (D2) presenting a clear halo on FMA (a) and its illustration of feather degradation in BSM (b).

Table 4. Feather degradation rate of the isolates D1, D2 and F1.

Strain	Degradation rate (%)
D2	71.11 ± 3.05^{a}
D1	57.78 ± 2.81^{b}
F1	44.44 ± 3.85^{c}

Means with different letter in superscript are significantly different at $p < 0.05$.

keratinolytic activity increased during the 120 h of cultivation and showed the peak (13.76 $U.mL^{-1}$) at the stationary microbial growth phase. During the production of keratinases, we also observed an increase in pH of the culture broth during the 144 h which decreases after this time (Figure 3). Likewise, the amount of soluble proteins released in the medium during the incubation increased up to 4.54 $mg.mL^{-1}$ during the 144 h of incubation and decreased later on (Figure 3). The keratinolytic activity of isolate D2 was maximal at pH8 and at 45°C (Figure 4). As for her bacterial growth, it was maximal at pH 8 and at 37 to 45°C.

DISCUSSION

A screening approach was employed to obtain feather-degrading bacteria able to produce keratinase. Three isolated strains obtained from two locations were able to grow on FMA and degrade feather in BSM. Among these strains, the isolate D2 showed the highest feather degradation efficiency. The degradation rate has been proposed by several authors as an indicator positively correlated to the keratinolytic activity of a microbial strain (Veenayohini and Sangeetha, 2016; Bach et al., 2015). The characterization revealed that isolate D2 is Gram-

Table 5. Morphological and biochemical characteristics of isolate D2.

Morphological characteristics		
Form		Rod
Gram stain		Positive
Biochemical characteristics		
Motility		+
Oxidase		-
Catalase		+
Casein hydrolysis		+
Citrate hydrolysis		-
Urea hydrolysis		+
H_2S production		-
O_2 requirement		+
Fermentative test		Heterofermentative
Sugars fermentation	Lactose	+
	Saccharose	+
	Glucose	+
	Fructose	+
	Galactose	+
	Arabinose	+
	Xylose	+
	Ribose	+
	Mannose	+
	mannitol	+

+ = positive; - = negative.

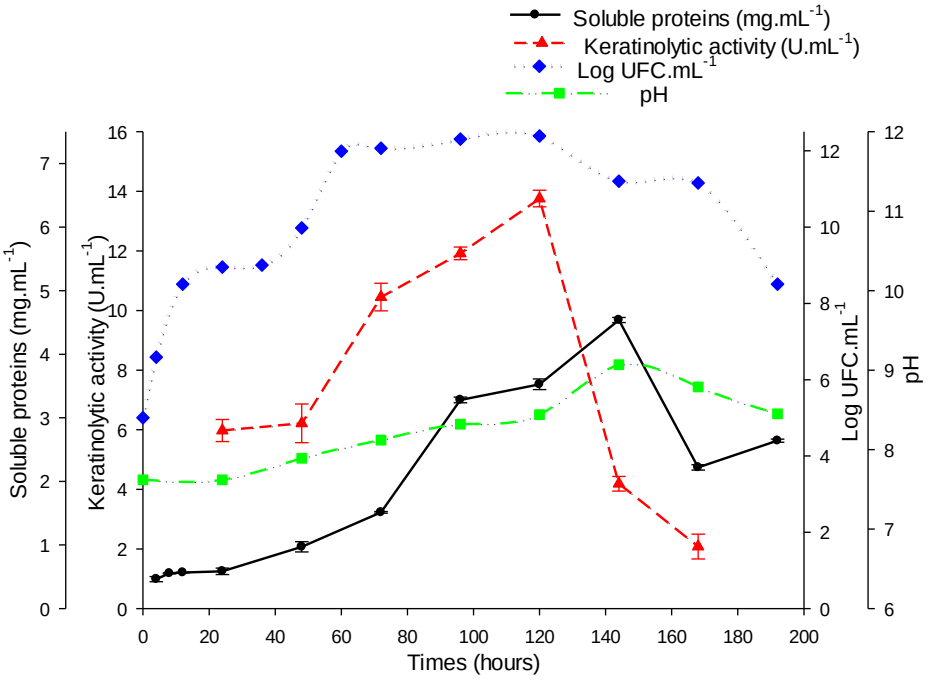

Figure 3. Keratinolytic activity, microbial growth, soluble proteins and pH changes during the degradation of feathers by the isolate D2 (pH 8, 37°C).

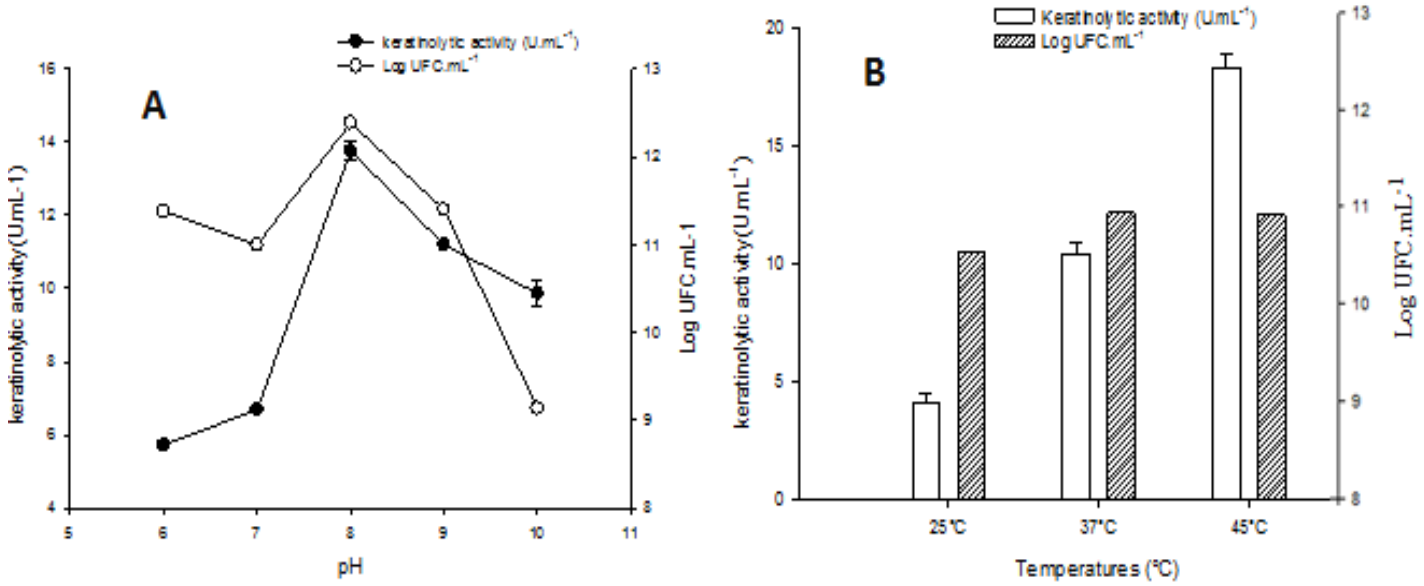

Figure 4. Effect of pH (A) and temperature (B) on Keratinolytic activity and growth of the isolate D2.

positive Bacillus sp. This result is similar to those reported by several authors who showed that Gram-positive bacteria, including Bacillus, Streptomyces can hydrolyze keratin (Gupta and Ramnani, 2006). Likewise, species of Bacillus is considered as the best producer of keratinase ((Govinden and Puchooa, 2012).

The increase in pH observed during the culture of Bacillus isolate D2 resulted from the release of alkaline molecules such as ammonia (NH_3^+) by deamination of peptides and amino acids. These are products of the keratinaceous feather protein degradation (Wilkesman and Kurz, 2009). These results corroborate with those of De Azeredo et al. (2006) and Kaul and Sumbali (1997), who showed that the increase in pH during culture is a consequence of the use of amino acids as sole source of carbon, since nitrogen has to be eliminated which involves deamination reactions and the keratinolytic potential of microorganisms especially Bacillus sp. However, the decrease in the pH during the culture after 144 h could result from the production of metabolites such as organic acids.

Many studies reported that most bacteria and fungi species produced keratinases in a large range of pH from 5 to 13 and the production is optimum at pH 7-8 (Sahoo et al., 2012; Gupta and Ramnani, 2006). The optimum temperature of keratinase production by most Bacillus strains has also been reported to be in the range of 30 to 80°C (Gupta and Ramnani, 2006). Similarly, the keratinolytic activity of isolate D2 was maximal at pH 8 at 45°C. Enzymes with optimum activity at alkaline pH and high temperature have advantages in application, both in degradation of chicken feathers, for production of animal feed and for management of poultry waste, as well as in leather industry (Godbole et al., 2017).

Conclusion

The soils of keratinous waste dumping in Ngaoundere have been shown to be a good source for isolation of microbial strains with keratinolytic activities. From the strains isolated, the Bacillus isolate D2 has its maximum keratinolytic activity in alkaline conditions (pH 8) and at rather high temperatures (optimum at 45°C). The strain D2 needs to be fully characterized about its taxonomic position, specifically in relation to relatedness to other Bacillus species known to be keratinolytic, such as *B. licheniformis*. If the D2 isolate appears to be novel, the following next two investigations would be highly interesting: Elucidation (at molecular level) of the enzyme composition of the D2 enzyme secretome; and testing of the end product of enzymatic (D2) bacterial degradation of the keratinaceous feather biomass for its nutritional value (in non-ruminant species, e.g. pigs). Furthermore, it would be of interest to adjust the protocol for the least energy requiring pretreatment and to test the resulting processing (pretreatment and microbial/enzymatic degradation) under upscaled conditions.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Mangroves render several ecosystem services globally, one of which is pharmacological products. This study was done to verify whether *Acrostichum aureum*, a species of mangrove, common in the Niger Delta has anti-bacterial property. Several leaf samples were plucked from the trees at Eagle Island and placed in a cooler and sent to the lab for further analysis. The leaves were dried and ground into fine powder with manual grinding machine and 100 g of the powdered sample was measured and placed in 1000 ml of each of the extraction solvents (hot water and methanol). Five different concentrations (32.25 mg/ml, 65.5 mg/ml, 125 mg/l, 250 mg/l and 500 mg/l) were made from each of the extracts using dimethyl sulfoxide (DMSO). Furthermore, bacterial species (*Escherichia coli*, *Salmonella paratyphi*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*) were isolated and a stock of each sample was made in agar slant and stored in the refrigerator at 4°C. Mueller Hinton Sensitivity Agar (MHA) medium was prepared in agar and triplicate discs of each of the concentrations made from the two extracts were placed on the medium (MHA). The zone of inhibition in diameter in all plates was measured and analyzed statistically using R statistics. The result revealed that there is a significant difference in the growth of the microbes on the different concentrations of *A. aureum* ($F_{4, 100} = 4.02$, $P = 0.01$). *A. aureum* had higher effect on *E. coli* and *S. aureus*. Similarly, the higher the concentrations of the extracts the more effective it is in controlling the bacteria. Finally, the study revealed that *A. aureum* has antibacterial properties that can be employed in drug production to treat common diseases prevalent in the region.

Key words: Antibiotics, bacteria, Niger Delta, mangrove, microbial species, medicinal products.

INTRODUCTION

Mangroves are herbaceous plants that grow in coastal areas in the tropical zones of the world. They are found in several countries such as Indonesia, China, Bangladesh, United States (Florida), and Australia (Kathiresan and Bingham, 2001). In Africa, mangroves are found in Nigeria, Cameroun, Ghana, and most West and Central African countries (Spalding et al., 2010; Adams and Rajkaran, 2021). However, the mangroves of Nigeria are the largest in Africa and the third largest in the world (Numbere, 2018a). Nigeria's mangroves are concentrated

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in the southern part of the country called the Niger Delta. Although, the population of mangroves are declining because of deforestation caused by exploration and urbanization activities and use of their stems for firewood. There are over hundred species of mangroves globally, but in the Niger Delta region the species commonly found are red (*Rhizophora* species), black (*Avicennia germinans*), white (*Laguncularia racemosa*), and golden leather fern (*Acrostichum aureum*) mangroves (Numbere and Camilo, 2018). Mangrove ferns are often found in sandy soil away from the river edge towards upland areas or areas with low river tidal height. They usually grow in few bunches of forest stands that are not greater than 1.8 m tall and 3-4 m wide par stand. They are also indicators of a recovering site. Mangroves provide numerous ecosystem services that are useful to humans and the environment such as air purification, food, aquaculture, and medicine. Mangrove parts have pharmacological properties that are of medical importance. For instance, a species of black mangrove twigs found in Asia (*Avicennia marina*) has antimicrobial properties against *Mycobacterium* species, *Staphylococcus aureus* and *Candida albicans* (Han et al., 2007). Similarly, leaf extracts from *A. marina* displayed antimicrobial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Salmonella aureus* (Devi et al., 2015). *A. marina*, a mangrove species, has cytotoxic activity against cancer cells (Eswaraiah et al., 2020). Other properties include mosquito repellence, anti-bacteriophage, anti-viral, anti-arthritis and antinociceptive properties. Extracts from the leaves and roots of *A*. *aureum* is an antioxidant, and has antibacterial and anti-inflammatory properties (Thomas, 2012). It is also shown that *A. aureum* can be used as an analgesic (that is, pain treatment) when ethanol extract of its products was used on mice (Khan et al., 2013). It has also been demonstrated that extracts of *A. aureum* has inhibitory effect on cancer cells (Dai et al., 2005; Uddin et al., 2011). Further studies had shown that *A. aureum* has wound healing and anti-diarrheal qualities. Similarly, *A. aureum* is shown to have antiviral and anti-parasitic properties (Devi et al., 2015). They also have wound healing properties when liquid from their leaves is squeezed on the surface of open wounds; this was tested on rats by Kimura et al. (2017).

A. aureum is a mangrove species found in many locations across the Niger Delta, but because of the ignorance in its pharmacological properties only few studies had been conducted to test its medicinal properties (Numbere, 2018b). Field observations show that their population is declining rapidly because of deforestation activities such as sand mining, urbanization, and oil exploration (Numbere, 2021). It is thus, important to investigate the potentials of developing drugs from the parts of this species to solve public health problems. The major purpose of the study is to determine whether *A. aureum* extract has anti-bacterial property.

This study is conducted to fill the data gap on the use of mangrove parts for the manufacture of medicinal products in the Niger Delta. This is because there are limited studies done to test the anti-bacterial properties of the mangrove species, *A. aureum.* The study will thus provide data for future studies that will be helpful in the conversion of mangrove parts into medicinal drugs. The objective of this study, therefore, includes the following: (i) to determine if different concentrations of *A. aureum* extract influence the zone of inhibition (diameter) of bacterial species, (ii) to determine if there is a significant difference in the zone of inhibition, and (iii) to determine if different solvents (that is, water and methanol) influence the zone of inhibition of bacterial species.

MATERIALS AND METHODS

Description of study area

The leaf samples were collected from a section of a deforested and sand filled mangrove forest at Eagle Island in the Niger Delta (N04°47; E006°58) and sent to the laboratory for identification and processing. The location is a dense mangrove forest that was cut and later used as a dumping ground for dredged sand. The area experiences rainfall in every month of the year with an annual mean of 3567.4 mm (Gobo, 2001). The mean monthly temperature ranges from 28 to 34°C. The adjoining river is an estuary with a salinity range of 1.45 to 1.62%. The soil is sandy to muddy (swampy) and grades from white to dark brown in color. The soil pH ranges from 6 to 7. *A. aureum* grow at the sandy side of the forest or shallow river zone.

Description of bacterial species

The shape and activities of the different bacteria species used for the study are shown in the following.

E. coli

It is a large and diverse group of bacteria species that are Gram negative. It is a facultative anaerobe that is rod-shaped (Eckburg et al., 2005; Tenaillon et al., 2010). It is usually harmless, but pathogenic strains can cause illnesses (Vergara et al., 2020). It causes diarrhea, urinary tract infections, pneumonia, and respiratory illnesses. *E. coli* is a common species that is found almost everywhere such as meat, vegetable, and other food sources; it is also part of the normal gut flora and found predominantly in fecal matter.

Salmonella paratyphi

This is a genus of rod-shaped (bacillus) Gram negative, and of the family Enterobacteriaceae. It is non-spore forming and motile. It is a facultative anaerobe which produces ATP with oxygen (Fàbrega and Vila, 2013). *S. paratyphi* causes typhoid fever (Ryan and Ray, 2004).

Staphylococcus aureus

This is a Gram-positive cocci (round-shaped) bacterium. It is a

member of the microbiota of the body, mostly found in the upper respiratory tract and on the skin (Todar, 2010). It is a facultative anaerobe that grows without the presence of oxygen (Masalha et al., 2001). Humans are regarded as carriers of *S. aureus* (Tong et al., 2015). It causes skin infections, respiratory diseases, and food poisoning (Todar, 2010).

P. aeruginosa

This is an encapsulated Gram-negative and rod-shaped bacterium that can cause disease in plant, animal and humans. It has strong anti-biotic resistance ability and ubiquitous in the environment. It is an opportunistic organism that invades during a preexisting disease condition such as during cystic fibrosis disease. It can colonize critical part of the body such as the lungs, urinary tract, and the kidney (Wagner et al., 2016).

Collection and preparation of plant sample

Fresh leaf samples of *A. aureum* were collected in a mangrove forest at Eagle Island in April 2020. The leaves were sent to the laboratory and identified by a plant taxonomist. Large quantities of the fresh leaves were air-dried without exposure to direct sun light. The dried leaves of *A. aureum* were cut into small pieces and ground with manual grinder (Corona). The powder of the plant sample was packed in small transparent polyethylene bags and stored in cool, dry cupboard for further use.

Extraction procedure

The powdered leaf sample was extracted using hot water and methanol solvents. Methanol solvents were used because it is bipolar and dissolve in lipids. It is widely used in studies for drug purification (Zhao et al., 2020) and formation of solvate (Yuan et al., 2020), whereas water is a universal solvent that is generally used in the laboratory. The protocol proposed by Mikayel et al. (2017) for the extraction of medicinal plant was followed. In each extraction process, 100 g of dry powder sample of *A. aureum* leaves was soaked in 1000 ml of each solvent with ratio of 1:10 (W/V) of the plant material to solvents. The mixtures were extracted up on shaking with DREHZAHL electronic magnetic stirrer (IKAMAG REO 79219 Staufen, Germany) for 48 h. The extract mixture from the hot water (100°C) was filtered and concentrated by heating the filtrate on a hot plate while the filtrate from the methanol extract was kept standing at room temperature (26°C) for 3 days to eliminate the extraction solvent. Extracts of hot water and methanol yielded 1.8 g (1.8%) and 2.0 g (2.0%), respectively. This occurred after the removal of the extraction solvents and leaf shaft.

Preparation of sensitivity discs from plant extracts

The disc diffusion method was used for this study. With the aid of a micropipette, five different concentrations of the plant extract solutions (500, 250, 125, 62.5 and 31.25) and one control solution using water were prepared with a concentrated solution of Dimethyl sulfoxide (DMSO) for each of the extracts. Sterile discs made using Whatman No. 1 Filter Paper, of 8-mm diameter were embedded with 20 μl/disc of the various concentrations made from plant extracts. The discs were dried in the incubator at 40°C.

Isolation of microorganisms

The test bacteria used for this study were isolated from patients' samples in a medical diagnostic laboratory (Cheesbrough, 2000). The characterization of these bacteria was done using culture dependent techniques and biochemical tests results (Pelczer et al., 2003). The culture dependent technique was used for this study because it is less expensive as compared to the molecular technique. The culture technique is widely used for comparing and isolating individual microorganisms, which are further identified using morphological features, microscopy, and biochemical tests. The four bacteria characterized and used were *E. coli*, *S. paratyphi*, *S. aureus*, and *P. aeruginosa*. The isolated microorganisms were sub-cultured on nutrient agar slants, incubated at 37°C for 24 h and stored in a refrigerator at 4°C. Nutrient agar is selective but classified as general purpose because it allows the growth of many different non-fastidious species such as the bacteria used for this study. To derive the bacterial count using the McFarland turbidity test would have been better but was not used because of the cost implication. However, future studies would consider using this method.

Antimicrobial activity test of plant extracts

The measurement of zone of inhibition of the selected bacteria by the extracts (hot water and methanol) of *A. aureum* leaves was achieved by following the disc diffusion method illustrated by Bauer et al. (1966) and Barry et al. (1979). After sterilization of Mueller-Hinton Sensitivity Agar (MHA) medium, it was poured into sterile Petri dishes and allowed to solidify. Homogeneous day-old bacterial suspensions of the selected bacteria prepared in test tubes with sterile nutrient broth were used. With the aid of sterile cotton swab, each bacterial culture was inoculated separately on the surface of dry, sterile, solid MHA under aseptic conditions. Then the impregnated discs with 20 μl/disc of the various concentrations made from the two extracts were placed on the surface of MHA plates inoculated with a microbial culture. Each plate contained triplicate disc of each of the concentrations and standard antibiotic discs of chloramphenicol, and gentamycin which served as positive control (Figure 1). After 24 h of incubation, the plates were observed for the zone of inhibition and the diameter of the inhibition zone was measured in millimetre. There were four bacterial species (n=4) and five extracted concentrations (n=5) making a total of 20 replicates. Therefore, the number of replicates for both ethanol and water solvents were 40 (that is, n = 2×20).

Statistical analysis

An analysis of variance (ANOVA) was done (Logan, 2010) to determine whether there was any significant difference in the growth of microbes in diameter on different concentrations of *A. aureum* following the example of Quinn and Keough (2002). A Tukey HSD test was done to determine where the significant difference lies (Logan, 2010). Logarithmic transformation of the data was performed to meet assumptions of normality and homoscedasticity (Logan, 2010). Similarly, a post-hoc Tukey's HSD test was done to investigate pair wise mean differences between groups. All analyses were performed in *R* statistical environment, 3.0.1 (R Development Core Team, 2014).

RESULTS

Effect of different concentrations of *A. aureum* on microbial zone of inhibition

The ANOVA result indicates that there is a significant

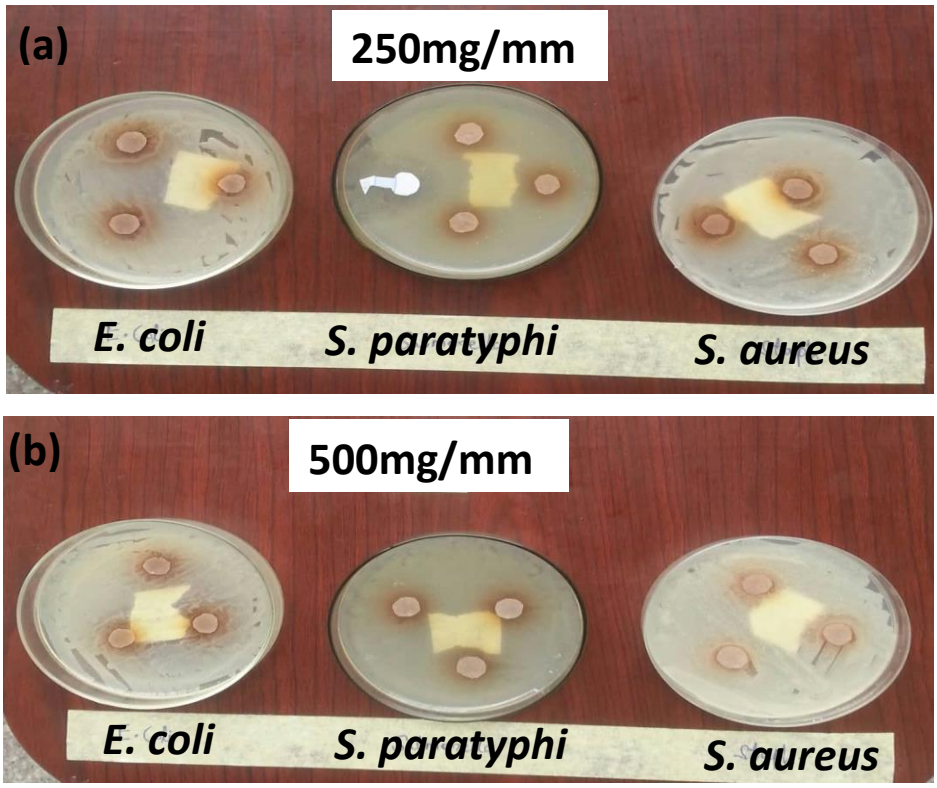

Figure 1. Culture plates with zones of inhibition (diameter in millimetre) for concentrations of 250 mg/mm and 500 mg/ml with hot water used as solvent.

difference in the zone of inhibition of the microbes on the different concentrations of *A. aureum* ($F_{4, 100}$ = 4.02, P = 0.01) (Figure 2 and Table 1). Similarly, the Tukey HSD test shows that the greatest difference in the zone of inhibition was observed in *A. aureum* concentrations of 62.5 and 500 and 62.5 and 250 mg/ml, respectively at P < 0.05. *A. aureum* concentrations of 500 and 250 mg/mm had the largest zones of inhibition and thus, the greatest inhibitory effect on all bacterial growth (Figure 1). Among the four bacterial species used for the study, *A. aureum* extract had the highest inhibitory effect on *E. coli* and *S. aureus* (Table 1) whereas the lowest inhibitory effect was recorded on *S. paratyphi*.

The result shows that higher concentration of *A. aureum* was more potent in inhibiting the growth of the microbes. There is thus a reduction in diameter as the concentration of *A. aureum* extracts decreases. It shows that *A. aureum* concentration of 500 mg/ml has the average highest zone of inhibition between the concentrations.

Secondly, the ANOVA result indicates that there is a significant difference in the zone of inhibition (diameter) between the microbial species ($F_{3, 101}$ = 12.08, P = 0.001). Similarly, the Tukey HSD test shows that the greatest difference was observed between *S. paratyphi* and *E. coli* at P<0.05 being species that has the lowest and greatest impact of the *A. aureum* extract, respectively.

The effect of *A. aureum* extractive solvents on microbial zone of inhibition

The ANOVA result indicates that there is a significant difference in microbial zone of inhibition (diameter) between the two extracts (that is, methanol and water) used ($F_{1, 103}$ = 6.43, P = 0.01) (Figure 3). The concentrations of all *A. aureum* extracts prepared with methanol has higher zone of inhibition than those prepared with hot water (Table 2). Furthermore, the concentrations with the highest zone of inhibition were the methanol extracts of 500 mg/mm (11.08±0.58 mm) and 250 mg/mm (11.33±0.48 mm), whereas the least zone of inhibition was recorded for water extract for concentration 62.5 mg/mm (3.25±1.18 mm). The zones of inhibition for extracts of 31.25 to 125 mg/ml are statistically significant as compared to 250 and 500 mg/ml that are not statistically different as shown by the error bars (Figure 3).

DISCUSSION

Studies show that *A. aureum* contains flavonoids, phthalates, sterols and terpenoids, and has some anti-bacterial properties because of its strong effect on *E. coli.* However, this is contrary to a study by Lai et al. (2009)

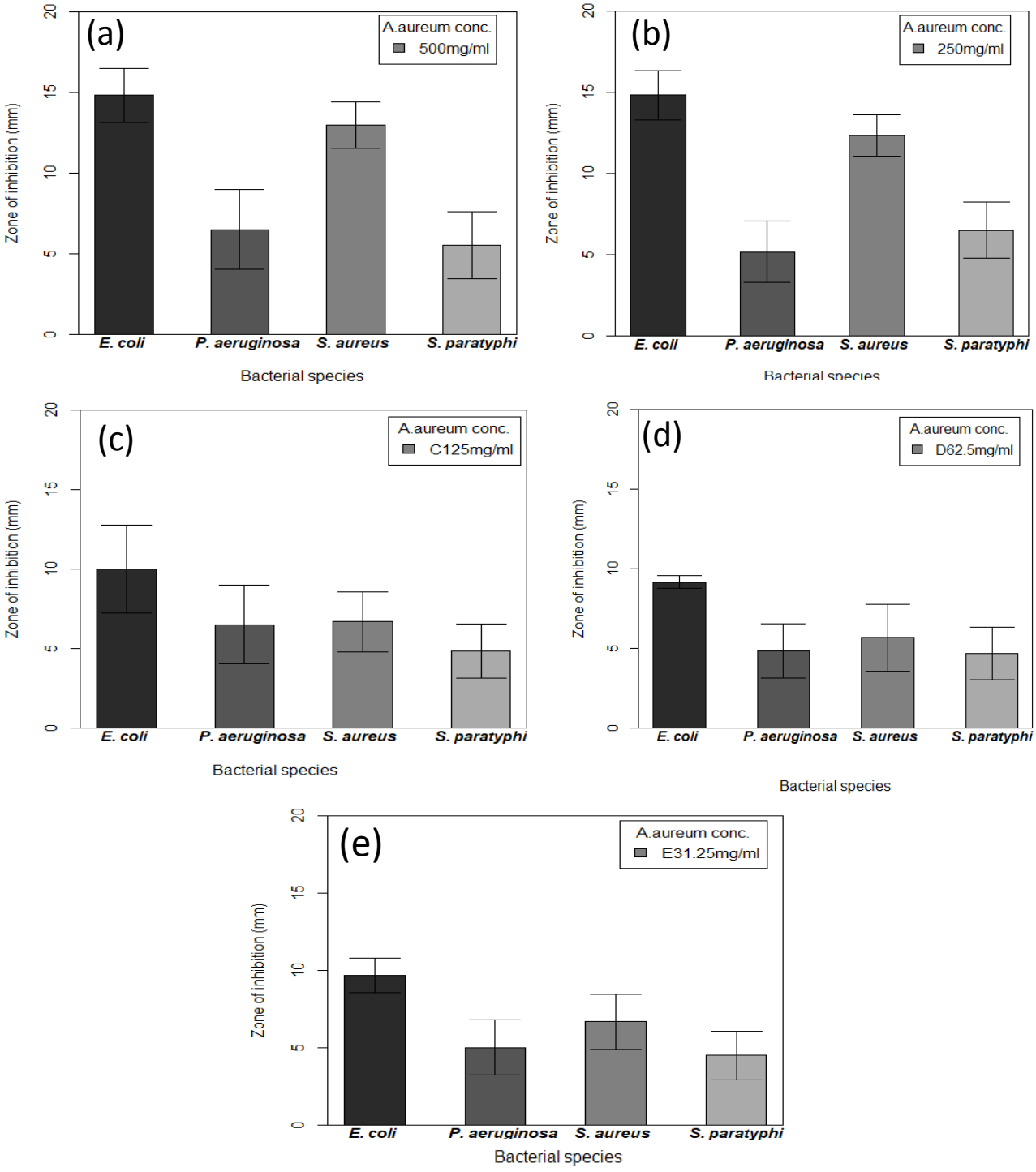

Figure 2. Zone of inhibition (mm) of bacteria species on different concentrations of *A. aureum* (a) 500 mg/ml, (b) 250 mg/ml, (c) 125 mg/ml, (d)62.5 mg/ml and (e) 31.25 mg/ml.

who showed that *A. aureum* has no anti-bacterial properties on some bacterial species such as *Micrococcus luteus*, *Bacillus cereus*, and *S. aureus*. The same study shows that the methanol extracts inhibited *Serratia marcescens* and *E. coli*. Furthermore, *A. aureum* extract has amino group and other chemicals that allows it to penetrate the cell walls of Gram-negative bacteria (Lode, 2001) making it a therapeutic agent (Nugraha et al., 2020). It is also a protein synthesis inhibitor and it inhibits enzyme reactions, and thus, controls the growth

Table 1. Mean zones of inhibition (mm) by bacterial species at different concentrations of *A. aureum* extracts ± 1 SE.

Bacterial species	Concentrations of *A. aureum* extracts (mg/ml)					P-value
	500 mm	250 mm	125 mm	62.5 mm	31.25 mm	
E. coli	14.83±1.68	14.83±1.52	10.00±2.76	9.17±0.40	9.67±1.12	0.01
S. paratyphi	5.50±2.08	6.5±1.75	4.8±1.72	4.7±1.65	4.5±1.56	
S. aureus	13.00±1.44	12.33±1.28	6.67±1.87	5.67±2.11	6.67±1.80	
P. aeruginosa	6.50±2.47	5.17±1.91	6.50±2.47	4.83±1.72	5.00±1.79	

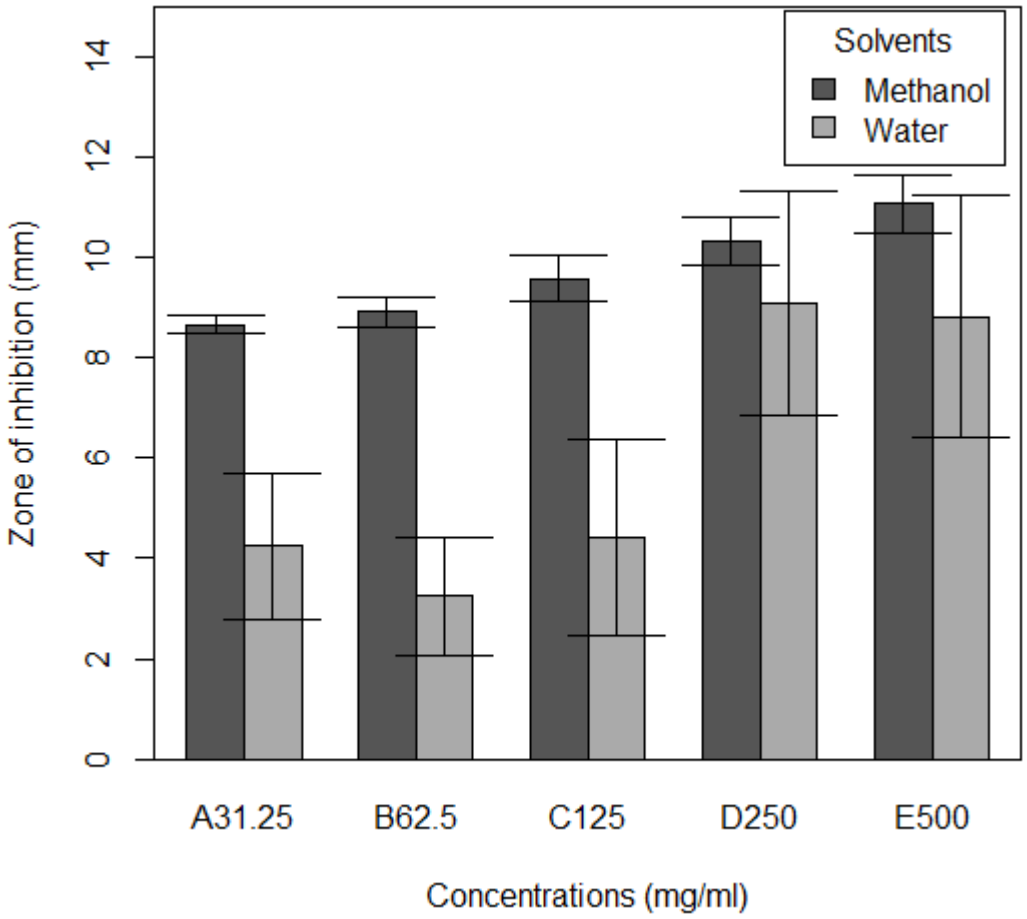

Figure 3. Effect of different solvents (that is, water and methanol) on bacterial growth on different concentration of *A. aureum*. Dark bars represent methanol while light bars represent water.

of microorganisms. *E. coli* is a Gram negative bacteria and the inhibition of its growth in the agar plates containing different concentrations of *A. aureum* is made possible because *A. aureum* can penetrate its cell wall and control its growth. Other studies used different species of *E. coli* to test the effectiveness of different antibiotics (Hernández-Porras et al., 2004). Some pathogenic bacteria also develop resistance against antibiotics, which makes them spread fast and difficult to control (May et al., 2009). Out of all the bacterial species *E. coli*, which is a Gram-negative bacteria had the highest growth inhibition by *A. aureum* extracts as compared to the Gram-positive bacteria (Figure 2 and Table 1). Martin (1995) reported that Gram negative bacteria are more resistant than Gram positive, but in this study the Gram-negative *E. coli* was the least resistant than the Gram positive. This can be attributed to the thin peptidoglycan layer of the cell wall of the Gram-negative bacteria. This layer as it grows eventually give way to a softer lipopolysaccharide outer membrane layer (Martin, 1995;

Table 2. Mean zone of inhibition of different extracts treated with two solvents, that is, water and methanol ± 1 SE.

Reagent	Concentrations of *A. aureum* extracts (mg/ml)					P-value
	500 mm	250 mm	125 mm	62.5 mm	31.25 mm	
Methanol	11.08±0.58	11.33±0.48	9.58±0.45	8.92±0.31	8.67±0.19	0.01
Water	8.83±2.42	9.08±2.24	4.42±1.95	3.25±1.18	4.25±1.46	

Guardabassi and Courvalin, 2001) that is easily penetrated, and its growth inhibited by the *A. aureum* extracts. Other studies have shown that the leaves of *A. aureum* has pharmacological properties, and contain anti-cancer, anti-microbial and anti-inflammatory agents that can be used to manufacture drugs (Badhsheeba and Vadivel, 2020; Ningsih et al., 2019). The lipopolysaccharide layer of the Gram-negative bacteria confers lower permeability than the Gram-positive bacteria (Arthur et al., 1996). Generally, antimicrobial agents attack basically three aspects of bacteria, namely, cell wall, protein, and nucleic acid biosynthesis. The effect of *A. aureum* on *E. coli* is because it acts on a single species as compared to extracts of other mangrove species that acts on many species called multiple spectrum effect (Helmerhorst et al., 1997). The current study also revealed that the higher the concentration of *A. aureum* extract the more effective it is in controlling bacteria, especially for *E. coli* and *S. aureus* (Figure 2). Higher concentrations of antibiotics have higher killing ability but must be checked regularly to ensure that it does not have adverse effect on humans when used as drugs to control diseases.

The methanol leaf extract of *A. aureum* had greater effect than the hot water extract, which is in line with studies done by Khan et al. (2013) who showed that ethanol leaf extract of *A. aureum* exhibited significant free radical scavenging activity with IC50 value of 42 µg/mL with higher effect on bacteria species. Similarly, Arora et al. (2019) showed that *A. aureum* has antimicrobial metabolites. Furthermore, other studies show that the methanol extract of *A. aureum* shows anti-cancer properties (Uddin et al., 2011). Results of previous and present studies show that there is high possibility of using *A. aureum* to manufacture medicinal products if the pure active ingredients of the extracts are tapped.

CONCLUSION AND RECOMMENDATION

This study shows that the mangrove species *A. aureum* has antibacterial properties, which means it can be used to produce antibiotics. It further revealed that *A. aureum* is more effective in eliminating *E. coli*, which is a major causative agent of food poisoning and many other diseases such as diarrhea urinary tract infections pneumonia, and respiratory illnesses prevalent in the Niger Delta region. There is thus a possibility of producing drugs with extracts of *A. aureum* if the extraction processes are effectively done to derive the undiluted active antibiotic ingredient. For example, micro-wave assisted extraction method (Mandal et al., 2007) can be used to extract better concentration that will give better antibiotic effect. For this study, the leaves alone were used for the extraction, but future studies will consider the use of other parts of the mangrove, such as root, bark, stem, and seeds to compare the parts with the best antibacterial properties. Finally, it was revealed that higher concentrates of *A. aureum* extracts (250 and 500 mg/ml) were more effective in controlling bacterial growth. Future studies will compare even higher concentration to determine the best threshold that will eliminate more bacterial species and in general other microbes.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Composition and balance of the analytical fractionation of desert date (*Balanites aegyptiaca* L.) seeds harvested in Senegal

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The desert date (*Balanites aegyptiaca* L.) can be one of the most common trees in Senegal. Thus, the objective is to carry out an analytical fractionation of these seeds to consider prospects for valuing the different constituents and a diagram of fractionation processes. The physical characterization of the seeds made it possible to know the fruit-seed and kernel-hull proportions. The chemical composition of the seeds shows that three main fractions represent 90% of its dry matter: The fibrous (68%), the lipid (14%) and the proteins (nearly 9%). The kernel is made up of 34.50% (80% of the lipid) and 28.75% of proteins (or 93% of the protein) while the hull is essentially fibrous, 90% contains 85% of the fibers. The high proportion of unsaturated fatty acids oleic and linoleic (73%) associated with those of saturated fatty acids (palmitic and stearic) in high proportions (27%) and the high contents of sterols (2.11 g/kg), and in tocopherols (512.40 mg/kg), make desert date oil an excellent food oil. On the other hand, it has qualities that make it suitable for use in various non-food applications. Proteins are made up of three main amino acids: glutamic acid (20.35%), arginine (14.42%) and aspartic acid (11.29%). The amino acid composition is close to that of oil seeds whose cakes are widely used in animal or even human food. Given their essentially lignocellulosic composition, the hulls obtained from shelling can be used as fuel or be extruded and used for the manufacture of composite agro-materials.

Key words: Desert date, *Balanites aegyptiaca*, seeds, analytical fractionation, proteins, lipids.

INTRODUCTION

Pollution concerns the high cost of fossil fuels and the depletion of non-renewable resources provide the impetus for scientists to explore other sources of energy and materials. Biofuels, as a source of renewable energy, are

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a good alternative to non-renewable fuels. Besides, the use of materials of biological origin, easily biodegradable and eco-compatible, offers an alternative to synthetic materials. Thus, exploring the potential of desert date seeds can be envisaged. The desert date (*Balanites aegyptiaca* L.) belongs to the Zygophyllaceae family which thrives in arid and semi arid regions of Africa, the Arabian Peninsula, and in the driest areas of Pakistan and India (Hall and Walker, 1991; Chapagain et al., 2006). Desert date is a species that generally lives on the plains up to 1000 m above sea level in areas where the annual average temperature varies between 20 and 30°C, and those where annual rainfall ranges from 250 to 400 mm (Schmidt and Joker, 2001). This species can be one of the most common in Senegal (Ndoye et al., 2004). The tree produces fruits at 5 to 7 years reaching a maximum in 15-25 years (Mohamed et al., 2002). The annual production of a tree is estimated to be about 100 to 150 kg (Elamin and Satti, 2013). It can live for 100 years including 75 years of fruiting. The fruit is a drupe 2.5 to 7 cm long and 1.5 to 4 cm diameter comprising a epicarp (5-9%), mesocarp (28-33%), endocarp (49-54%) and of the kernel (8-12%; Mohamed et al., 2002). The kernel may contain 30-60% oil and 20-30% protein (Aviara et al., 2005). Fruits are green or yellow and become smooth at maturity (Chothani and Vaghasiya, 2011). Desert date has many uses including food, medicinal or cosmetic with fruits, kernels and oil used for human consumption. Various extracts are used in traditional medicine, in wound healing and as a laxative but also to treat many diseases such as jaundice, yellow fever, syphilis, diarrhea, epilepsy, and hemorrhoid (Chaudhry and Khoo, 2004). *B. aegyptiaca* also has anti-inflammatory, anthelmintic, insecticide, antifungal, antibacterial, molluscicide properties and is used as an antidote against snake bites (Mohamed et al., 2002; Chothani and Vaghasiya, 2011).

The objective of the present study is to carry out an analytical fractionation of the desert seeds, to establish theoretical material balances and to propose diagrams of fractionation and possibilities of valorization of the extracts, by-products and raffinates obtained during the extraction.

MATERIALS AND METHODS

Plant

The plant material used in this work consisted of desert date seeds obtained from mature dry fruits harvested in the central and northern regions of Senegal. The seeds were dried with sun exposure at the open air and then in an oven at 40°C and the kernels and the hulls were separated by shelling.

Solvents and reagents

All chemical reagents, standards and solvents are of analytical (HPLC) grade (Sigma-Aldrich, France).

Dry matter

The dry matter content was determined according to French standard NF V 03-903. It is calculated after drying a sample of about 1 g in an oven at 103°C until constant mass.

Minerals content

The ash content was determined by measuring the mass loss of a sample through its incineration in a muffle oven, electrically heated at 550°C for 3 h (NF V 03-922). After incineration, white, light gray or reddish ashes are obtained, visibly devoid of charcoal. The sample was then cooled in a desiccator and weighed at room temperature.

Lipid extraction

The lipid content was determined by using the standardized soxhlet method (NF ISO 734-1) which consists of extracting the lipids contained in the matter with cyclohexane for minimum of 6 h. An amount of about 30 g of seeds was used. The soxhlet extractor is equipped at its base with a 250 mL flask into which 200 mL of solvent has been introduced. The oil used for the tocopherols analysis was extracted by cold centrifugation using cyclohexane.

B. aegyptiaca physicochemical properties

The physicochemical propreties were determined according to standardized methods: density (AFNOR T60-214), viscosity (ASTM D 445), flash point (ASTM D 93), freezing point, pour point (ASTM D 97), cloud point (ASTM D 2500), acid value (AFNOR T60-204), saponification value (AFNOR T60-206), iodine value (AFNOR T60-203), peroxide value (AFNOR T60-220), ash content (ASTM D 482), carbon residue content (ASTM D 189), sulphur content (ASTM D 4294), sediment content (ASTM D 4052) and water content (ASTM D 4052) (ASTM, 2011). However, the refractive index was measured at 25°C by direct reading with a refractometer ABBE RMT model (EXACTA + OPTECH France 77646 CHELLES France) while the calorific value was estimated using the following empirical relationship (Haidara, 1996):

Calorific value = 11380 - Iodine value - 9.15 × saponification value.

Fatty acids analysis

The fatty acid profile was determined by analysis of fatty acids methyl esters (FAME) in gas chromatography (GC) according to the French Standard NF ISO 5508 standard. The esterification was carried out in two steps, solubilization of the oil by tert-butyl methyl ether (TBME) and uploading trimethyl sulphonium hydroxide 0.5 M in methanol (TMSH). The analysis was performed in type GC 3800 equipped with a Varian CP-select column for FAME fused silica WCOT (length 50 m, internal diameter 0.25 mm, film thickness 0.25 µm) coupled with a flame-ionization detector (FID) heating the components at 250°C. The carrier gas was helium (flow rate of 1 mL/min). The injection was Split (1: 100 µL 1 250°C for 55 min). The temperature programming was 185°C for 40 min and then rose from 185 to 250°C at 15°C/min and finally 250°C for 10.68 min (analysis time 55.01 min). The standard used was the MGFA (SI) and the data was processed with Varian Star software.

Glycerides and triglycerides analyses

Analysis of glycerides and triglycerides were carried out after the

Table 1. Dimensions, masses, average proportions of fruit, seed, kernel and hull in the desert date (*B. aegyptiaca*).

Operation	Component	Length (cm)	Width (cm)	Mass (g)	Proportion (% DM)
Fruit-seed separation	Fruit	-	-	2.24	52.17
	Seed	2.27	1.27	2.04	47.83
Seed shelling	Kernel	1.58	0.80	0.56	27.67
	Hull	2.27	1.27	1.44	72.33

glycerides silylation by 50 µL of methyl imidazole with 1 mL N-Methyl-N-trimethyl silyl-Hepta Fluoro butyramide (MSHFBA). The analyses were performed by gas chromatography (Perkin Elmer) equipped with a CP Sil column 8CB Low Bleed MS Varian, length 15 m, internal diameter 0.32 mm, film thickness 0.25 µm. The injection was on column type 1 µL. The temperature program was 55°C for 0.5 min, then 200°C min^{-1} to 340°C and 340°C for 40 min. Helium was the carrier gas (column head pressure 15 psi). The injection into the oven was performed under the following conditions: 55°C for 0.5 min, 45°C min^{-1} to 80°C, 10°C min^{-1} up to 360°C and 360°C for 16 min. FID carried out detection at 365°C. The compounds were identified through comparison of the retention time with standards reference and the quantification was carried out by external calibration.

Sterols analysis

Sterols were analyzed on the unsaponifiable fraction after silylation by Methyl trimethylsilyl heptafluorobutyramide (MSHFBA + 50 µL 1 methyl imidazole). The analysis was performed by GPC (Perkin Elmer lane 2) coupled to an FID (365°C) and equipped with a column CPSil 8 CB (Varian) of length 30 m, diameter 0.25 mm and film thickness 0.25 µm. The injection was on column type (1 µL), the carrier gas was helium and the column head pressure was 100 kPa.

The injector temperature programming was 55°C for 0.5 min, then increase from 55 to 340°C at 200°C min^{-1} and stabilization at 340°C for 30 min. The temperature of the oven was 160°C for 0.5 min, then rose from 160 to 260°C at 20°C min^{-1} and stabilization at 260°C for 5.5 min then rise from 260 to 300°C at 2°C/min then maintaining the temperature for 10 min at 300°C finally a rise from 300 to 350°C at 45°C min^{-1} and stabilization at 350°C for 3 min.

Tocopherols analysis

The analysis of the tocopherols of desert date oil, using the standards of α, γ and δ-tocopherols by external calibration was performed according to EN ISO 9936. Exactly 10 mg of oil were diluted with 1 mL of cyclohexane. The sample was analyzed by HPLC Dionex type equipped with a Kromasil 100 column SIL 5 µ (250 × 4 mm) and a fluorescence detector (λex = 290 nm and λem = 317 nm). The eluent was composed of mixture isooctane/ isopropanol (99.5%/0.5%) at a flow rate of 1.1 mL/min.

Proteins analysis

The proteins concentrations were determined by the Kjeldahl method according to French standard NF V 18-100. It consists of determining the total nitrogen content in the sample to obtain an ammonium salt. The proteins concentrations was performed using an automatic device consisting of a Kjeltec 8400 Analyzer and Kjeltec 8420 Treader and consists of transformation by mineralization of organic nitrogen in the treated sample (400 mg) and also consists of an acid-base determination of inorganic nitrogen (ammonia).

Amino acids analysis

The amino acids dosage of the proteins of the desert date seeds was performed according to the European Standard. The analysis was performed by ion-exchange liquid chromatography using Biochrom 30+ equipped with a packed column, of the PEKK type, filled with cation exchange resin, under the following conditions: 20 to 99°C, use of pressure 24 to 150 bars, injection volume 1 to 5000 µL, and detection by photometer 440 to 570 nm.

Parietal constituents analysis

The method of Van Soest and Wine (1968) also known as ADF-NDF assay makes it possible to determine lignins, celluloses and hemicelluloses. It is based on the difference in solubility of the components. The Neutral Detergent Fiber (NDF) attacks and solubilizes all the compounds except the cellulose, hemicellulose and lignin. The first Acid Detergent Fiber (ADF) permanganate attack solubilized the compounds except cellulose and lignin. The second ADF attacks left cellulose only. These attacks are carried out in a device called a Tecator Fibertec M1017.

RESULTS AND DISCUSSION

Physical characteristics of the seeds

The physical characteristics obtained (Table 1) from 50 samples are in accordance with the data found in the literature. 100 kg of fruit can provide almost 48 kg of seeds. Manual shelling of the seed shows a very clear difference in the hull/kernel distribution. This result shows that shelling 100 kg of seeds will provide approximately 27.5 kg of desert date kernel.

Chemical composition of the seeds

Apart from many minority compounds, in particular from metabolic origin and which are valuable in traditional medicine, three main fractions represent 90% of the dry matter of the seeds. These fractions are the fibrous fraction (cellulose, hemicelluloses and lignin; 68%), the

Table 2. Fibers composition of whole seeds of desert date.

Parietal Fibers	Cellulose (% DM)	41.66
	Hemicelluloses (% DM)	20.31
	Lignin (% DM)	5.76
	Total (% DM)	67.73

Table 3. Distribution of fibers between the kernel and the hull of the desert date.

Parameter	Cellulose (% DM)	Hemicelluloses (% DM)	Lignin (% DM)	Fibers (% DM fibers)
Seed	62	29	9	100
Kernel	75	22	3	15
Hull	60	31	9	85

Table 4. Lipid content and distribution in the kernel and the hull of desert date seeds.

Parameter	Seed	Kernel	Hull
Lipid content (% DM)	14.08	34.46	3.38
Potential lipid mass (% DM seed)	-	9.5	2.4

lipid fraction (14%) and the protein fraction (consisting of all proteins and polypeptides; 8%) characterized by the protein nitrogen content and the amino acid composition.

The fibrous fraction

The parietal fibers represent nearly 68% of the dry matter (Table 2). The hulls are essentially fibrous in nature (almost 90%) and contain most of the fiber (85%) of the entire seed (Table 3). The hull and kernel fibers are of different compositions. Hull fibers are more lignified (9%) than kernel fibers (3%). Mainly cellulosic, the cellulose-hemicelluloses proportion is different; hull fibers contain 60% cellulose, while kernel fibers contain 22% hemicelluloses and 75% cellulose.

The lipid fraction

The extractable lipid potential per gram of dry matter is 14.08% for the seed, 34.46% for the kernel and 3.38% for the hull (Table 4). The assays carried out show that 80% of the lipids in the seed come from the kernel.

Unlike the oil extracted from kernels, which is light yellow, oil extracted from the hulls is transparent and more viscous, reflecting the presence of waxes as in the case of sunflower hulls (Briffaud and Melcion, 1986; Brisson, 1996; Dekker and Wallis, 1983; Evon, 2008; Thibault et al., 1989).

Composition and physico-chemical characteristics of the oil

Composition of fatty acids

Desert date oil has four (4) major fatty acids: linoleic acid C18: 2 (37.58%), oleic acid C18: 1 (34.35%), palmitic acid C16: 0 (13.74%) and C18 stearic acid: 0 (13.34%) (Table 5). These results are comparable to those obtained in other previous studies (Chapagain et al., 2009; Deshmukh and Bhuyar, 2009; Gutti et al., 2012; Mohamed et al., 2002).

The simultaneous presence of unsaturated oleic and linoleic fatty acids in high proportion (72.55%) associated with that of saturated palmitic and stearic acids in high and equivalent proportions (27.45%) makes desert date oil an original oil for food. This fatty acid composition is not found in the main oils produced industrially (coconut, cotton, peanuts, palm, rapeseed, soy, sunflower), nor in minor oils (Gustone and Pandley, 1997). Only sesame oil (C16: 0 = 9%; C18: 0 = 6%; C18: 1 = 38%; C18: 2 = 45%) and Brazil nut oil (*Bertholletia myrtaceae:* C16: 0 = 14%; C18: 0 = 9%; C18: 1 = 29%; C18: 2 = 47%), for example, have similar profiles.

Composition of glycerides and triglycerides

Triglycerides represent 98.01% of the desert date oil (Table 6). This triglyceride content is consistent with that

Table 5. Composition of fatty acids of desert date oil (%).

Fatty acids	%
C14: 0 Myristic acid	0.19
C16: 0 Palmitic acid	13.74
C16: 1 Palmitolic acid	0.18
C17: 0 Heptadecanoic acid	0.12
C18: 0 Stearic acid	13.34
C18: 1n9c Oleic acid	34.35
C18: 2n6c Linoleic acid	37.58
C20: 0 Arachidic acid	0.4
C18: 3n3a Linolenic acid	0.04
Saturated fatty acids	27.45
Unsaturated fatty acids	72.55

Table 6. Proportion of glycerides from desert date oil (%).

Glycerides	%
Triglycerides	98.01
Diglycerides (C16 and C18)	1.28
Monoglycerides C18	0.00
FAA (C16 et C18)	0.70

Table 7. Proportion of triglycerides (%) of desert date oil.

Triglycerides	%
PPlL (Palmitic, Palmitolic, Linoleic)	4.83
POL (Palmitic, Oleic, Linoleic)	33.87
SOL (Stearic, Oleic, Linoleic)	61.31

P: Palmitic acid C16: 0; Pl: Palmitolic acid C16: 1; L: Linoleic acid C18: 1; O: Oleic acid C18: 1; S: Stearic acid C18: 0.

of most seed oils and fats of animal origin, generally more than 98% (Gustone and Pandley, 1997). The diglycerides C16 and C18 are in a low proportion (1.28%). Free fatty acids (FAA), responsible for acidity represent 0.70% of the desert date oil.

The analysis of the triglycerides shows that the desert date oil consists mainly of two major triglycerides (Stearic acid, Oleic acid, Linoleic acid [SOL]: 61.31%) and (Palmitic acid, Oleic acid, Linoleic acid [POL]: 33.87%) (Table 7). These two triglycerides (SOL + POL) represent 95.18% of the total triglycerides of the desert date oil. This triglyceride profile is consistent with the fatty acids composition of the oils. All these results confirm that this oil is made up of reserve lipids. It also contains a minority fraction of metabolic lipids.

Phytosterols composition

Desert date oil has a fairly high sterols content (2.11 g/kg of oil or 0.21%). This content is lower than the average of peanut oil (1.6%), of neem oil (3.34%) (Diedhiou al., 2015), of sunflower oil (3.4%), rapeseed oil (7.3%) or corn germ oil (13.8%) known for their richness in phytosterols (Gustone and Pandley, 1997). The major phytosterols are β-sitosterol (0.75 g/kg or 35.54%), stigmasterol (0.6 g/kg or 28.43%) and cycloarterol (0.24 g/kg or 11.37%) (Table 8).

Table 8. Phytosterols composition of desert date oil.

Composition	g/kg	%
Cholesterol	0.09	4.26
Campesterol	0.03	1.42
Stigmasterol	0.6	28.43
β-Sitosterol	0.75	35.54
Δ^5-Avenasterol	0.2	9.47
Cycloarterol	0.24	11.37
Citrostadienol	0.02	0.95
Not identified	0.18	8.53
Total	2.11	100

As in desert date oil, high levels of β-sitosterol are found in most phytosterols from vegetable oils such as olive oil (84.3%), peanut oil (62.3%), sunflower oil (61.9%), rapeseed oil (45-61%), soybean oil (47-59%) and sesame oil (59-62%) (Besbes et al., 2004; Feinberg et al., 1987; Merrien et al., 1992). β-sitosterol is the most studied sterol because of its importance and its physiological effects on health (Yang et al., 2001).

Numerous clinical studies have shown that the consumption of approximately 2 g per day of β-sitosterol lowers LDL-cholesterol by approximately 10% (Lütjohann et al., 1995) and several scientific publications have focused on the anti-tumor effects of phytosterols and especially β-sitosterol (Awad et al., 2000). Thus, it has been proven that phytosterols can reduce the risk of certain types of cancer (Shahzad et al., 2017; Blanco-Vaca et al., 2019 ; Hannana et al., 2020; Le Goff et al., 2019), in particular that of the lung (Mendilaharsu et al., 1998), the breast (Ronco et al., 1999), the esophagus (Stefani et al., 2000), stomach (McCann et al., 2000), colon (McCann et al., 2003) and ovary (Stefani et al., 2000). They could also stimulate immune responses in people infected with HIV (Breytenbach et al., 2001). Phystosterols are known for their many therapeutic virtues such as reducing cholesterol levels, obesity, diabetes or inflammatory diseases (Feng et al., 2020; Hannana et al., 2020; Le Goff et al., 2019; Shahzada et al., 2017) and their properties such as antioxidant, antibacterial or antifungal (Burčová, 2018; Hannana et al., 2020).

Composition of tocopherols

The tocopherols content of desert date oil is 512.40

Table 9. Tocopherol content of desert date oil.

Tocopherol	mg/kg	%
α-tocopherol	343.4	67.18
β-tocopherol	51	9.95
γ-tocopherol	73	14.25
δ-tocopherol	45	8.78
Total	512.4	100

mg/kg. The α-tocopherol is the major compound with a content equal to 343.4 mg/kg (67.18% of the total tocopherols) (Table 9). This tocopherols content is close to that found in sunflower oil (546 mg/kg). This value remains much lower than that of rapeseed oil (1153 mg/kg) or wheat germ oil (2571 mg/kg) (Gustone and Pandley, 1997).

Due to their antioxidant activity (Yang et al., 2020), tocopherols play an important role in the stabilization of oil during its conservation (Demir and Cetin, 1999). They are also known for their positive health effects. Indeed, they prevent the oxidation of polyunsaturated fatty acids in the blood and protect Low Density Lipoproteins (LDL) from oxidation induced by free radicals causing the development of arteriosclerotic lesions (Morris et al., 2005; Schneider, 2005). They can also participate in the reduction of cardiovascular diseases and have anti-cancer properties (Beardsell et al., 2002; Bramley et al., 2000; Yang et al., 2020).

Physico-chemical characteristics of the oil

The results of the physico-chemical characterization reveal a low acidity (0.5 mg/g) and a saponification index of 182.2 mg/g. Its high Iodine Number Saponification value (INS) 116 confirms its quality for soap production. Its iodine value (66.7 g/100 g) is related to the unsaturated nature of the oil (72.5%).

The relatively high calorific value (40.3 MJ/kg) is close to that of diesel which is 43.8 MJ/kg (Vaitillingom, 2006). This result confirms that this oil is an interesting raw material for the production of biodiesel, considering that the use of vegetable oils or animal fats as biofuel is linked to 80% to their lower calorific value (LCV) (Kulkarni et al., 2007; Stavarache et al., 2007). The high flash point value (132°C) confirms that there is no risk of ignition or explosion during handling or storage under normal conditions (ASTM, 2011).

The protein fraction

Based on an average nitrogen content of 16%, the protein content of desert date seed can be estimated at 8.74%, at 27.76% in its kernel and 2.17% in its hull (considering the nitrogen to protein conversion factor of 6.25). This protein content of desert date grains is of the same order of that of rapeseed grains (17 to 23%) (Kowalska et al., 2019). A comparison of the protein content in the seed, the kernel and the hull shows that, as with the lipid fraction, the majority of the proteins is in the kernel.The fractionation obtained by shelling seeds and extracting the kernel oil will lead to a protein-rich kernel (42%). As this value is comparable to that of most industrial cakes available on the market, its recovery for animal and even human feed would be interesting.

The analysis of the amino acid composition of the protein fraction shows its proximity to that of oleoproteaginous. The essential amino acids composition remains very close to that of sunflower, rapeseed, soybean (Godon, 1985), peanut and sesame (Babiker, 2012) obtained after extraction of lipids (Table 10).

Theoretical results of the fractionation by dehulling of desert date seed

The composition and distribution of the fibers of desert date seeds thus make it possible to estimate the theoretical balance in lipids, proteins, fibers (cellulose, hemicelluloses and lignin) and other constituents in the kernel and the hull obtained from100 kg of seeds. The results of the composition reveal that shelling 100 kg of desert date seeds makes it possible to produce nearly 27.5 kg of kernel capable of providing 9.5 kg of oil, 7.6 kg of proteins, 4.2 kg of fibers (cellulose, hemicelluloses and lignin) and 6.2 kg of other constituents, and 72.5 kg of hulls containing 65 kg of fibers (Table 11). Compared to the theoretical yield of oil extraction, without shelling, the loss linked to shelling can be estimated between 15 and 20% compared to the potential of extractable oil from the seed, knowing that 80% of the lipids of the seed come from the kernel. The oil obtained from kernel can have several fields of applications such as food, cosmetics and soap, formulation of emulsion, energy, etc.

After the solvent extraction, the seeds leave a solid raffinate consisting mainly of fibers (86%). The preliminary shelling of the seeds makes it possible to reduce the fiber content of the meal even if it results in a loss of oil obtained from the separated kernels. The oil cakes obtained after extracting the oil from the kernels are rich in proteins (42.36%) and their amino acids composition (Table 10) shows that they can be used in animal feed.

The separated hulls, mainly lignocellulosic, could be converted into fibrous granulate usable as fuel for domestic uses or to produce the energy necessary for operation in a biomass boiler. Based on the calorific value of the main constituents (cellulose, hemicelluloses and lignin), a significant energy production can be estimated. In fact, a ton of desert date hull (1.38 tons of seeds) will provide an amount of heat equal to 18,285.3 MJ. Other applications of the fibrous fractions of the hulls

Table 10. Amino acids composition of desert date seeds and comparison with other oilseeds.

Amino acids	Desert date (%)	Godon (1985)		
		Sunflower (%)	Soybean (%)	Rapeseed (%)
Aspartic Acid (Asp)	11.01	8.2	10.6	7.1
Threonine (Thr)	3.27	3.7	4	4.8
Serine (Ser)	4.46	4.5	5.6	6.,5
Glutamic acid (Glu)	19.87	15.9	18.9	14.9
Glycine (Gly)	7.16	5.9	4.3	4.6
Alanine (Ala)	4.08	4.7	4.5	4.3
Cysteine (Cys)	3.14	1.8	0.2	2.5
Valine (Val)	4.76	4.6	5	5.2
Methionine (Met)	0.56	2.3	1.5	2.2
Isoleucine (Ile)	3.64	4.1	4.7	4
Leucine (Leu)	6.60	5.9	9.8	6.4
Tyrosine (Tyr)	2.24	2.8	3.9	2.9
Phenylanine (Phe)	6.25	4.3	4	3.8
Histidine (His)	2.19	2.5	3.1	2.7
Triptophan (Trp)	-	1.2	-	1.3
Lysine (Lys)	3.64	3.9	6.3	6.2
Arginine (Agr)	13.96	7.9	7.8	6
Proline (Pro)	4.71	4.3	3.5	6.3

Table 11. Theoretical balance of mechanical fractionation by shelling of the desert date seeds.

Parameter		Kernel	Hull
Dry matter (kg)		27.5	72.5
Lipids (kg)		9.5	2.4
Proteins (kg)		7.6	1.6
Fibers	Cellulose (kg)	3.3	39
	Hemicelluloses (kg)	0.8	20
	Lignin (kg)	0.1	5.8
	Total Fibers (kg)	4.2	65
Other constituents (kg)		6.2	3.7

could be envisaged and studied in the field of materials, in particular particle and fiber panels.

Conclusion

The results of the analytical fractionation of desert date seeds confirm several possibilities of valorization and allow to consider the processes of their transformation. This fractionation shows that the shelling of the seeds and the pressing of the kernels allows the production of an oil (yields between 30 and 60% depending on the lipid potential). Its high proportion of unsaturated fatty acids especially oleic and linoleic representing 72% of total fatty acids makes desert date oil a quality edible oil. Its composition and physicochemical properties open up various possibilities for non-food applications such as soap making, biofuels, agrochemicals, especially in the formulation of biopesticides. The cake from oil extraction with a high protein content (42.3%) can be used as animal feed. The aqueous fractionation of kernels makes it possible to coextract the oil and the secondary metabolites, in particular the saponins (whose presence is confirmed in the literature) as an emulsion which can be used in agrochemistry, pharmacy, cosmetics or food.

The essentially fibrous hulls can be transformed into aggregates (calorific value of 18.3 MJ/kg) and used as fuel for domestic uses or biomass boilers. These hulls

could also be extruded and mixed with other vegetable materials for the manufacture of composite agromaterials.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Molecular characterization of bacteria strians isolated from "JIKO": A herbal preparation consumed in some parts of Kaduna metropolis, Nigeria

Ebipade Kereakede[1*], Mohammed Sani Abdulsalami[1], Nkechi Eucharia Egbe[1], Victoria Moltong Yilwa[2], Enimie Endurance Oaikhena[1], Elizabeth Omolola Oladapo[1], Asabe Danjuma[1] and Kingsley Onuh[1]

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Herbal products are considered as phytomedicines which are used as complementary medicine and without adequate monitoring they are leased straightaway into the market. Preparation, transportation and handling of these products can lead to high microbial contamination which can be harmful to human health. The aim of this research was to molecularly characterize some multiple resistance bacteria obtained from JIKO which is a herbal preparation consumed in some parts of Kaduna metropolis. The genomic DNA was extracted from each bacteria isolates, 16s rRNA genes were amplified and sequenced. The sequenced obtained from the amplified genes were analyzed using Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Institute database to compare the genes with the exist genes with ones in GENbank. The results obtained confirmed the identities of four bacteria isolates: Three *Bacillus cereus* and a *Serratia* species. It was concluded that herbal products can contain some bacterial contaminants if not properly handled.

Key words: Herbal, phytomedicines, contaminants, genomic.

INTRODUCTION

Globally, herbal medicine is practiced, and most people rely on plants as source of food and for medical reasons since time immemorial. Most of the people in developing countries including Nigeria make use of herbal medicines to satisfy their health desire. Herbal preparations is also called botanical medicines or phytomedicines, herbs, herbal materials, herbal medicines, and finished herbal products that contain parts of plants or other plant materials as active ingredients (WHO, 2011). The plant materials used include seeds, berries, roots, leaves, bark or flowers (Ehrlich, 2010). Local herbal products and their preparations have been globally used for the thousands of years in developing and developed countries owing to its natural origin and lesser side effects (Anjoo, 2012). Herbal medicine contains active ingredients of plant materials which comprise different biological activities. Despite the widespread use of herbal preparations globally and their health benefits, they are not completely

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harmless. The indiscriminate, irresponsible or non-regulated use of several herbal medicines may put the health of their consumers at risk of toxicity (Kloucek et al., 2005; Abt et al., 1995). Due to their excessive use and disposal, contaminants from environmental sources may even be present if an herb is organically grown (WHO, 2007). Harmful contaminants may also originate from the soil in which the herbs are cultivated, post-harvest treatment of herbal material e.g. fumigants, and finished product manufacturing stages (*e.g*. organic solvent residues) (Chan, 2003). Microbial contamination of herbs and/or products may result from improper handling during production and packaging process. The most likely sources of contamination are microbes like bacteria, fungi and virus from the soil and processing facilities (contaminated air, microbes of human origin). Cross contamination is also possible from extraneous materials such as packaging plastics and other materials which come in contact with herbal medicines, herbal preparations or products. Other contaminants may also be introduced during harvesting, handling and production of different herbal remedies since no awareness are made to decontaminate the herbs other than by washing them (Anyanwu, 2010). This study was aimed at molecularly characterizing some bacteria from herbal preparation consumed in some parts of Kaduna metropolis, Nigeria.

MATERIALS AND METHODS

Study site

The study was conducted in Kaduna metropolis, the capital of Kaduna State. Kaduna comprises two local government areas: Kaduna South and Kaduna North and also extends to Chikun and Igabi local government areas. The metropolis holds a region of about 260 km^2, and the distance between the Eastern and Western limits of the city is estimated 13.7 km (Figure 1).

Herbal sample collection

A total of 30 samples of JIKO herbal preparation were collected 15 from Kakuri and 15 from Ugwan Tanko market were collected into sterile bottles. Samples collected were transported to the laboratory of Biological Sciences Department, NDA in an ice-box container for bacterial isolation.

Preparation of media

Nutrient agar

Nutrient agar (Antec/USA) was prepared according to manufacturer's specification and sterilization of materials was done in an autoclave at 121°C for 15 min.

Isolation of bacteria

Serial dilution of the herbal samples was done in sterile distilled water. Then 0.1 ml of the sample in 10^{-2} and 10^{-4} dilution was transferred into nutrient agar plates and spread on the agar surface using sterile bent glass rod. This was done in triplicate and was incubated at 37°C for 24 h. After incubation, the bacterial colonies on the agar plates were sub-cultured and pure culture obtained.

Molecular identification of the bacteria isolates

Extraction of DNA

The DNA of the bacteria isolates were extracted using phenol chloroform (Sambrook et al., 2012).

Amplification of 16S rRNA Gene using PCR

The amplification of the 16S rRNA gene from extracted bacteria DNA was done using the primer pairs Forward 5'-GGACTACAGGGTATCTAAT-3' and Reverse 3'-AGAGTTTGATCCTGG-5'. The amplification was carried out in an Eppendorf thermocycler TTC-100^{TM} (USA) using the following parameters: predenaturation at 90°C for 5 min, 25 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 7 min. The amplified 16S rRNA gene of each isolate was visualized by agarose gel electrophoresis.

RIBOSE-1 GGACTACAGGGTATCTAAT 16S Primer and Forward RIBOSE-2 AGAGTTTGATCCTGG 16S Primer reverse

Sequencing of PCR products

The amplified 16S rRNA gene of each isolate was processed for sequencing. The sequencing kit (Applied Biosystems) with the product was analyzed with ABI prism DNA sequence (ABI). The gene sequence of each of the isolate obtained in this study was confirmed by similarity of 16s rRNA gene sequence with that in the Gene Bank database as described by Jyothi et al. (2012).

RESULTS

16S rRNA gene amplification of bacteria isolates

The band size of 800 bp was observed on the agarose gel for the bacteria isolates from Kakuri and Unguwan-tanko (Plate 1). BLAST analysis confirmed the identities of the bacteria isolates.

16S rRNA gene sequences

The BLAST uses the National Center for Biotechnology Information. BLAST analysis of the gene sequence from three of the isolates showed 99.73, 97.45 and 98.86% similarity with *Bacillus cereus*, respectively while the fourth sequence showed 86.26% similarity with *Serratia* species shown in Table 1

DISCUSSION

Local herbal products and herbal remedies have been globally used for the thousands of years in developing and developed countries owing to its natural origin and

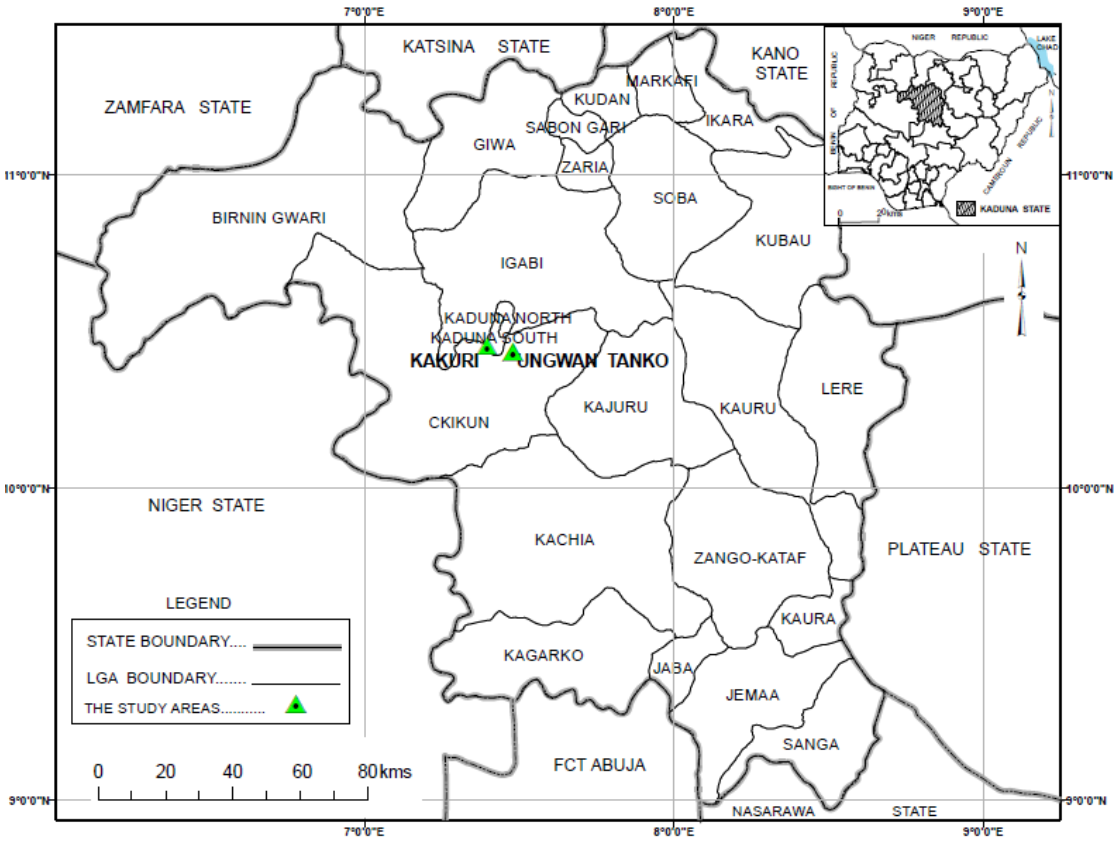

Figure 1. Map of Kaduna State showing the study area (Kakuri and Ugwan-Tanko).
Source: Geography Department NDA Kaduna.

lesser side effects (Anjoo, 2012). However herbal preparations usually carry enormous number of microorganisms originating from the soil. Microorganisms of different types are usually attached to roots, leaves, seeds, stems and flowers.

In the present study, four bacterial isolates obtained from the local herbal preparation (JIKO) were characterized by molecular techniques. The 16s rRNA gene sequence of the bacteria were aligned to the sequences in the GenBank using the BLAST tool. *B. cereus* was revealed as the dominant bacteria isolate. Similarly, Oyetayo (2008) and Heejin Ham (2017) recorded microbial contamination of agricultural herb products with *B. cereus* as the most dominant pathogenic species. This result is also in agreement with Nwankwo and Olime (2019) who reported that among the *Bacillus* species, the most prevalent was *Bacillus subtilis,* this was followed by *B. cereus* in their herbal preparation.

B. cereus is a widespread aerobic spore-forming bacteria naturally occurring micro-flora of medicinal plants and it is widely distributed in soil and water. The presence of *B. cereus* probably come from the soil, since the herbal preparation usually contain more than one plant or plant parts that have been procured from multiple harvest sites, from plants grown on soil which is the natural habitat for *Bacillus*. Other possible sources of contaminants could be improper drying, unhygienic handling of product as well as water, those processing the herbs and other contaminated equipment. Microbial contamination can render plant materials toxic either by transforming the chemicals in the plant material or from the production of toxic compounds by the microbes.

Also, *Serratia* spp. was recovered from the herbal drink. This finding agrees with the work of Abdela et al., (2016) which revealed that bacterial isolates such as *Serratia, Shigella*, *Streptococcus*, *Staphylococcus, Bacillus*, *Escherichia*, *Klebsiella*, *Clostridium*, *Enterobacter*, *Clostridium*, *Pseudomonas*, *Salmonella* and *Citrobacter* was recovered from herbal medicinal preparation. Balvindra and Neelam (2019) recorded *Serratia*, *Salmonella, Klebsiella,* and *Proteous* species from fruit juice.

Serratia spp. are widely spread in the environment, but they are not usual constituents of the human faecal flora (Carrero et al., 1995).

The microflora of the final product may represent contaminants from the raw materials, equipment, water, and atmosphere and from personnel (Oyetayo, 2008).

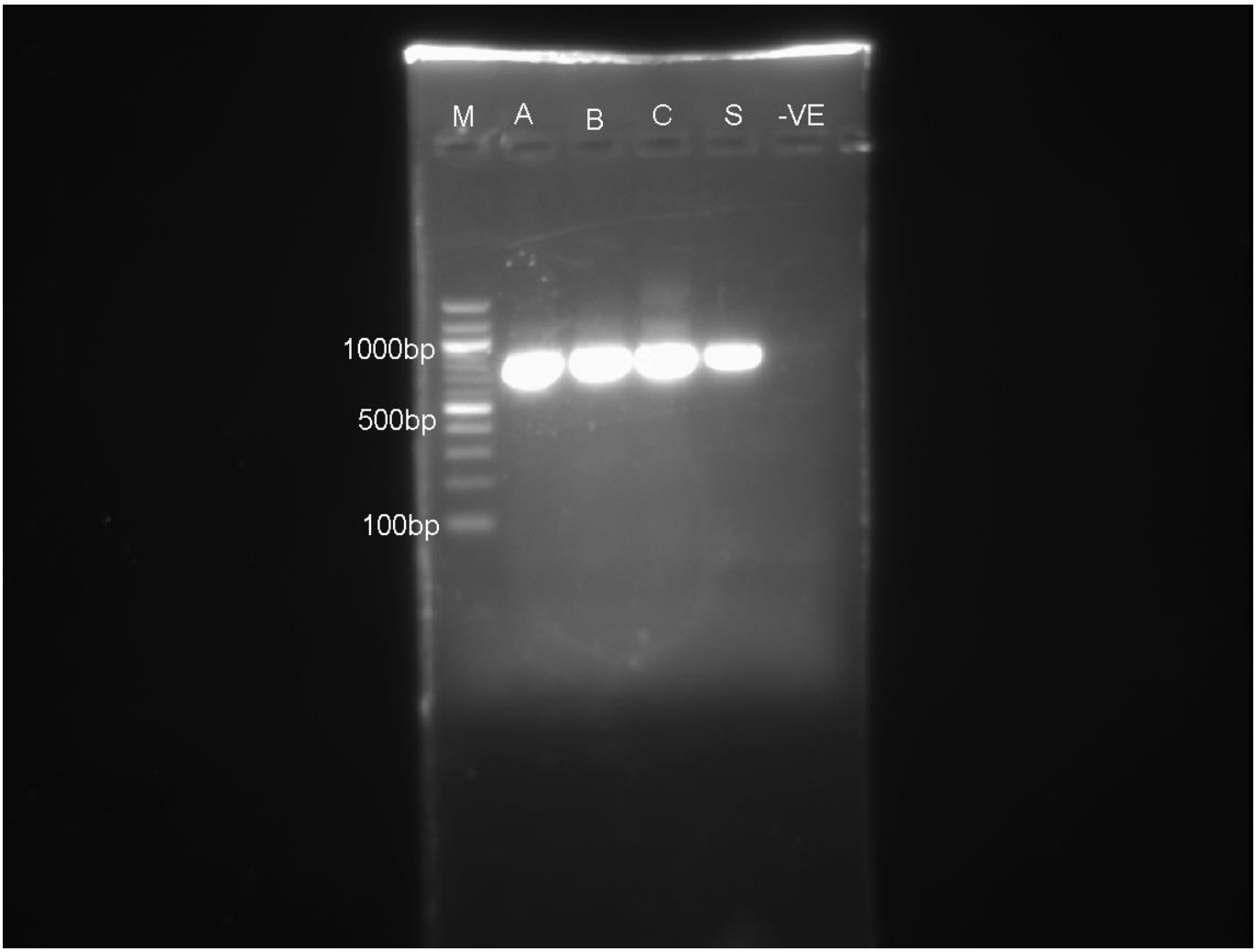

Plate 1. Agarose gel electrophoresis pattern showing PCR amplification of 16S rRNA gene of four bacteria isolates from JIKO from Kakuri and Ungwan-Tanko. M: DNA marker, -VE, 800base pair, A-2A, B-2B, C-2C, S- 2S, Negative control.

Table 1. BLAST results of 16S rRNA sequenced gene for bacteria identification.

Isolate code	Accession number	Length	Identity (%)	Identified organism
A-2A	KY435707.1	741	99.73	*Bacillus cereus*
B-2B	MH938327.1	692	97.45	*Bacillus cereus*
C-2C	KP701021.2	729	98.86	*Bacillus cereus*
S-2S	HQ238869.1	447	86.26	*Serratia* spp.

Conclusion

Herbs are extensively used in Nigeria because some plants possess vital curative properties, which can actually be used to treat human and animal diseases. The sequenced analysis of the 16S rRNA genes suggested that molecular identification of bacteria is the most accurate approach, especially for identifying organisms. Therefore, plants should be properly screened and also Good Manufacturing Practice (GMP) should be adopted by producers to ensure safe and good hygiene of herbal products.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Serum total protein concentration and liver enzymes activities in albino rats model administered with ethanolic leaf extract of *Ficus capensis*

Chukwudoruo, Chieme Sunday*, Osuji-Kalu-Ibe, Nnenna Chinenyenwa, Igwe, Kalu Okereke, Iheme, Callistus Izunna, Favour Ntite Ujowundu and Mba, Blessing Amarachi

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There is an increased use of the *Ficus capensis* plant and concerns about safety alongside efficacy have been raised. The aim of this work is to study the total protein, bilirubin and serum liver enzyme activities in albino rats administered graded concentrations of ethanol extract of *F. capensis* leaf. Twenty-four albino rats were shared into four groups and different concentrations of the leaf extract were administered with respect to body weight (250, 150 and 100 mg/kg) respectively while the fourth group (Control) was administered distilled water only. The serum liver enzymes activities were determined using the Randox Laboratory Kit while the total protein and bilirubin concentrations were determined by Spectrophotometric methods. The results showed p≤0.05 decrease in total protein and globulin concentrations, with an increase in bilirubin and albumin/globulin concentration and a slight decrease in the albumin concentration in 250 and 150 mg/kg. Furthermore, the administration of the extract caused p≤0.05 increase in the enzyme activity of alkaline phosphatase and alanine transaminase at 250 and 150 mg/kg. For aspartate transaminase, there was p≤0.05 increase in 250 mg/kg. From the study, the liver is implicated in the administration of high doses of ethanol extract of *F. capensis* hence lower doses are recommended.

Key words: *Ficus capensis*, liver enzymes, total protein, bilirubin, spectrophotometer.

INTRODUCTION

The use of medicinal plants in West Africa is probably as old as the duration of human settlement in the region (Bakkali et al., 2008). Medicinal plants are important sources of pharmaceutical manufacturing. Medicinal plants and herbal medicines account for a significant percentage of the pharmaceutical market (WHO, 2014). The therapeutic properties of medicinal plants are conditioned by the presence of active substances which include alkanoids, flavonoids, glycosides, vitamins, tannins and comarin compounds in their organs which physiologically affect the bodies of humans and animals (Thomas, 2001). Most researchers have focused on plants that have been traditionally used for various therapeutic reasons as these plants are more abundantly

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or readily available; thus, they are found to be less expensive and seemingly pose lesser side effects than synthetic drugs (Yakubu et al., 2007).

Ficus capensis which belongs to the Moraceae family is a tropical or subtropical plant that contains some phytochemicals traditionally used for various therapeutic benefits. Many of these photochemicals have beneficial effects on long term health when consumed by humans and can be used to effectively treat human diseases (Darshan, 1996). The leaves of *F. capensis* are found to contain flavonoids, tannins, cardiac glycosides, saponins, steroids, terpenoids and alkaloids, and these phytochemicals have shown effectiveness in antimicrobial, anti-diarrheal, antiallergic and anticancer treatments as well as treatment of cardiac problems (Uzoekwe and Mohammed, 2015). Serum liver enzymes are predominantly contained within the liver cells. If the liver is injured or damaged the liver cells spill these enzymes into the blood, raising their levels in the blood and signaling liver disease (Daniel and Marshall, 1999). Total protein and bilirubin could be implicated in liver function in humans and animals. In this research, the ability of well directed ethanol extracts of *F. capensis* to cause changes to the serum liver enzymes and total protein, and bilirubin levels in albino rats *in vivo* hence determining its safety and possible hepatotoxicity at different concentrations will be studied.

MATERIALS AND METHODS

Sample collection and identification

Fresh leaves of *F. capensis* were randomly harvested from Okporo Community, Orlu Local Government Area of Imo State, Nigeria. It was identified by the School of Agriculture and Agricultural Sciences (SAAT), Federal University of Technology, Owerri, Imo State with voucher identification number 001/FWT/FUTO/2015. The leaves were washed and dried at room temperature, crushed, allowed to cool, then bottled and deposited at the university herbarium.

Sample preparation

The crude extract of *F. capensis* was obtained by soaking crushed leaves in 98% ethanol for 72 h, thoroughly shaken, filtered and filtrate was put in the Soxhlet extraction unit. The final leaf extract obtained after Soxhlet extraction was labeled Extract F.

Experimental animals

Twenty-four albino rats weighing between 90-120 g were obtained from Department of Biochemistry FUTO and housed in a well ventilated metal cage. They were fed with rat feed twice daily and left to acclimatize for 14 days and their average weight was taken note of preceding commencement of administration.

Experimental design

The randomized complete block design was used. Rats were grouped into four (4) groups of six (6) rats each; Groups A-C served as groups administered various fractions of *F. capensis* leaf extract while Group D served as the control.

The grouped albino rats were administered doses of the plant extract with respect to their body weight (250, 150 and 100 mg/kg). The administration of the extract was done on a daily basis for 14 days after which the blood samples were collected in triplicates. The statistical analysis of data obtained from test groups and control groups were evaluated by ANOVA, applying the level of significance ($P \leq 0.05$) using SPSS 8.1.

Preparation of serum

The method of Yakubu et al. (2005) was adopted for the preparation of serum. Twenty-four hours after the last extract administration, briefly under ether anaesthesia, the neck area of the rats was quickly shaved to expose the jugular veins. The veins after being displaced (to avoid contamination with interstitial fluid) were cut with sterile scapel blade and an aliquot of the blood was collected into labeled vacutainer sample tubes.

A table top centrifuge was used to centrifuge the blood samples at 700 rpm for 15 min after which the serum was transferred from the clotted blood into plain tubes with the aid of a pasture pipette. The collected serum was stored at 4°C till the next day for analysis.

Determination of serum total protein

The commonly used method for measuring serum protein is the biuret reaction (Gornall et al., 1949). The principle of the reaction is that serum proteins react with Cu^{2+} (Copper Sulphate) in sodium hydroxide to form a purple/violet complex. The intensity of the purple/violet colour is proportional to the total protein present. The absorbance of sample and standard were read against reagent blank at 540 nm using a DRE 3000 HACH Spectrophotometer.

Determination of serum albumin

The measurement of albumin is generally by a dye binding technique that utilizes the ability of albumin to form a stable blue coloured complex with bromocresol green dye (Tietz et al., 1994). The intensity of the colour is proportional to the amount of albumin present. The absorbance of sample and standard were read against reagent blank at 630 nm using a DRE 3000 HACH spectrophotometer.

Determination of serum globulin

Owing to the fact that bromocresol green-albumin complex absorbs light at different wavelengths from the unbound dye, the method may overestimate albumin by binding to other proteins (Willacy, 2019). As such, the total globulin fraction is generally determined by subtracting the ALBUMIN fraction from the total protein fraction.

Determination of serum bilirubin

The dimethylsulphoxide method as described by Tietz et al. (1994) was used to determine serum bilirubin concentration. The principle of the reaction is that bilirubin reacts with diazotized sulphanic acid in the presence of dimethylsulphoxide to form a coloured complex whose colour intensity is proportional to the amount of bilirubin present. The absorbance of the sample and standard were read against the blank at 550 nm using a DRE 3000 HACH

Table 1. Serum total protein and bilirubin concentration of ethanolic leaf extract of *Ficus capensis.*

Group	Total protein concentration (umol/L)	Albumin concentration (umol/L)	Globulin concentration (umol/L)	Bilirubin concentration (umol/L)	Albumin/Globulin ratio concentration (umol/L)
A (250 mg/kg)	52.81±2.61[a]	31.57±1.14[a]	21.24±2.50[a]	36.23±3.46[a]	1.50±0.20[a]
B (150 mg/kg)	62.20±1.93[b]	32.52±1.11[a]	29.68±2.83[b]	32.31±3.46[b]	1.11±0.14[b]
C (100 mg/kg)	72.41±2.22[c]	33.46±1.13[a]	38.95±1.52[c]	17.08±0.78[c]	0.86±0.03[b]
D (Control Distilled water)	74.88±1.62[c]	34.18±1.33[a]	40.70±1.11[c]	15.81±0.67[c]	0.84±0.04[b]

Values are Means + standard deviation of triplicate determination. Superscripts with different alphabets = p≤0.05; Superscripts with same alphabets = p>0.05.

Table 2. Serum liver enzymes activities of ethanolic leaf extract of *Ficus capensis*.

Group	A (250 mg/kg)	B (150 mg/kg)	C (100 mg/kg)	D (Distilled water)
ALP activity(IU/L)	49.32±2.19[a]	38.53±4.26[b]	24.91±2.16[c]	19.57±0.90[c]
ALT activity(IU/L)	27.70±0.64[a]	24.43±1.86[b]	20.24±1.04[c]	17.77±0.54[c]
AST activity(IU/L)	46.17±4.77[a]	35.45±1.78[b]	30.23±0.48[c]	28.78±0.61[c]

Values are Means + standard deviation of triplicate determination. Superscripts with different alphabets = p≤0.05; Superscripts with same alphabets = p>0.05.

spectrophotometer.

Determination of aspartate transaminase activity

The Reitman and Frankel method was used to determine the serum aspartate transaminase activity (Reitman and Frankel, 1957). The principle of the reaction is that the AST catalyses an exchange reaction of an amino group between aspartate and a-ketoglutarate forming oxaloacetate and glutamate. The oxaloacetate thus formed reacts with 2,4-dinitrophenylhydrazine in sodium hydroxide to form oxaloacetatehydrazone which has a reddish brown colour.

The absorbance of the sample was read against the reagent blank at 546 nm using a DRE 3000 HACH spectrophotometer.

Determination of serum alanine transaminase activity

The Reitman and Frankel method was used to determine serum alanine transaminase activity (Reitman and Frankel, 1957). The principle of the reaction is that ALT catalyses the transfer of the amino group between L-alanine and a-ketoglutarate to form pyruvate and glutamate. The pyruvate formed reacts with 2,4-dinitrophenylhydrazine in sodium hydroxide to give a complex with a reddish-brown colour. The absorbance of the sample was read against the reagent blank at 546 nm using a DRE 3000 HACH spectrophotometer.

Determination of alkaline phosphatase activity

The method of Wilkinson and Vodden was used in determining serum alkaline phosphatase activity (Wilkinson and Vodden, 1966). The principle of the reaction is that ALP acts upon phenolpthalein monophosphate in 2-amino-2methylpropan-1-ol buffer at pH of 10.15. The addition of an alkaline reagent stops enzyme activity and simultaneously develops a blue chromogen. The absorbance of the sample was read against the reagent blank at 590 nm using a DRE 3000 HACH spectrophotometer.

RESULTS

As represented in Table 1, the total protein and bilirubin concentrations and serum liver enzyme activity in albino rats administered graded concentrations of ethanol extract of *F. capensis* leaf was studied. The results showed a significant p≤0.05 decrease in total protein and globulin concentrations in albino rats administered 250 and 150 mg/kg of plant extract (52.81±2.61 umol/L; 62.20±1.93 umol/L; and 21.24±2.50 umol/L; 29.68±2.83 umol/L) when compared with 100 mg/kg and Control. There was a significant p≤0.05 increase in bilirubin concentration in albino rats administered 250 and 150 mg/kg of plant extract (36.23+3.46 and 32.31+3.46 umol/L respectively) and a significant p≤0.05 increase in the albumin/globulin concentration in albino rats administered 250 mg/kg of plant extract (1.50±0.20 umol/L) when compared with other concentrations of administration and Control. There was also a slight decrease in the albumin concentration in albino rats administered 250 and 150 mg/kg of plant extract (31.57±1.14 and 32.52±1.11 umol/L respectively) when compared with 100mg/kg and control.

As represented in Table 2, the administration of the extract caused significant p≤0.05 increase in the enzyme activity of Alkaline Phosphatase in albino rats administered 250 and 150 mg/kg of plant extract (49.32±2.19 IU/L and 38.53±4.26 IU/L) when compared with the 100 mg/kg and Control. There was also a significant p≤0.05 increase in the activity of Alanine

Transaminase (ALT) in albino rats administered 250 and 150 mg/kg of plant extract (27.70+0.64 and 24.431±1.86 IU/L) when compared with 100 mg/kg and Control. For Aspartate Transaminase (AST), there was a significant $p \leq 0.05$ increase in albino rats administered 250 mg/kg of plant extract (46.17+4.77 IU/L) when compared with the other concentrations of administration.

DISCUSSION

The liver maintains homeostasis in living system. It is involved in biochemical pathways necessary for growth and fighting against diseases (Ward and Daly, 1999). The *F. capensis* plant is used for a number of therapeutic purposes, and this raises the concern of safety and possible toxicity. This study pointed towards possible hepatotoxicity which is shown in the decreased serum total protein concentrations and may be attributed to toxicants in the phytochemical constituents of the leaves or may have been due to increased release of tissue specific enzymes and other intra cellular proteins secondary to parasite-induced cell membrane disruption. Total protein (albumin and globulin) are produced by the liver and in the case of a liver damage, production of these proteins are reduced or completely ceased. The concentrations of the total protein, bilirubin and albumin may indicate the state of the liver and the type of damage (Yakubu et al., 2005). The study also suggests that toxic metabolites in *F. capensis* extract at high concentrations may be responsible for the significantly high value of bilirubin of rats administered extracts at 250 mg/kg. This assertion is supported by the work of Ovuru et al. (2004) who reported an increase in the total serum bilirubin concentration and attributed this to a metabolic disturbance in the liver arising from a defective conjugation and/or excretion of bilirubin. The albumin/ globulin ratio concentration significantly increased indicating conditions causing underproduction of globulins which include liver cirrhosis. There was also significant increase in the serum liver enzyme activities at higher levels of administration. Alkaline phosphatase is a hydrolytic enzyme which is responsible for removal of phosphate group from many types of molecules including nucleotides, proteins etc. ALP is particularly concentrated in the liver, bile duct, kidney bone and placenta. An increase in ALP is an indication that there is an obstruction of bile duct consequently affecting the liver. An increase in ALP may also be as a result of celiac diseases (Tamas et al., 2002). Alanine transaminase usually increases where liver has been diseased or damaged and is also a test used for screening liver problems. Aspartate transaminase is a commonly measured clinical marker for liver health (Ghouri et al., 2010). Hence the marked increases in the serum liver enzyme activity, at high levels of administration of plant extract indicate possible hepatotoxicity at high doses.

CONCLUSION AND RECOMMENDATION

From the study, it is shown that high doses of administration of the ethanol extract of *F. capensis* leaf caused hepatotoxicity in albino rats and if the therapeutic power of this plant must be utilized, lower doses would be recommended.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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