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Phytochemical, High Performance Liquid Chromatography and Antimicrobial Evaluations of the Ethanolic Root Extract of *Phoenix dactylifera L.*

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Abstract

Bioactive compounds from plants have received a great deal of interest from scientists all over the globe for development of drugs. This study was aimed at evaluating phytochemicals, High Performance Liquid Chromatography (HPLC) and antibacterial activities of ethanolic root extract of *Phoenix dactylifera L*. (date plant) against some clinical isolates. Roots of date plant were dried and extracted with ethanol using the cold maceration method before concentrating it with water bath at 45 ^{o}C. The phytochemicals were identified using HPLC based on their individual retention time. Antibacterial activities of the methanolic root extract were assessed against some clinical isolates including *Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pyogenes,* and *Pseudomonas aeruginosa* utilizing agar disc diffusion method. The percentage yield of ethanol extract was 0.3892%. The result of the phytochemical screening revealed the presence of carbohydrate, phenols, tannins, saponins, flavonoids, cardiac glycosides, steroids, alkaloids and terpenes at various retention time of 2.853, 3.235, 4.129, 4.713, 6.722, 8.954, 11.228 and 22.912 min. Compounds identified include phenolic acid (caffeic acid) and two flavonoids (rutin and quercetin). The zone of inhibition diameter ranged from 15 to 19 mm. The maximum zone of inhibition was detected against *Streptococcus pyogenes,* (19 mm). *Staphylococcus aureus* and *Klebsiella pneumoniae* were inhibited with 17 mm and 16 mm zone of inhibition respectively, followed by *Pseudomonas aeruginosa* which had the least zone of inhibition (15 mm). The result of the present study suggests that the root of *Phoenix dactylifera L*. possesses important phytochemical components with antibacterial activity that could be possibly exploited for pharmaceutical development.

Keywords: Phytochemicals, Antibacterial activities, ethanolic root extract, Clinical isolates, *Phoenix dactylifera L*

1. Introduction

Since prehistoric periods, individuals have looked for medicines from nature to salvage their illness [1]. These medicines are typically obtained from plants and are conventionally referred to as herbs. The term "herb" was derived from the Latin word; "herba" and an old French word "herbe" which mean "grass" [2]. In the past, the term "herb" was only used for non-woody plants, including those that originate from shrubs and trees [2]. However, today, herbs refer to any part of plant that is used for therapeutic purposes. The presence of various bioactive compounds in plants has made them to be employed for other purposes such as in condiments and food, perfume or medicine as well as some certain cultural and divine events.

Bioactive compounds/phytochemicals such as alkaloids, glycosides, tannins, terpenes, phenols and varying essential oils are among many phytochemicals found in plants and have received increasing attention over the last three decades from scientists all over the globe for the development of various drugs [3]. Moreover, the manufacture of natural medicines or antimicrobials from plant origin has become a critical component in developing countries like Nigeria since they are cheap and easy to sort for [1]. The increasing rate of life-threatening infections, caused by pathogenic bacteria, are the leading cause of increased morbidity and mortality around the world [4]. Despite efforts put in place to manage this menace, however the causative microbes are gradually becoming resistant to multiple drugs making the drugs ineffective. As such, an improvement on the existing drugs or the development of new ones is essential to effectively eradicate infectious microorganisms. Bearing this in mind, botanist, pharmacologist, microbiologists among other disciplines have been combing the earth for solutions to tackle the menace posed by multidrug resistant bacteria [3].

Phoenix dactylifera L. is among the myriad plants exhibiting various nutritional and therapeutic benefits to man and animals. *Phoenix dactylifera L*. belong to the family Aceraceae, and is mostly found and grown in subtropical and tropical regions of the world [5,6]. Date plant, commonly called *Dobino* in Hausa

language in Nigeria is popularly known for its vital part in the everyday life needs for both human beings and animals in providing high nutritional and therapeutic values [5,7]. Because of these viable attributes and its invaluable nutritional benefits and its prolonged shelf life, date plant had since been declared as the 'tree of life' [8]. Various parts of date plant have been extensively utilized in folk medicine for treatment and alleviation of various ailment including; memory disturbances and nervous disorders, inflammation, paralysis, fever, unconsciousness and memory disturbances among many neural dysfunctions [5]. The roots are used by traditional healers in the treatment of various toothaches. These therapeutic benefits are often associated with the bioactive compounds present in date plant such as flavonoids, alkaloids, terpenes, saponins, glycosides among many others [3, 8-12]. Nevertheless, there is paucity of information on the bioactive compounds responsible for the therapeutic activities of the roots of date plant and thus, this study was aimed at evaluating phytochemicals, High Performance Liquid Chromatography (HPLC) and antibacterial activities of ethanolic root extract of date plant against some clinical isolates.

2. Materials and Methods

2.1 Collection and Preparation of date Plant Sample

Fresh samples of date plant (*P. dactylifera*) root were collected in October, 2019 from a fully grown date tree at Gbedako village, Lapai Local Government Area, Niger State, Nigeria. The root was authenticated at the National Institute for Pharmaceutical Research and Development (NIPRD), Idu, Abuja, at the herbarium unit and was assigned with voucher Specimen Number NIPRD/H/7075. The identified roots were washed with distilled water and were air-dried at room temperature for four (4) weeks. The dried roots were then crushed into fine particle size using clean mortar and pestle and further crushed into fine powder using electric milling machine. The powdered sample was stored in a firmly closed polythene bag and taken for extraction, phytochemical and other microbiological analysis at Department of Medicinal Plant Research and Traditional Medicine (MPR & TM) and Department of Microbiology and Biotechnology (MB & BT), respectively of the NIPRD.

2.2 Preparation of Ethanolic extract

One thousand millilitres (1000 mL) of 95% ethanol was poured into 100 g of powdered root of *P. dactylifera* and shaken for 6 h with rotary shaker. Thereafter, the mixture obtained was filtered utilizing a muslin cloth and passed through a sterile Whatman No. 1 filter paper. The filtrate was concentrated by method described by Orji *et al.* [13] using water bath at 45°C to eliminate the moisture content into dried form by evaporation. The yield of concentrated extract was calculated utilizing the formula,

Yield (%) = $(W_1 \times 100)/W_2$

Where, W_1 was the extract's weight before evaporation and W_2 was the dry weight of the extract. The extract was stored in a container that is air proof for further analysis.

2.3 Phytochemical screening of the extracts

Qualitative phytochemical screening was performed on the concentrated root extract of *P. dactylifera* to test for the presence or absences of some secondary metabolites. These tests are described as follows:

2.3.1 Molisch's Test for Carbohydrates

Method reported by Sofowora [14] was used to test for carbohydrates, in which concentrated *P. dactylifera* root extract (0.5 g) was dissolved in a test tube containing 5 mL of distilled water before filtering it. Molisch's reagent (5 drops) was added to the filtrate before adding 1 mL of concentrated H_2SO_4 by the side of the test tube and left to stand for 3 min. Distilled water (5 mL) was further used to dilute the solution and the production of a red colour or dull violet between the interphase produced was taken as a positive result.

2.3.2 Test for Phenols

The phenolic content of the extract was determined in accordance to the method described by Evans [15], in which half gram (0.5 g) of ethanolic extract was added with three (3) drops of the same concentration of 1% (w/v) solution of ferric chloride ($FeCl_2$) followed by 1% (w/v) gelatine in sodium chloride ($NaCl_2$). The precipitate formation suggested the presence of phenols.

2.3.3 Test for Tannin

The tannin content in the extract was assessed in accordance to the method documented by Amorim *et al.* [16], in which 0.1 mL of the ethanolic root extract of *P. dactylifera* were added into a test tube containing 7.5 mL of distilled water after which 1 mL of 35% sodium carbonate solution and 0.5 mL of Folin-Ciocalteu phenol reagent was added. The production of blackish blue colouration showed the presence of tannins.

2.3.4 Test for Saponin

The saponin content of the extract was determined by dissolving one gram of extract into 4 mL of distilled water. Production of 10 min persistent foam was suggestive of the presence of saponins [15].

2.3.5 Test for Cardiac glycosides (Keller-Killani test)

One millilitre (1 mL) of crude extract was preserved with two millilitres (2 mL) of glacial acetic acid containing a drop of $FeCL_2$ solution, which was under laid with 1 mL of concentrated sulphuric acid (H_2SO_4). The formation of brown ring layers is an indication to the presence of cardenolides a feature of deoxy sugar. Sometimes, below the brown rings may be appearance of violet ring. However, in an acetic layer a thin greenish ring will be formed gradually [14].

2.3.6 Test for Flavonoid

The presence of flavonoid in the extract was assessed following the method documented by Kale *et al.* [17], in which a test tube was filled with 0.5 ml of the extract following the addition of methanol (1.5 mL) and 0.1 mL of aluminium chloride (10%), 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. The production of orange or red colouration shows the presence of flavonoids.

2.3.7 Test for Terpenes

The terpene content of the extract was determined using the method described by Evans [15]. Approximately half a gram (0.5 g) of the *P. dactylifera* root extract was mixed with 3 mL of concentrated H_2SO_4 and 2 mL of chloroform was cautiously added to form a layer. The formation of reddish-brown colouration at the interface suggested the presence of terpenes.

2.3.8 Test for Alkaloid

The total alkaloid content of the extract was determined using the method described by Singh *et al.* [18]. In this method, 1 mL of aliquoted extract was evenly mixed with 1 mL of 0.025 M $FeCl_2$ in 3 0.5 M HCl and 1 mL of 0.05 M of 1, 10-phenanthroline in ethanol. After which, incubation was done using a hot water bath at 70±2ºC for a period of 30 minutes. The indication of white precipitation showed the presence of alkaloids.

2.3.9 Liebermann-Burchard test for steroids

About 0.2 g of crude powder of the sample was dissolved in 2 mL of acetic acid and the solutions were cooled in ice water and concentrated H_2SO_4 was added carefully. Colour production from violet to bluish-green or blue showed the presence of a steroidal ring as described by Sofowora [14].

2.4 HPLC Analysis

The ethanolic root extract of *P. dactylifera* was subjected to HPLC analysis. The chromatographic separation was done on a Shimadzu HPLC system containing Ultra- Fast LC-20AB prominence equipped with SIL- 20AC autosampler; DGU-20A3 degasser; SPD-M20A UV diode array detector (UV-DAD); column oven CTO-20AC, system controller CBM-20A lite and Windows LC solution software (Shimadzu Corporation, Kyoto Japan). A reverse phase column (VP-ODS C18) with a particle size of 5µm, length of 150 mm and an internal diameter of 4.6 mm was used for HPLC. The method described by Adamu *et al*. [19] was utilized with some few modifications. The chromatographic conditions included mobile phase solvent A: 0.2% v/v formic acid in HPLC grade water and solvent B: HPLC grade acetonitrile; mode: isocratic; flow rate 0.6 mL/min; injection volume 10 µL of 10 mg/mL solution of extracts solution in the mobile phase; detection was at UV 254 nm wavelength. Reference standards catechin hydrate, gallic acid, chlorogenic acid, rutin, caffeic acid, quercetin, ferulic acid and luteolin were analysed independently under similar conditions with the extract. The operational conditions of the HPLC were set to give the following: column oven temperature of 40 °C and solvent B: 20% with a total run time of 30 min.

2.5 Clinical Isolates Collection and Maintenance

The clinical isolates (test organisms) used were obtained from the Department of Microbiology and Biotechnology, National Institute for Pharmaceutical Research and Development (NIPRD), Idu, Abuja, Nigeria. These organisms include: *S. aureus, P. aeruginosa, K. pneumoniae* and *S. pyogenes.* They were well-maintained on nutrient agar (NA) slants. These slants were refrigerated at 4 ºC. The microbes were inoculated in normal saline and stored for further use in accordance with the method described by Bamidele *et al.* [20].

2.6 Preparation of Chemicals and Media

All chemicals and reagents used were of analytical grade manufactured by BDH Chemicals, UK. Distillation of all organic solvents was carried out prior to use. The culture media, Mueller Hinton Agar (MHA) and Nutrient agar (NA) were prepared according to the manufacturer's guide.

2.7 Antimicrobial Sensitivity Testing

The sensitivity test on the extract was carried out based on standard protocol described by Bamidele *et al.* [20], Saddiq and Bawazir [21], and Paudel *et al.* [22] in which agar well diffusion method was employed. The test organisms were *P. aeruginosa, K. pneumonia, S. pyogenes* and *S. aureus.* The organisms were spread on MHA using a glass rod. Impregnation of filter paper discs (6 mm in diameter) was done in 20 µL ethanolic root extract and the discs were oven dried for 1 h at 37 °C before aseptically placing them on surfaces of inoculated MHA plates with respective test isolates. The inoculated MHA plates were then incubated at 37 °C for 24 h. Control plates were inoculated with streptomycin (30 µg) antibiotic discs as positive control. After incubation period has elapsed, the zones of inhibition were measured and recorded using a meter rule and the results of the antimicrobial activities were evaluated using the positive control. Streptomycin, a standard broad-spectrum antibiotic disc was used as a positive control in this study.

2.8 Statistical Analysis

The data obtained from this study was analysed using SPSS statistical package, version 20.0. ANOVA was used to compare the association between the zones of inhibition. The values were considered significant at $p < 0.05$.

3. Results and Discussion

3.1 Percentage (%) Yield of Crude Extract

The percentage yield of the crude root extract of *P. dactylifera* was found to be 0.3892%. This result is similar to the findings of Ado *et al.* [23] who in his study documented that bioactive compounds occur usually in low concentration. However, Dhanani *et al.* [24] reported in their study that extraction method that gives higher yield and minimal changes to the purposeful properties of the extract has to be employed. The ethanolic root extract yield in this study was not comparable to that reported by Dhanani *et al.* [24] in which date seed extract gave improved and higher yield of 32.8%.

3.2 Phytochemical Screening

The result of the phytochemical screening indicated the presence of carbohydrate, tannins, phenols, saponins, flavonoids, cardiac glycosides, terpenes, steroids, phenols, and alkaloids in the ethanol extract of *P. dactylifera* root as depicted in Table 3.1. The secondary metabolites revealed from this study were recognised for their broad spectrum biological and pharmacological activities in therapeutic applications [25]. They have been found to be active and effective in treating most common pathogenic strain infections caused by microbes such as *S. aureus* [26, 27]. The phytochemical components present were also comparable to the result reported by Delphin *et al.* [28] in their study using date seeds. However, in a similar study, the petroleum ether extract of date seed only revealed the presence of diterpenes, whereas the ethyl acetate extract revealed the presence of all other compounds except carbohydrates and chloroform, which demonstrated that ethyl acetate is a poor extraction solvent for bioactive compounds [29].

Flavonoids in plants have been used in the past to curb the spread of infectious diseases in outbreaks and are very popular due to their anti-cancer, antiviral, anti-allergic and anti-inflammatory activities [26]. Thus, it should not be shocking that they have been observed to be active and effective antimicrobial constituents against a varied array of microbes, as confirmed by Mierziak *et al.* [30] in-vitro. Biological activities of the date fruits, seeds and roots such as antimicrobial, antidiabetic, hypoglycaemic, anticarcinogenic, antioxidant, anti-inflammatory, antimalarial, and anticholinergic activities were reported to be due to momentous contribution of these secondary metabolites [31]. Tannins were also described by Negi *et al.* [31] as not only technologically valuable compounds, but also possess several physiological effects such as antisecretolytic, antiphlogistic, antimicrobial, antiparasitic and anti-irritant properties. Tannin containing plants were reported to be used as Phyto therapeutics to treat nonspecific diarrhoea, slightly injured skins and inflammations of mouth and throat [32]. The plant phenols are vibrant compounds used in eradicating the origins and proliferation of skin diseases, skin aging, and skin damage such as burns and wounds [33]. However, flavonoids and phenols play an essential role in plants antimicrobial activities [34]. The carbohydrate contents found in this study can confer daily energy requirements for bodily activities, particularly the nervous system and the brain [35], as well as reducing the sugar contents in the body, which are crucial for brain function and physical energy.

Table 3.1: Phytochemical Screening of The Root Ethanolic Extract of *P. dactylifera*

Phytochemicals	**Ethanol extract of *P. dactylifera* root**
Carbohydrate	+
Phenols	+
Tannins	+
Saponins	+
Flavonoids	+
Cardiac glycosides	+
Terpenes	+
Steroids	+
Alkaloids	+

Key: + = Present

3.3 HPLC Analysis

From the HPLC analysis, eight (8) peaks were detected from the ethanolic root extract of *P. dactylifera*. The identification of the peaks was based on retention times of 2.853, 3.235, 4.129, 4.713, 6.722, 8.954, 11.228 and 22.912 min respectively as shown in Figure 3.1 and Table 3.2. Caffeic acid appears at 4.713 min, whereas rutin and quercetin appeared at 6.722 and 22.912 min of retention time. The compounds identified in this study corresponded to some known phytochemicals such as phenolic acid (Caffeic acid) and flavonoids (rutin and quercetin) reported in *Laurus nobilis* by Kaurinovic and Vastag [36].

Table 3.2: Compound Table of the *P. dactylifera* from HPLC Analysis

Ethanol extract of *P. dactylifera root* retention time	**Identity**
2.853	Unknown
3.235	Unknown
-	Unknown
-	Unknown
4.129	Unknown
-	Unknown
-	Unknown
4.713	Caffeic acid
-	Unknown
-	Unknown
6.722	Rutin
8.954	Unknown
11.228	Unknown
22.912	Quercetiin

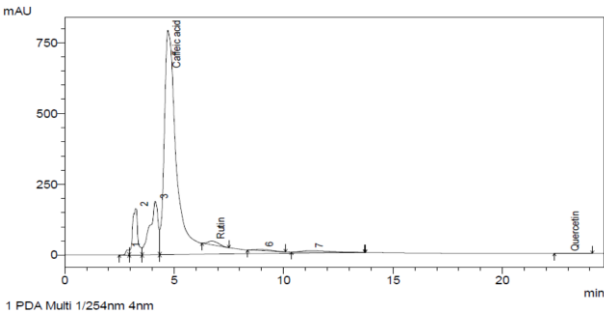

Figure 3.1: Chromatogram of Ethanolic Root Extract of *P. dactylifera*

3.4 Antimicrobial Activities of Crude Ethanolic Extract of P. dactylifera

In a study carried out by Paudel *et al.* [22], it was reported that the inhibition zone expressed in millimetre (mm) depend on diameter of the zone and divided by the antimicrobial activities in four intervals based on the inhibition zones recorded. They include low activity (diameters ≤ 10 mm), moderate (diameters between 10 and 15 mm), strong (diameters between 15 and 20 mm) and extremely strong (diameter ≥ 20 mm). The findings of the antimicrobial screening of the crude ethanolic extract of *P. dactylifera* roots against four (4) selected bacteria, which included two Gram negative *bacteria (K. pneumoniae* and *P. aeruginosa*) and two Gram positive (*S. aureus* and *S. pyogenes)* revealed that the zone of inhibition across tested isolates ranged from 15 to 19 mm in diameter. There were significant differences ($P \leq 0.05$) between treatments and control (Tables 3). The maximum zone of inhibition was detected against *S. pyogenes*, (19 mm). *S. aureus* and *K. pneumoniae* were inhibited with 17 mm and 16 mm zones of inhibition respectively. The least zone of inhibition (15 mm) was obtained for *P. aeruginosa*.

The findings from this study when compared to other naturals extracts, the root extract of date plant revealed to exhibit quite similar effective activities when compared to the control (Streptomycin) used. Although the ethanolic extract of the roots of date plant was able to reduce microbe's growth but was not effective enough as the microorganisms were able to grow to some certain extent. It is note-worthy to mention here that organic extracts of date roots represent an essential source of phenolic compounds involved as antimicrobial agents. Nevertheless, several similar research works indicating the presence of phytochemicals isolated from date roots have inhibitory potentials against the growth of various pathogenic bacteria [36]. Thus, the obtained bioactive extract from the root of date plants may serve as an alternative agent in reducing the proliferation of related pathogenic bacteria that serve as the causative agents in infectious diseases.

In this present study, the crude extract of date plant root was more efficient in inhibiting growth of all Gram-negative bacteria which is in concordance with the report from the earlier study by Saddiq and Bawazir [21] as they described antibacterial activity of aqueous extract of date plant pit against Gram negative bacteria such as *Escherichia coli* and *K. pneumonia*.

Table 3.3: Antimicrobial Activity of Ethanolic Root Extract of *Phoenix dactylifera* L.

Test organisms	Zone of inhibition (mm)	
	Streptomycin	**Date plant root extract**
Klebsiella pneumoniae	21	16
Pseudomonas aeruginosa	23	15
Staphylococcus aureus	24	17
Streptococcus pyogenes	18	19

4. Conclusion

Based on our findings from this study, it can be concluded that the roots of date plant (*P. dactylifera*) possess some important phytochemical components and antibacterial activity. Thus, further research work is needed for the extraction of various biologically active components existing in the date plant root that could be possibly exploited for pharmaceutical development.

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