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Antibacterial Activities of *Hyphaene thebaica* (Doum Palm) Fruit Extracts against Intestinal Microflora and Potential Constipation Associated Pathogens in Yola Metropolis, Nigeria

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Abstract

This study aimed at determining the antibacterial activities of *Hyphaene thebaica* fruit extracts against some intestinal constipation associated bacteria. Qualitative analysis of some phytochemical constituents, agar well diffusion and broth dilution methods were used to determine the zones of inhibition and minimum inhibitory concentration (MIC) of the plant extracts. Phytochemical components viz flavonoids, saponins, terpenoids, tannins, phenols, alkaloids, glycosides and steroids were detected in the plant extracts, and the test organisms were susceptible to the plant extracts. The diameter zone of inhibition (DZI) obtained with n-hexane extract ranged from 15.10 ± 0.51 mm to 2.0 ± 0.55 mm against *K. pneumoniae*, 10.20 ± 0.57 mm to 2.00 ± 0.35 mm against *P. aeruginosa* and 8.00 ± 0.35 mm to 1.00 ± 0.55 mm against *S. typhi*, while the DZI for aqueous extract was 7.10 ± 0.23 mm to 2.0 ± 0.35 mm against *K. pneumoniae*, 6.20 ± 0.31 mm to 2.00 ± 0.35 mm against *S. typhi* and 5.42 ± 0.55 mm to 2.05 ± 0.75 mm against *P. aeruginosa.* The MIC and minimum bactericidal concentration of the extracts were 100 mg/mL and 200 mg/mL, respectively. *Hyphaene thebaica* fruit possessed antimicrobial activities against the test organisms. Therefore, toxicity and clinical studies are recommended for possible drug development.

Keywords: Doum; Constipation; Intestinal; Microflora; Fibre; *Hyphaene thebaica.*

Introduction

Medicinal plants play vital roles in the control and prevention of various diseases such as cancers, diabetes, and cardiovascular diseases (Mohanta et al. 2003). Some medicinal plants have been used for production of various drugs and as principal raw materials for production of other conventional medicines due to their antimicrobial activities (Srivastava et al. 2005). Antimicrobial activity involves complex mechanisms such as prevention of cell membrane, cell wall formation and inhibition of nucleic acid and protein metabolism.

Hyphaene thebaica is an oval fruit that belongs to the mint family (Arecaceae). It is a desert palm fruit native to Nigeria, Senegal, Egypt, Tanzania, Kenya, Arabian Peninsula and West India (Orwa et al. 2009). This fruit

serves as source of minerals, (calcium, potassium, phosphorus, sodium, and magnesium), carbohydrate, B-complex vitamins, and fibres that are essential for good nutrition (Hsu et al. 2006). The root is used in the treatment of bilharzia, while the fruit pulp is used in controlling hyperlipidaemia and hypertension (Coopoosamy and Magwa 2007). Aboshora et al. (2014) reported on the antioxidant, anticancer, antimicrobial, and anti-inflammatory activities of *Hyphaene thebaica* fruit due to the high amounts of phenol, and flavonoids.

Constipation is a condition of digestive system that makes bowel movements become less frequent and result in hard stools that are difficult to excrete. Studies by Zhao and Yu (2016) have shown that constipation is caused by disturbances in composition and stability of gut microbiota. Zhao and Yu (2016) stated that changes or alterations of intestinal microbiota in patients with chronic constipation can be characterised by a relative decrease in obligate bacteria (e.g., *Bifidobacterium, Lactobacillus* and *Bacteroides species*) and parallel increase of potentially pathogenic microorganisms such as *Campylobacter jejuni* and *Pseudomonas aeruginosa*. Thus, this alteration might have influenced on intestinal mobility and secretory functions by changing the available amount of physiological active substances and metabolic environment (Khalif et al. 2005). Constipation can result from inadequate intake of fibres, and changes in diet (Khalif et al. 2005).

The increase in resistance of microorganisms to many commercial synthetic products has led to search of plant materials that can be used in developing new antimicrobial agents that can solve this problem (Zhao and Yu 2016). Hence, this study determined the antibacterial activities of doum palm (*Hyphaene thebaica*) fruit extracts against intestinal microflora and potential constipation associated pathogens.

Materials and Methods

Preparations of plant materials

The fresh fruits of *Hyphaene thebaica* were harvested during the rainy season, precisely in May, 2019 at Jambutu farmland Yola, Adamawa State, Nigeria. The samples were kept in polyethylene bags, transported to the laboratory, and were identified by Dr Changya of the Department of Plant Science in Modibbo Adamawa University of Technology, Yola, Nigeria. The epicarp of doum palm fruit was cleaned, scrapped with sterile (70% ethanol cleaned) scapel, and dried at room temperature (26 °C) until constant weight was obtained. The fruits were pulverized by pounding with mortar and pestle to increase the surface area with the aim of enhancing the penetration and extraction by the extracting solvents.

Sample collection

A total of 50 stool samples from patients with cases of constipation were collected from Peace Hospital, Jimeta, Adamawa State, Nigeria and transported to the laboratory using transport medium (Cary-Blair) for analysis.

Extraction

Cold maceration method as described by Obeidat (2011) was used for aqueous extract with distilled water as extracting solvent and Soxhlet extraction procedure for the n-hexane extract as described by Ewansiha et al. (2017). For aqueous extract, 250 g of plant material were weighed and transferred into 600 mL of distilled water, the mixture was homogenised and left at room temperature (26 °C) for three days. After three days, the aqueous solution was filtered with muslin cloth, and the filtrates were evaporated to dryness in a water bath set at 60 °C. The dried extracts were stored in screw capped bottles and kept in the refrigerator at 4 °C until further analysis.

Identification of phytochemical constituents

The presence of flavonoids, tannins, phenols, alkaloids, and saponins were identified according to the methods reported by Akoma and Olawepo (2002). The flavonoids were

identified by dissolving 0.5 mg of the extract into 1 mL of ethanol and filtered. After filtration, 2 mL of 1% HCl and magnesium ribbon were added. Colour changes of red or pink colour indicated the presence of flavonoids. Tannins were identified by dissolving 0.5 mg of dried plant extract into 1 mL of distilled water, filtered and 2 mL of $FeCl_3$ was added to the filtrate. Appearance of blue or black colour indicated the presence of tannins.

***Determination of alkaloids*:** The Alkaloids were determined by adding 0.5 mg of plant extract to 1 mL of methanol, filtered and 1% of HCl was added to the filtrate and the solution was heated. Colour changes from red to pink after the addition of Mayor's reagent confirmed the presence of alkaloids.

***Determination of phenols*:** Phenols were also determined by dissolving 50 mg of dried extract into 5 mL of distilled water, 2-3 drops of neutral ferric chloride solution was added, and a dark green colour indicated the presence of phenols.

Determination of saponins: Saponins were identified by dissolving 0.5 mg of dried plant extract into 1 mL of methanol and filtered. Distilled water was added, the filtrate was shaken for few minutes and persistent frothing indicated the presence of saponins.

Isolation and identification of test organisms

Samples were prepared based on a method described by Pradhan and Tamang (2019) with brief modifications. Briefly, 1 g of stool sample was homogenised with 9 mL of distilled water and 1 mL of the diluent was inoculated into MacConkey agar, *Salmonella-Shigella* agar, cetrimide agar and incubated at 37 °C for 24 hours. After incubation, the plates were observed for growth. Colonies from each plate were sub-cultured unto freshly prepared medium to obtain pure isolates for identification.

Identification of test organisms

***Gram staining*:** Gram stain procedures were done according to Chessbrough (2002). The overnight culture of bacteria was gram stained, observed through the microscope and was grouped based on Gram's reaction and morphology.

Biochemical characterization

The biochemical identification was done on the isolates using the method described by Chessbrough (2002). The tests included catalase, lactose, oxidase, indole, citrate, mannitol tests and were done after microscopic identification of the isolates to confirm their genus and species.

Antibacterial susceptibility test

Standardization of inoculum

McFarland standard (0.5) was made by mixing 0.05 mL of 1.175% barium chloride dihydrate ($BaCl_2.H_2O$) with 9.95 mL of 1% sulfuric acid (H_2SO_4) and its turbidity was used as a standard for the test organisms. This was done according to Chessbrough (2002).

Preparation of extract concentrations

Different concentrations of the plant extracts were made by using distilled water as diluent and the n-hexane extract in 10% dimethyl sulfoxide. Each concentration of extracts (100 mg/mL, 200 mg/mL, 300 mg/mL ,400 mg/mL, and 500 mg/mL) were prepared by dissolving 1000 mg, 2000 mg, 3000 mg, 4000 mg and 5000 mg of extracts in 10 mL of water, respectively.

Antibacterial assay

The antibacterial test was done using agar well diffusion method as described by Akoma and Olawepo (2002). Mueller Hinton agar was used and 0.1 mL of standardized inoculum was inoculated on the agar with even distribution. A well was made on the medium using a 6 mm diameter sterile cork bearer. 0.2 mL each of concentration of plant extracts was pipetted into the well of the test organism. Distilled water was used as negative control, 3 mg/mL of ciprofloxacin as positive control, and the plates were left on the bench for 5 minutes for proper diffusion before incubating at 37 °C for

24 hours. After incubation, the plates were checked for zones of inhibition.

Determination of minimum inhibitory concentration and minimum bactericidal concentration

These were determined according to the method described by the Clinical and Laboratory Standards Institute (CLSI) (2008). Nutrient broth was dispensed into test tubes and 2 mL each of n-hexane and plant extract was serially dispensed, 0.1 mL of the test organisms was added to the test tube containing the extract. Broth containing the organism was used as positive control and the broth without inoculation of the organism but with the extract included was used as negative control. The tubes were incubated at 37 °C for 24 hours and after incubation, the tube with no visible growth, with the lowest concentration having the same appearance with that of the negative control was taken as minimum inhibition concentration**.** The tube with no visible growth was streaked on nutrient agar and incubated at 37 °C for 24 hours. The lowest concentration of both the n-hexane and aqueous extract that totally stopped the growth of the organism was taken as the minimum bactericidal concentration.

Statistical analysis

Results values were expressed as mean ± SEM (standard error of mean). Statistical analysis was carried out using statistical package for social sciences (SPSS version 20) and one-way analysis of variance (ANOVA) followed by Post Hoc multiple comparisons and Duncan test to establish significant differences where p-values < 0.05 were considered significant.

Results

Isolated organisms from stool samples

The isolated organisms from stool samples are presented in Table 1. The isolated organisms were identified based on cultural characteristics, Gram's reactions and biochemical characteristics. *Salmonella typhi* was identified based on the isolate inability to ferment lactose, oxidase, and indole but could use citrate, mannitol and catalase. *Pseudomonas aeruginosa* was identified based on the isolate inability to ferment lactose, citrate, and metabolise indole but could ferment catalase, oxidase and mannitol. *Klebsiella pneumoniae* was identified based on the isolate inability to ferment oxidase and indole but could use catalase, lactose, citrate and mannitol thus could not use oxidase and indole. All isolates were Gram negative and a total of three bacteria species were isolated and identified as *Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *Salmonella typhi*.

Table 1: Gram stain and biochemical characteristics of isolated organisms

Gram Reaction	Catalase	Lactose	Oxidase	Indole	Citrate	Mannitol	Bacteria
-	+	-	-	-	+	+	*Salmonella typhi*
-	+	-	+	-	-	+	*Pseudomonas aeruginosa*
-	+	+	-	-	+	+	*Klebsiella pneumoniae*

Key: "-" = negative, "+" = positive.

Phytochemical constituents of doum palm fruit extracts

The results of the phytochemical screening of aqueous extracts and n-hexane extract of doum palm fruits are presented in Table 2. The results showed 8 components, viz. flavonoids, saponins, terpenoids, tannins, phenols, alkaloids, glycosides and steroids. Of the 8 components, 6 (flavonoids, saponins, tannins, phenols, alkaloids and steroids) were present in

aqueous extracts, while 5 (flavonoids, saponins, tannins, phenols and steroids) were present in n-hexane extracts.

Table 2: Phytochemical constituents of the extracts

Compounds	Aqueous extract	n-Hexane
Saponins	+	+
Tannins	+	+
Terpenoids	-	-
Alkaloids	+	-
Flavonoids	+	+
Steroids	+	+
Glycosides	-	-
Phenols	+	+

Key: "+" = Present, "- "= Absent.

Antibacterial activities of n-hexane extracts of doum palm fruit extracts

The antibacterial activities of n-hexane extracts of doum palm extracts are presented in Table 3. The results show that n-hexane extracts of doum palm fruit were active at 500, 400, 300, 200 and 100 mg/mL concentrations but the activities were concentration dependent; i.e., the activities decreased as the concentrations decreased. The highest activity range of 15.10 ± 0.51 mm–2.0 ± 0.55 mm was obtained at 500 mg/mL–100 mg/mL against *K. pneumoniae*, followed by 10.20 ± 0.57 mm–2.00 ± 0.35 mm at 500 mg/mL–200 mg/mL against *P. aeruginosa* while the least activity range of 8.00 ± 0.35 mm–1.00 ± 0.55 mm at 500 mg/mL–200 mg/mL was obtained against *S. typhi.* The organisms were more susceptible to the standard drug (ciprofloxacin) which exhibited at least 20.0 ± 0.35 mm diameter zone of inhibition (DZI), with significant difference ($P < 0.05$) compared to the plant extract except for *P. aeruginosa* and *S. typhi* at 100 mg/mL that were resistant to the plant extract at 100 mg/mL.

Table 3: Mean zones of inhibition exhibited by n-hexane extracts of doum palm fruit in millimetre

	n-Hexane (mg/mL) extract					Control	
Organism	500	400	300	200	100	CPX (30 µg)	$D^{10\%}$
S. typhi	8.00 ± 0.35^{c}	5.20 ± 0.65^{b}	4.30 ± 1.25^{b}	1.00 ± 0.55^{ab}	0.00^{a}	20.0 ± 0.52^{d}	0.00^{a}
P. aeruginosa	10.20 ± 0.57^{c}	5.00 ± 1.75^{b}	3.20 ± 0.75^{ab}	2.00 ± 0.35^{ab}	0.00^{a}	20.0 ± 0.35^{d}	0.00^{a}
K. pneumoniae	15.10 ± 0.51^{d}	12.05 ± 0.55^{cd}	7.10 ± 1.15^{c}	4.00 ± 0.01^{b}	2.0 ± 0.55^{ab}	20.0 ± 0.35^{e}	0.00^{a}

Key: CPX = ciprofloxacin; $D^{10\%}$ = 10% dimethyl sulfoxide; mm = millimetre; values on the same row with different superscript are significantly different ($P < 0.05$).

Antibacterial activity of aqueous extracts of doum palm fruit

The antibacterial activity of aqueous extracts of doum palm extracts is presented in Table 4. The result showed that aqueous extracts of doum palm fruit is active at 500, 400, 300, 200 mg/mL concentrations but the activities were concentration dependent; i.e. the activity decreased as the concentration decreased while *P. aeruginosa* and *S. typhi* were not susceptible at 200 mg/mL and 100 mg/mL. The highest activity range of 7.10 ± 0.23 mm–2.0 ± 0.35 mm was obtained at 500 mg/mL–100 mg/mL against *K. pneumoniae*, followed by 6.20 ± 0.31 mm–2.00 ± 0.35 mm at 500 mg/mL–200 mg/mL against *S. typhi*, while the least activity range of 5.42 ± 0.55 mm–2.05 ± 0.75 mm at 500 mg/mL–300 mg/mL was obtained against *P. aeruginosa*. The organisms were more susceptible to the standard drug (ciprofloxacin) which exhibited at least 20.0 ± 0.35 mm diameter zone of inhibition (DZI), with significant difference ($P < 0.05$) compared to the plant extracts.

Table 4 Mean zones of inhibition exhibited by aqueous extracts of doum palm fruit in millimetre

	Aqueous (mg/mL) extract					Control	
Organism	500	400	300	200	100	CPX (30 µg)	$D^{10\%}$
S. typhi	6.20 ± 0.31^{c}	3.50 ± 0.05^{b}	2.08 ± 0.45^{b}	2.00 ± 0.35^{b}	0.00^{a}	20.0 ± 0.52^{d}	0.00^{a}
P. aeruginosa	5.42 ± 0.55^{c}	3.38 ± 1.15^{b}	2.05 ± 0.75^{b}	0.00^{a}	0.00^{a}	20.0 ± 0.35^{d}	0.00^{a}
K. pneumoniae	7.10 ± 0.23^{c}	6.27 ± 0.34^{c}	5.80 ± 1.35^{c}	3.00 ± 1.39^{c}	2.00 ± 0.35^{d}	20.0 ± 0.35^{d}	0.00^{a}

Key: CPX = ciprofloxacin; $D^{10\%}$ = 10% dimethyl sulfoxide; mm = millimetre; values on the same row with different superscript are significantly different ($P < 0.05$).

The minimum inhibitory and bactericidal concentration of doum palm fruit extracts

The aqueous and n-hexane extract had a MIC value of 100 mg/mL against all isolates as shown in Table 5. The MBC of the aqueous and n-hexane extracts against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were 100 mg/mL and 200 mg/mL, respectively, also 100 mg/mL and 200 mg/mL against *Salmonella typhi*.

Table 5: Minimum inhibitory and bactericidal concentrations of doum palm fruit extracts

Doum palm fruit extracts	Concentrations (mg/mL)	Organisms *S. typhi*	*P. aeruginosa*	*K. pneumoniae*
n-Hexane extract	400	-	-	-
	200	-	-	-
	100	-	-	-
	50	+	+	+
	25	+	+	+
	12.5	+	+	+
Aqueous extract	400	-	-	-
	200	-	-	-
	100	-	-	-
	50	+	+	+
	25	+	+	+
	12.5	+	+	+
n-Hexane extract	MIC	100	100	100
	MBC	200	100	100
Aqueous extract	MIC	100	100	100
	MBC	100	200	100

Key: NH = n-hexane extract; AQ = aqueous extract; "+" = presence of turbidity, "-" = absence of turbidity.

Discussions

This study evaluated the antibacterial effectiveness of doum (*Hyphaene thebaica*) fruit extracts (aqueous and n-hexane) against *Salmonella typhi, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. The results obtained

showed that doum palm fruit contains at least 8 phytochemical components namely flavonoids, phenols, tannins, terpenoids, saponins, glycosides, alkaloids and steroids which correlate with the findings of Mohamed et al. (2010) who also identified these phytochemicals from doum palm fruit. Each phytochemical component is reported to exert inhibitory activity against isolated organisms (Hsu et al. 2006). Flavonoids have been reported to exert their antimicrobial activities by inhibiting the synthesis of nucleic acids, tampering with the integrity of the cytoplasmic membrane functions and energy metabolism process (Cushnie and Lamb 2005). Phenols, alkaloids, steroids, and saponins, have also been reported by Umeh et al. (2005) to exert their antibacterial properties against some pathogens by interfering with metabolic processes in microorganisms. Previous studies showed that doum fruit extract possesses antioxidant and anticancer activities due to substantial amounts of water-soluble phenolic contents (Hsu et al. 2006).

Results of the antibacterial screening of both for aqueous and n-hexane extracts of the fruit against the test organisms indicated that the extracts caused zones of inhibition indicative of antibacterial activities. The aqueous extracts showed higher activity against *Klebsiella pneumoniae* compared to *Pseudomonas aeruginosa* and *Salmonella typhi* in which growth was not inhibited at 100 mg/mL, and this could be as a result of the discrepancies among the metabolic structures and nature of microorganisms. The zones of inhibition exhibited by the n-hexane extracts against *K. pneumoniae* are in agreement with the report of Aboshora et al. (2014) who stated that methanol and ethanol extracts of doum palm fruit had more inhibitory activity than the aqueous extract. Gram-negative bacteria are generally more resistant to antibacterial agents than the Gram-positive bacteria due to their complex cell wall structures. Also, Gram negative bacteria such as *Pseudomonas aeruginosa* maintain antibiotic resistance plasmids and can transfer these genes by means of bacterial processes of conjugation and transduction to other bacteria in their immediate vicinity (Lal et al. 2008). The presence of thick murine layer in the cell walls of Gram negative bacteria prevents the entry of environmental substances such as plant-based biocides and antibiotics (Kandhasamy et al. 2008). Therefore, the reduced activities exhibited by the lower concentrations of the extracts could also be attributed to the inability of the extracts to effectively penetrate the bacterial cell due to the structural make-up of the cell wall and its inherent components. Furthermore, the test organisms were more susceptible to the standard drug with DZI of up to 20.0 ± 0.35 mm, which is significantly higher ($P < 0.05$) than that of the fruit extracts (15.10 ± 0.51 mm). The MIC and MBC results obtained for both solvent extracts, showed that the viability of the test organisms were inhibited by the extract at 100 mg/mL while killing began at 200 mg/mL.

These results are similar with the work done by Junaid et al. (2006) who stated that concentration of 100 mg/mL crude extract of several plants is required to possibly commence inhibition of the viability of *Pseudomonas aeruginosa* and some other Gram negative microorganisms.

Conclusion

This research supports the view that doum palm fruit may be efficient as preventive agents of gastrointestinal diseases. Therefore, it can be concluded that doum palm fruit possessed antibacterial activities against the test organisms isolated from stool at higher concentrations due to the presence of some phytochemicals.

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