EVALUATION OF ANTIMICROBIAL ACTIVITIES OF CRUDE METHANOL EXTRACT OF *Phoenix dactylifera* SEEDS ON CLINICAL ISOLATES OF DIFFERENT STRAINS OF *E.coli*

ABSTRACT

Aim: This study was conducted to evaluate the antimicrobial activities of seed extract of *Phoenix dactylifera*

Study design: Extraction of active ingredients of *Phoenix dactylifera* seeds using methanol and its effects on selected clinical isolates and isolated strains of *E. coli.*

Place and Duration of Study: Department of Phatrmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical, University of Nigeria, Nsukka between march 2017 and October 2018

Methodology: The seed of *phoenix dactylifera* were washed thoroughly to get rid of any adhering date flesh, air dried and ground. The ground powders were subjected to extraction by cold maceration using methanol. Preliminary sensitivity test was carried out against eight microorganisms, namely: *Pseudomonas aeruginosa, E-coli, Enterococcus, Klebsiella, Staph. spp, Salmonella spp, Bacillus sp and Candida albicans* using agar diffusion method. Antimicrobial susceptibility tests were carried out on isolated strains of E. coli from urine samples using agar diffusion method. The minimal inhibitory concentrations of crude methanol extract of seeds of *Phoenix dactylifera* were determined using agar diffusion method. The phytochemical analysis was conducted to determine the secondary metabolites.

Results: The preliminary sensitivity test performed shows that the crude methanol extract of date seed were sensitive against all organisms tested. The active ingredients of the extract showed activities against all strains of *E.coli* tested though their zones of inhibition vary (18-21mm). The minimum inhibitory concentration (MIC) value ranges from 10-19.9 mg/ml. The phytochemical analysis of crude methanol seed extract of *P. dacterifera* indicated the presence of alkaloids, flavonoids, tannins, carbohydrates, proteins, reducing sugar, sterols and Terpenes, Anthraquinone glycosides.

Conclusion: The present study provides the scientific information about *Phoenix dactylifera* seed activity.

Keywords: Phoenix dactylifera, , **antimicrobial activity, phytochemical analysis and methanol extract.**

Introduction

Health is the most precious of all things and it is the foundation of all happiness [1]. Traditional medicine has developed in various communities in Nigeria in response to the health needs of the people. African traditional medicine provides holistic treatment [2]. The type of treatment varies and includes the use of vegetative organs (leaves, barks, roots, seeds e.t.c) or products (latex, resins), whole or parts of animals and mineral substances (alum, kaolin). Others are fasting and dieting, treatment of burns, massage, psychotherapy, and faith healings [3]. Attempt have been made by scientists to justify or rationalize on a scientific basis many aspect of the practices of the African traditional medical practitioners, some of these medical practices are inexplicable whereas others like the use of many of the herbs, can be rationalized [4].

In recent times, the rapid development of multi-resistance strains of chemically imported pathogens attracts the interest of scientists to develop newer broad spectrum antimicrobials. The development of synthetic antibiotics such as the third and fourth generation of cephalosporin appear to be potent but are scarce, costly and not affordable particularly in developing countries and make compliance difficult [5]. These necessitate looking for substances from alternative medicine with claimed antimicrobial activity. Efforts in this regards have focused on plant because of their uses historically, and the fact that a good portion of the world's population (80%) particularly in developing countries rely on plants for the treatment of infectious diseases and non infectious diseases [6]. Therefore, any deduction of the therapeutic effect of a given plant parts is backed by an identification and characterization of the active ingredients in the plant. A proper study of African plants antimicrobial properties, constituents, bactericidal and bacterostatic activities would help and facilitate the development of a desired effect of a given plant. In this work, screening was conducted using seed of Date plant*; phoenix dactylifera* for its antibacterial activity against a clinical isolated strains of *Escherichia coli* and some bacterial species such as *Pseudomonas aeruginosa, Enterococcus, Klebsiella, Staph. Spp, Salmonella, Bacillus and Candida albicans.*

Materials and Methods

Bacterial and fungal strains used in preliminary studies

Preliminary screening of the crude extract was carried out using clinical isolates of *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa, Bacillus subtilis, Salmonella typhi, Candida albican* and *Enterococcus* collected from department of Pharmaceutical Microbiology and Biotechnology Laboratory while 19 different strains of *E,coli* were isolated from 35 urine samples collected from Bishop Shanahann Hospital, Nsukka. The urine samples were inoculated unto sterile MaCconkey agar and Eosine methyl blue agar and the isolates were characterized using standard microbiological techniques

.

Nutrient media

Nutrient agar (FLUKA limited) for bacteria were used for inoculation on plates, slants and tubes. Sabouraud Dextrose agar (FLUKA Limited) for fungi were used for inoculation of plates, slants and tubes as well.

Antibiotic

Ciprofloxacin

Solvents

Methanol (Merck,Germany), Dimethyl sulphoxide (DMSO) (May & baker, England), Distilled Water (Laboratory grade).

Materials used

Test tubes, petri -dishes, pipette, measuring cylinder, flatbottom flask, burnsen burner, autoclave, refrigerator, cotton wool, Weighing balance, foil, wire loop, masking tape

Collection and authentication of plant material

Date fruits (*phoenix dactylifera*) were bought from Jos, Plateu state of Nigeria season (February – March 2016). Identification and authentication were done by Mr Alfred Ozoiko, a taxonomist with the International Center for Ethnomedicine and Drug Development (Inter CEDD) Nsukka with Voucher no interCEED 042.

Processing of *Phoenix dactylifera* seeds

Fruit pulp was removed and the seeds were washed thoroughly to get rid of any adhering date flesh, and air dried. They were further dried at temperature of 30 ^{0}C for 5 hrs. The seeds were milled powdered and sieved through a 2.0 mm sieve size to remove larger particles and fiber. The processed root was then stored in sterile and air-tight container for further use.

Preparation of extract

A One kilogram (1 kg) ground powdered seeds were weighed subjected to extraction with 80% methanol by cold maceration. A 500 ml volume of methanol was poured into the container containing the powdered seed and left for 24 h. This was filtered with gauze and a funnel into a vessel. The filtrate in a closed container was poured into an evaporating dishes and left to dry at 30^{0} C. After extraction, 4 g of dried extract were gotten and was poured into a clean container and placed in the refrigerator at 4 ^{0}C.

Antimicrobial screening

The antimicrobial activity of methanol extracts of *Phoenix dactylifera* seeds was determined by agar well diffusion method and agar disc diffusion method for standard antibiotics. The test organisms were sub cultured into a fresh nutrient agar and the concentration of working stock culture was assessed as 10^{-6} Cfu/ml. For susceptibility test, 100µl of inoculums mixed with 19 ml of sterilized Mueller Hinton agar and poured immediately into the sterile petri dishes. The petri dishes were left to solidify for 10 minutes. A sterilized 6 mm cork borer was used to make wells in the centre of the divided areas. Few drops of each extract were pipetted into the wells. The petri dishes were incubated at 37ºC for 24 h. The experiment was done three times to minimize error. After incubation period, the antimicrobial activity was evaluated by measuring the inhibition zone.

For *candida albicans*, Sabouraud agar was included. The inoculated Petri dishes were incubated at 25ºC for 48 h

The bacterial inhibitions were compared with ciproflaxacin disc (5µg). Few drops of DMSO were pipetted into each well for bacteria and fungi as negative control. The extracts that exhibited inhibition zones were subjected to minimum inhibitory concentration (MIC) assay using diffusion method.

A quantity of 1 g of the extract was dissolved in 3 ml of DMSO which yield initial concentration of 333.33mg/ml. Subsequently, two –folds serial dilution was made from the stock to obtain 166.65, 83.32 and 41.66 mg/ml concentrations.

The agar plates was prepared and allowed to solidify. A 0.1 ml of the test organisms was smeared on the surface of the mueller Hinton agar in the petri dishes using a sterile glass rod and allowed to stand for five minutes in aseptic condition. Five wells were made on the agar using cork borer of 6 mm [15][16]. Six drop of each concentration was transferred into each and labeled. The positive control which is standard drug (ciprofloxacin) and negative control DMSO which was used to fill one of the wells. A pre- diffusion time of 30 mins was allowed before incubation. Bacteria were incubated at 37ºC for 24 h. Zone of inhibition was determined using measuring ruler.

Zones of inhibition were determined using measuring ruler.

The MIC was estimated as follows;

- Obtaining the mean of inhibitory zonal diameter (IZD) of each wells strains of *E.coli*.
- Calculating logarithm of the concentrations.
- Plotting the graph of mean IZD against Log concentration.
- Finding the intercept and calculating anti-log of the intercept which is the MIC

Phytochemical analysis of the extract

The phytochemical analysis was conducted at the department of pharmacognosy university of Nigeria Nsukka using harborn method.[17]

Test for Alkaloids

A quantity 0.5 g of *Phoenix dactylifera* seed extract was stirred with 5 ml of 1% aqueous hydrochloric acid on a water bath. 1ml of the filtrate was treated with a few drops of Wagner's reagent; a reddish brown precipitate was observed. The second 1ml portion was treated similarly with dragendorff's reagent. A reddish brown precipitate was observed

Test for flavonoids

A quantity 0.1 g of the extract was dissolved in 1ml ethanol. 1ml of 10%ferric chloride was then added to it. A brown solution with dirty green precipitate was observed.

Test for tannins

About 0.5 g of the extract was stirred with 1 ml of distilled water and filtered. 2ml of ferric chloride reagent was added to the filtrate. A blue-black precipitate was observed indicating presence of hydrolysable tannins

Test for saponins;

Frothing test

A quantity 0.5 g weight of the extract was shaken with water in a test tube. A honey comb froth which does not persist on standing was observed.

Haemolysis test

A 0.5 g of the extract was dissolved in 3 ml of distilled water and filtered. 0.5 ml of animal blood was added to the filtrate in the test tube. Another 0.5ml of animal blood was added to 0.5 ml of normal sodium chloride solution (0.9%) for control and allows standing for 10mins. The solution in the test tube containing extract and blood turned dull- red with no precipitate. While the tube containing normal sodium chloride and blood maintained the initial red color.

Test for reducing sugar

A quantity 0.2 g weight of the extract was dissolved in 5 ml distilled water, 5 ml of equal mixture of Fehling solutions A & B was added and boiled. A precipitate of brick-red color was formed.

Test for carbohydrate

Molish test: A quantity 0.5 g of the extract was dissolved in 3 ml of water and heated. 3 drops of molish reagent was added and small amount of concentrated sulphuric acid was carefully added from the side of the test tube to form a lower layer. A reddish colored ring at the interfacial ring was observed .

Test for sterols and triterpenes

A 2 ml extract was taken in a test tube, few drops of acidic anhydride and concentrated sulphuric acid was added to the test tube slowly. Formation of reddish brown ring was observed.

Test for anthraquinone glycoside

A 2 ml extract was taken in a test tube; 1 ml of ammonia was added and stirred. Appearance of reddish brown color in aqueous layer and green color in the bottom was observed.

RESULTS

Preliminary sensitivity test

The preliminary sensitivity test result shows that the methanol extract of seeds of *P. datylifera* has activity against *Bacillus subtilis, E. coli*, *Klebsiella pneumonia Enterococcus Pseudomonas aeruginosa and Candida albicans* (Table 1)

Table 1: Preliminary Susceptibility of the organisms to methanol extracts of *P.datylifera* seeds

Organisms	IZD(mm)
p. aeruginosa	20
E.coli	18
Enterococcus	20
Klebsiela Pneumonae	21
Staphylococcus auerus	20
Salmonella	20
Bacillus	21
Candida	20

Phytochemical analyses of crude methanol extract of *P. datylifera* seed

The solvent was removed under pressure to obtain 3.8 g of the crude extract. Phytochemical screening of the extract reveals the presence of alkaloids, tannins, steroids, glycosides, flavonoids, reducing sugar, carbohydrates, proteins, anthraquinone glycoside, sterol and triterpenes

Table 2: The phytochemical analysis of crude methanol extract of *P. datylifera* seeds.

Secondary metabolites	Composition
Alkaloids	++
Flavonoids	+++
Tannins	+++
Steroids	-
Carbohydrates	++
Proteins	++
Reducing sugar	+++
Sterol and triterpenes	++
Anthraquinone glycoside	+++

Key:

+++: Abundantly present

++: Present

- : Absent

The susceptibility test result

The susceptibility test result shows that the active ingredients of the methanol extract of *P datylifera* showed activities against all the strains of *E.coli* tested though their zones of inhibition vary (Table 3)

Table 3: Susceptibility Test of *E.coli* strains to methanol extracts of *P datylifera* seeds

Strains of *E.coli*	Zone of inhibition (mm) Concentrations of the extract (mg/ml)				Negative Control DMSO	Positive Control Ciprofloxacin (5µg)
	333.33 mg/ml	166.65 mg/ml	83.33 mg/ ml	41.66 mg/ml		
UE_9	19	16	10	5	0	28
UE_{13}	16	13	12	7	0	22
UE_{15}	6	5	4	2	0	23
UE_{16}	8	6	4	2	0	31
UE_{20}	12	11	10	7	0	20

UE_{25}	19	16	10	6	0	27
UE_{26}	22	19	14	11	0	19
UE_{30}	20	15	14	10	0	18
UE_{35}	19	16	13	7	0	22

Table 4: MIC of phoenix dactylifera.

Isolated strains of *E-coli*	MIC (mg/ml)
UE_{9}	15.8
UE_{13}	15.8
UE_{15}	19.4
UE_{16}	19.9
UE_{20}	15.8
UE_{25}	15.8
UE_{26}	14.1
UE_{30}	10
UE_{35}	14.1

Discussion

This study investigated antibacterial effect of *Phoenix dactylifera* methanolic seeds extract against different isolated strains of *Escherichia coli* from urine samples and clinical samples of some selected strains of organisms. Dates have medicinal uses including anticancer, antihyperlipidemic, hepatoprotective activities and thereby serving as an essential healthy food in the human diet [18]. The date fruit is used in folk medicine to treat the different infectious diseases probably because of their antibacterial ability, immunomodulatory activity and antifungal property [19].

Our study showed that the date seed powder contains alkaloids, flavonoids, tannins, saponins, phenol, and sterols and triterpenes. These phytochemicals were quantitatively analyzed in the study by Abiola *et al*[20]. Furthermore, study demonstrated that administration of date seed extract significantly increased the paraoxonase and arylesterase activity in hypercholesterolemic rats [21]. Eimad *et al.* also suggested that date seeds have potential to be used as ingredient in food additives, cosmetics, and pharmaceutical industries. All these reports prove that date's seed has potential to be used in pharmaceutical, cosmetic, and industrial applications. The bacterial inhibition may be attributable to date seed extract's heatlabile bioactive components attaching to bacterial surface. The bioactive component, namely, protein and some derived polyphenolic compounds such as polysaccharides, lignans and bioflavonoids, are present in reasonable amounts

in date seed [22]. Tannin in date seeds also known as antimicrobial agent have contributed in the antimicrobial properties of the plant due to its ability to precipitate protein thereby denaturing the peptidoglycan components of the bacteria cell wall.[23]. Therefore, further research is needed to characterize isolated components and search for bioactive constituents with antimicrobial, antioxidant and other health- promising activities. The current study on the antibacterial activity adds significance to the medicinal property of the date seeds.

Conclusions

Based on the observed results of this study, it can be concluded that date seeds have potential for antibacterial activity and suggested that it may be used in treatment of the infections caused by *E.coli*.

COMPETING INTERESTS

The authors have not declared any conflicts of interests.

REFERENCES

1. Adenisa SK, Idowu O, Ogundaini AO, Oladimeji H, Olugbade TA, Onawunmi GO, Pais M. Antimicrobial constituents of the leaves of *Acalypha wilkesiana* and *Acalypha hispida*. Phytother. Res. 2000;14:371–374.

2. Ali A, Memon, MS. Incorporation of *Enteromorpha procera* Ahlner as nutrition supplement in chick's feed. Int. J. Biol. Biotechn. 2008;5 (3/4): 211-214

3. Sofowora A, Ogunbodede E, Onayade A. Afr J Tradit Complement Altern Med. 2013;10(5):210-229 http://dx.doi.org/10.4314/ajtcam.v10i5.2

4. Comican MG, Jones RN. Antibacterial activity and spectrum of LB20304, a novel fluoronaphthyridone. Antimicrob Agent Chemother. 1997;41:204–211.

5. Martínez-Pastor MT, Marchler G, Schuller C, Marchler-Bauer A, Ruis H, Estruch F. The Saccharomyces cerevisiae zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). *EMBO J*. 1996;15(9):2227-35

6. Abdelhak M, Guendez E, Eugene K, Kefalas P (2005). Phenolic profile and antioxidant activity of the Algerian ripe date palm fruit (Pheonix dactylifera). Food Chemistry. 2005;89:411-420.

7. Wainwright M, Swan H TC, Paine G. Moulds in ancient and more recent medicine. 1989;3 (1) Pages 21-23

8. Kingston W. Irish contributions to the origins of antibiotic. Irish journal of medical science. 2008;177(2); 87-92 dio; 10; 1007/S1/845-008- 0139.

9. Wolfgang S, Kingston W. Irish contributions to the origins of antibiotic. Irish journal of medical sciences. 1999;173(2);87-92. dio;10; 1007/S1/845-008-0139

10. Bonev B, Hooper J, Parisot, J. "Principles of assessing bacterial susceptibility to antibiotics using the agar diffusion method". The Journal of Antimicrobial Chemotherapy. 2008;61 (6):1295–301. doi:10.1093/jac/dkn090. PMID 18339637.

11. Andrews JM. Determination of minimum inhibitory concentrations.J Antimicrob Chemother. 2001;48 Suppl 1:5-16. Department of Microbiology, City Hospital NHS Trust, Birmingham B18 7QH, UK. Jenny.Andrews@cityhospbham.wmids.nhs.uk

12. Feng QL, Wu J, Chen GQ, Cui FZ, Kim TN, Kim JO. A mechanistic study of the antibacterial effect of silver ions on Escherichia coli and *Staphylococcus aureus*. J Biomed Mater Res. 2000;52:662–8.

13. Jorgensen J H, Howell AW, Maher LA. Antimicrobial susceptibility testing of less commonly isolated *Haemophilus* species using *Haemophilus* test medium. J. Clin. Microbiol. 1990;28:985-988

14. Nyenje M, Ndip RN. In-vitro antimicrobial activities of crude acetone extract of the stem bark of Combretum molle against selected bacterial pathogens J Med Plants Res. 2011;44:534-9.

15. Okore, V.C. Pharmaceutical Microbiology. Principle of the Pharmaceutical Application of Antimicrobial Agents. El demak Ltd, 9 Ani Stree, Ogui New Layout, Enugu. 2005; pp.186,191, 205-228.

16. Adesina SK. Traditional medicinal care. In; Ajaegbu, H.L St Mathew-Daniel, B.J, Uya, D.E (Eds). A people united, A future assured, A compendium. Millennium Edition. Publication of federal ministry of information. 2000;1:285-290.

17. Harbons J.B. Textbook of Phytochemical Method, Champman and Hall Ltd , London. 1973; pp 49-55

18. Biglari F, AlKarkhi AFM, Mat E A. (2009). Cluster analysis of antioxidantcompounds in dates (*Phoenix dactylifera*): Effect of longterm cold storage. Journal of Food Chemistry. 2009;998–1001.

19. Baliga S, Baliga V, Kandathil S. A review of the chemistry and pharmacology of the date fruits (*Phoenix dactylifera L.*). Journal of Food Research International. 2011; 44: 1812-22.

20. Abiola M, Adeosun M , Sarah O, Oni M, Osasenaga M. Ighodaro P, Okikiola H, Durosinlorun B, Omotayo M , Oyedele B. Phytochemical, minerals and free radical scavenging profiles of *Phoenix dactilyfera L.* seed extract. https://doi.org/10.1016/j.jtumed. 2015;11.006

21. Mohammedi Z, Atik F. Impact of solvent extraction type on total polyphenols content and biological activity from tamarix aphylla (L) karst. International Journal of Pharma and Bio Sciences. 2011; (2)1 609-615. Retrieved from http://ijpbs.net/volume2/issue1/pharma/_64.pdf.education

22. M, H. H., C, P., E, M., V, C., & H, I. W. Polyphenolic compounds in date fruit seed (Phoenix dactylifera): characterisation and quantification by using UPLC-DAD-ESI-MS.. PubMed. 2013; 94(6), 1084-9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/24

23. Al-Farsi M, Chang Yong L. Optimization of phenolics and dietary fiber extraction from date seeds. J Food Chem. 2008;3: 977-985