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PHYTOCHEMICAL AND ANTIMICROBIAL STUDIES OF METHANOL EXTRACT AND FRACTIONS FROM STEM BARK OF *UAPACA AMPLIFOLIA* (DENIS)

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ABSTRACT

Phytochemical screening conducted on thecrude methanol extract, ethyl acetate and hexane fractions of the stem bark of *Uapaca amplifolia* (Denis) revealed the presence of alkaloids, terpenoids, glycosides, cardiac glycosides, flavonoids and steroids in the methanol extract and fractions. Tannins and anthraquinones were absent in the crude methanol extract and fractions of hexane and ethyl acetate. However, saponins and phlobotannins were present in methanol extract and ethyl acetate fractions respectively. Antimicrobial activity showed inhibitions against both gram positive and gram negative bacterial and fungal species which ranged between 20-23 mm for hexane fraction, 26-29 mm for ethyl acetate fraction and 24-27 mm for methanol extract. The n-hexane fraction had minimum inhibitory concentration of 10 mg/ml against Vancomycin resistant *enterococci*, *Klebsiella pneumonia*, *Candida albicans*, *streptococcus pyogenes*, *Escherichia coli Pseudomonas aeruginosa*, and *Candida krusei*. The ethyl acetate fraction showed activity against *Pseudomonas aeruginosa* and *Candida krusei* at 10 mg/ml while Vancomycin resistant *enterococci*, *Klebsiella pneumonia*, *Candida albicans*, *Streptococcus pyogenes* and *E. coli* had MIC at 5 mg/ml respectively. The methanol extract also showed activity against Vancomycin resistant *enterococci*, *Candida albicans*, *Streptococcus pyogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida krusei* at 10 mg/ml while *Klebsiella pneumonia* was inhibited at 5 mg/ml. Findings from this study showed that the extract and fractions from the stem bark of *U. amplifolia* contained antimicrobial compounds that are potentially useful for further investigation. The results support the ethno medicinal uses of *U. amplifolia* for the treatment of skin diseases, female sterility, wounds, fever, and Rheumatism.

Key words: Antimicrobial activity, Methicillin resistant *staphylococcus aureus*, Minimum inhibitory concentration, phytochemical screening and Vancomycin resistant *enterococci.*

INTRODUCTION

Plant-derived substances have been of great interest due to their versatile applications. Medicinal plants are the richest biological sources of drugs for traditional systems of medicine, modern medicines, nutraceutricals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs [1]. Since ancient times, natural products from plant sources remain one of the major sources of preventive and curative items. These result in the large population of people still being dependent on medicinal plants for their preventive and curative properties. Traditional medicines, including herbal medicine, have been, and are being used in every country around the world in some capacity. In most of the developing world, 80-95% of the population depends on these traditional medicines for their primary healthcare needs [2]. Herbal formulations have achieved widespread acceptability as therapeutic agents for the treatment of diabetes, arthritis, liver disease, cough and memory enhancers. According to the World Health Organization (WHO) there are three kinds of herbal medicines: raw plant material, processed plant material and medicinal herbal products [3]. Herbal medicines are widely used in the healthcare sector in both developed and developing countries. They are complex chemical mixtures prepared from plants and are limited in their efficacy because they are poorly absorbed when orally taken [4].

As a result of indiscriminate use of antimicrobial drugs in the treatment of infectious diseases, microorganisms have developed resistance to so many antibiotics. There is therefore the need to develop new and alternative antibioticsfrom plants. One approach is to screen local medicinal plants which represent rich source of novel antimicrobial agents. *Uapaca* species are part of ethno medicinal plants used in African in treating various diseases. Some of the species in the genus are represented in West Africa [5]. Two of the species *Uapaca stipularis* and *Uapaca kirkiana* are commonly used in folkloric medicine, which is a practice by lay people in the West African sub region. *Uapaca heudelotii* is ethnomedicinally reported to treat skin infections, female sterility, as an emetic, pile and gargle for tooth-troubles. It also serves as a source of highly-priced hard timber [6]. *Uapacatogoensis* is reported in the treatment of female infertility, as a restorative wash against fatigue and for making charcoal [7]. The wood of *Uapaca staudtii*is termite-proof, difficult to work upon because of its chemical composition and strength; it is used for making furniture, railway sleepers and barrel staves. *Uapaca paludosa* and *Uapaca vanhouttei* have also been reported for making charcoal and are used as firewood. *Uapaca*

amplifolia (Denis.) with names including Kaffafago (Hausa), Obia (Igbo) and Ajebge (Yoruba) [5] belongs to the family *Euphorbiaceae*. Itis an indigenous tropical African plant, which can be found in Madagascar, Congo, Nigeria and other Africa countries. It isan evergreen tree that usually grows up to 20 metres tall, though those of 30 metres tall occurs [8]. The stem is around 20 cm in diameter usually with stilt roots; however, these stilt roots are usually absent when the tree grows in the Savannah. In both Central and West Africa, stem bark of *Uapaca amplifolia* is given as traditional remedies against malaria, boils, wounds, rheumatism, skin diseases toothache and female sterility [9,10]. The root is used in the treatment of erectile dysfunction, pile and menstrual pain [10]. This paper reports the phytochemical constituents in extracts and fractions from *U. amplifolia* and its antimicrobial activity against some pathogenic microbes.

EXPERIMENTAL SECTION

Plant Materials

Fresh leaves, stems and root of *Uapaca amplifolia* were collected from Dogorawa in Sabon Gari Zaria of Kaduna State, Nigeria, in January, 2019. The leaves were identified and authenticated by the Herbarium section of Botanical Science Department, Ahmadu Bello University Zaria – Nigeria. Voucher number was **1279** was deposited there for further reference.

Extraction of the Stem Bark

The stem bark was removed from the stem, air dried and crushed to coarse powder. The powdered stem bark (500 g) was extracted first with the polar solvent methanol (1L) by cold maceration. The methanol extract was reconstituted in warm distilled water and partitioned with n-hexane and ethyl acetate to obtain their various fractions. The methanol extract and fractions were evaporated in a rotary evaporator at 40^{O}C under reduced pressure [11].

Phytochemical Screening

Extract and fractions obtained were subjected to various phytochemical tests to identify the constituent secondary metabolites using standard methods [12, 13].

Test for Alkaloids

Wagner's test: the extract (0.2 g) was stirred with 1% hydrochloric acid (5 ml) on a water bath and filtered. To the filtrate (1 ml) was added, 2-3 drops of the Wagner's reagents (solution of

iodine in potassium iodide) were added. A reddish –brown precipitate indicated the presence of alkaloids.

Test for Saponins

Frothing test: The extract (0.2 g) was shaken with water (5m1) in a test tube for 30 seconds. A persistent froth for 15 minutes or when warmed on water bath indicates the presence of saponins.

Test for steroids/ terpenoids

Salkowski's test for steroids: The extract (0.5g) was dissolved in chloroform and concentrated sulphuric acid (2ml) was carefully added down the side of the test tube to form a lower layer. A reddish brown colour at the interface indicates the presence of steroidal ring.

Test for Tannins

Ferric chloride test: The extract (0.2g) was stirred with water (5ml) and filtered. To the filtrate (2ml) in a test tube, two drops of ferric chloride solution were added. A green or greenish black precipitate indicate the presence of condensed tannins, while a blue or bluish black precipitate shows the presence of hydrolysable tannins.

Test for Flavonoids

Shinoda's test: The extract (0.2 g) was diluted with ethanol. Some pieces of magnesium filing were added followed by five drops of concentrated hydrochloric acid. A pink or red colour indicates the presence of flavonoids.

Microorganisms

The microorganisms used include Methicillin resistant *staphylococcus aureus* (MR*SA*), *Vancomycin resistant enterococc i*(VR*E*), *Staphylococcus aureus, Bacillus subtilis, Streptococcus pyrogenes, Salmonella typhi, Pseudomonas aeruginosa, Klebsiella pneumonia, Escherichia coli, Shigella dysenteriae, Candida albicans and Candida krusei.* They were obtained from the Department of Microbiology, Ahmadu Bello University Teaching Hospital Zaria - Nigeria.

Antimicrobial Activity

Preparation of the extract and fractions for antimicrobial screening

Stock solutions were prepared by dissolving 0.4 g of the methanol extract, n-hexane and ethylacetate fractions in 10 ml of Dimethylsulphoxide (DMSO) to obtain a concentration of 40 mg/ ml. From the stock solution, serial dilution of various concentrations (40 mg/ml, 20 mg/ ml, 10 mg/ ml, 5 mg/ ml and 2.5 mg/ml) were obtained for the extract and fractions.

Sensitivity test: The agar well diffusion method was used [14]. The antimicrobial activities of the n-hexane, ethyl acetate fractions and methanol extract of the stem bark of *Uapaca amplifolia* were determined using stock concentrations of 40 mg/ml. The standardized inocula of the isolates were uniformly streaked unto freshly prepared Mueller Hinton agar plates with the aid of a sterile swab stick. Using a sterile cork borer (6 mm in diameter), five appropriately labeled wells were punched into each agar plate. 0.2 ml of the appropriate extract or fractions were placed in each well and then allowed to diffuse into the agar. The plates were then incubated at 37 ^{o}C for 24 hours. However, Sabouraud dextrox agar was used for the fungi and the incubation period was 48 hours at 25 ^{o}C .The antimicrobial activities were expressed as diameter of zones of inhibition produced by the plant fractions and methanol extract are expressed in millimeter (mm) [15].

Determination of Minimum Inhibitory Concentration (MIC)

MIC was carried out using micro broth dilution in accordance with National Committee for Clinical Laboratory Standards [16]. Serial dilution of the extract and fractions was prepared between 2.5 mg/ml and 40 mg/ml concentration. The tests tubes were inoculated with the suspension of the standardized inocula and incubated at 37°C for 24 h. MICs were recorded as the lowest concentration of the extract and fractions showing no visible growth of the broth.

Determination of Minimum Bactericidal/Fungicidal Concentrations (MBC/MFC)

MBC/MFC were determined by aseptically inoculating aliquots of culture from MIC tubes that showed no growth, on sterile nutrient agar plates and incubating at 37 °C for 48 h. MBC/ MFC were recorded as the lowest concentration of the extract and fractions showing no bacterial growth.

Isolation of active compound for antimicrobial activity

Column chromatography was the major technique used in the isolation of active compounds from the ethyl acetate fraction

Chromatographic separation of the ethyl acetate fraction

The ethyl acetate fraction (4.0 g) of *U. amplifolia* was eluted in a silica gel (60-120 mesh size) packed column of dimension 75 by 3.5 cm, the column was eluted continuously using 100% n-Hexane and later n-Hexane: Ethyl acetate mixtures. Thirty two fractions of 50 ml each were collected. The thirty two fractions were pooled together based on similarities in their TLC profile to give 9 major sub-fractions labeled as F1-F9. Fraction **F8** which formed a yellowish crystal and was consistent with two major spots was subjected to antimicrobial analysis.

Antimicrobial studies of the isolated compound F8

The antimicrobial activities of the isolated compound F8 and control (Ciprofloxacin and Fluconazol) were determined using some pathogenic microbes. 0.02 mg of the compound was weighed and dissolved in 10 ml of DMSO to obtain a concentration of 20 µg/ml which is the initial concentration used to determine its antimicrobial activity. From the stock solution, serial dilutions of the compound (10 µg/ml, 5 µg/ml, 2.5 µg/ml, 1.25 µg/ml and 0.62 µg/ml) were made. Mueller Hinton Agar was the medium used as the growth medium for the test microbes. It was autoclaved at 121°C for 15 minutes, cooled to 45 °C and 25 ml of the sterilized media was aseptically dispensed into sterilized Petri dishes. The media was covered and allowed to solidify. The antimicrobial activity was carried out using agar well diffusion method as described by Nostro, *et al.,* [17]. 0.1 ml standard inoculums of the test organisms were uniformly streaked unto freshly prepared Mueller Hinton Agar plates with the aid of a sterile swab stick. Using a sterile cork borer (6 mm diameter) wells were punched into each agar plate. Thereafter, 0.1 ml of the appropriate isolate concentration was placed in each well and allowed to diffuse into the agar. An extra plate was also streaked with the inocula and ciprofloxacin (5µg/disc) as reference drug was placed in it. The plates were incubated at 37°C for 24 hours. While for the fungus, Sabouraud dextrose agar was used at incubation period of 72 hours at 25°C.

RESULTS AND DISCUSSION

The crude methanol crude extract and fractions from the stem bark of *Uapaca amplifolia* were subjected to phytochemical tests and the results are shown in Table 1. The tests were positive for all the secondary metabolites tested except for flavonoids and tannins in the methanol extract. The ethyl acetate fraction revealed the presence of terpenoids, flavonoids, glycosides, cardiac glycosides, steroids, phlobatanins and saponins while anthraquinones and tannins were absent. The n-hexane fraction revealed the presence of all the tested secondary metabolites except phlobatanins, saponins and anthraquinones. In general, the accumulation and concentration of secondary metabolites are responsible for the observed antibacterial activity and this varies according to plant extracts depending on their polarity [18]. These metabolites actby different mechanisms and exert marked antimicrobial activities [19]. This result is similar to result obtained by Uwaiya *et al*., [20] and Atibioke *et al*., [21] which showed the presence of alkaloids, tannins, cardiac glycosides, steroids and terpenoids. Some glycosides and tannins have been reported to have hypoglycemic activities [22] and tannins are found to hasten the healing of wounds and inflammed mucous membrane. [23]. Rupasingh et al. reported the hypocholesterolemic and antidiabetic activities of saponins [24]. Terpenoids are also known to lower blood sugar in animal studies [25]. Alkaloids have been also known to exhibit marked physiological properties when administered to animals. Majority of plants used to cure diseases are reported to have traces of alkaloids in them [26]. Saponins and alkaloids are reported to possess ethno-pharmacological uses as analgesic, antiplasmodic and antifungal agents respectively [27].

Table 1: Phytochemicals constituents of the stem bark of *U. amplifolia*

Phytochemicals	nHF	EAF	ME
Alkaloid	+	+	+
Saponins	-	+	+
Tannins	-	-	-
C.Glycosides	+	+	+
Flavonoid	+	+	+
Glycoside	+	+	+
Phlobotannins	-	+	+
Steroids/triterpenes	+	+	+
Anthraquinones	-	-	-

Keys: HF = Hexane fraction; EAF = Ethyl acetate fraction;
ME = Methanol extract; + = present; - = absent

Table 2: Zone of inhibition in (mm) of the stem bark of *U. amplifolia*

Test organism	nHF	EAF	ME	Cipro	Fluco
MRS*A*	0	0	0	32	0
VR*E*	22	27	25	0	0
S.aureus	0	0	0	35	0
S.pyrogenes	20	28	24	0	0
E. coli	23	29	26	38	0
K. pneumonia	22	28	27	0	0
S. dysenterea	0	0	0	38	0
P.aeruginosa	20	26	24	30	0
S.typhi	0	0	0	40	0
C.albicans	22	27	25	0	35
C. krusei	20	26	24	0	34

Keys: nHF= Hexane fraction; EAF= Ethyl acetate fraction, mm= Millimeter
ME= Methanol extract; Cipro= ciprofloxacin; Fluco=fluconazol

Antimicrobial screening showed that the extract and fractions of *Uapaca amplifolia* exhibited moderate to good antibacterial activities. The result of Zones of inhibition (Table 2) shows zones of inhibitions which ranges from **20-23 mm** for the hexane fraction, **26-29 mm**for the ethyl acetate fraction and **24-27 mm** for the methanol extract against all the test organisms except Methicillin resistant *staphylococcus aureus*, *staphylococcus aureus,Shigella dysenteria*, and *salmonella typhi.* The results were comparable to the reference drugs used as positive control (Ciprofloxacin, **30-40 mm** and Fluconazol; **34–35 mm**). The result of the MIC in table 3 shows that the n-hexane fraction had MIC of 10 mg/ml against Vancomycin resistant *enterococci*, *Klebsiella pneumonia*, *Candida albicans*, *streptococcus pyogenes*, *E. coli*, *P. aeruginosa*, and *Candida krusei*, the ethyl acetate fraction showed activity against *Pseudomonas aeruginosa* and *Candida krusei*at a concentration of 10 mg/mlwhile Vancomycin resistant *enterococci*, *Klebsiella pneumonia*, *Candida albicans*, *streptococcus pyogenes* and *Escherichia coli* had MIC at 5 mg/ml respectively. The methanol extract also showed activity against Vancomycin resistant *enterococci*, *Streptococcus pyogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans* and *Candida krusei* at a concentration of 10 mg/ml while for *Klebsiella pneumonia* the MIC is at 5 mg/ml. The n- hexane fraction had MBC/ MFC of between 20-40 mg/ml; thee thyl acetate fraction had MBC/ MFC that is between 10-20 mg/ml while the methanol extract had MBC/MFC of 10-20 mg/ ml respectively. The ability of the crude extract and fractions to inhibit the growth and exert bactericidal effect on several bacteria is an indication of the antimicrobial potential of the stem bark of *U. amplifolia*, which makes the plant a good candidate for use as antibiotics. The ethyl acetate fraction showed the highest inhibitory activity against the test

organisms used compared to the methanol extract and hexane fraction. The sensitivity of the fractions to Vancomycin resistant *enterococci*, *Streptococcus pyogenes*, *Escherichia coli*, *Klebsiella pneumonia*, *P. aeruginosa*, *C. krusei* and *C. albicans* which are extremely difficult organisms to treat due to multiple drug resistance implies that the chemical compounds in the plant could be used to develop drugs to treat ailments caused by these organisms. Furthermore, the extract and fractions showed activity against gram positive and gram negative bacteria, as well as fungal species, these findings revealed that the fractions had broad spectrum activity against a wide range of pathogenic agents [28]. Previous antimicrobial studies on the roots extracts of *U .amplifolia* revealed that the methanol extract had MIC of 5 mg/ml against Vancomycin resistant *enterococci*, *Escherichia coli*, *Candida albicans* and *Candida krusei* whereas the n-hexane extract had MIC of between 10–20 mg/ml, a closely related result with the findings of these study [20].

Table 3: Results of MIC and MBC/ MFC of the stem bark extract of *U. amplifolia*

	MIC and MBC/ MFC (mg/mL)					
Test organisms	nHF	EAF	ME	nHF	EAF	ME
MRS*A*	0	0	0	0	0	0
VR*E*	10	5	10	40	20	20
S.aureus	0	0	0	0	0	0
S.pyrogenes	10	5	10	40	10	20
E. coli	10	5	10	20	10	10
K. pneumonia	10	5	5	40	10	10
S. dysenterea	0	0	0	0	0	0
P.aeruginosa	10	10	10	40	20	20
S.typhi	0	0	0	0	0	0
C.albicans	10	5	10	40	20	20
C. krusei	10	10	10	40	20	20

Keys: nHF = Hexane fraction; EAF = Ethyl acetate fraction; ME = Methanol extract; MIC= Minimum inhibitory concentration, MBC= Minimum bactericidalConcentration; MFC = Minimum fungicidal concentration

Table 4: Zone of inhibition in mm, MIC and MBC/ MFC of the isolated compound F8

Test organisms	F8	MIC µg/ml	MBC/MFC µg/ml	Cipro 5µg/disc	Fluco 5µg/disc
MRS*A*	35	1.25	2.5	0	0
S.aureus	32	1.25	2.5	32	0
S.pyrogenes	0	0	0	30	0
B. subtilis	27	2.5	10	0	0
E. coli	0	0	0	38	0
K. pneumonia	28	1.25	2.5	0	0
P.aeruginosa	0	0	0	31	0
S.typhi	0	0	0	40	0
C.albicans	26	5	10	0	34
C. krusei	0	0	0	0	31

Keys: Cipro= ciprofloxacin, Fluco =fluconazol, mm = Millimeters,MIC = Minimum inhibitory concentration; MBC = Minimum Bactericidal concentration; MFC = Minimum fungicidal concentration

The isolated compound F8 was also screened for biological activities using ten pathogenic microbes namely**,** Methicillin resistant *staphylococcus aureus*, *Staphylococcus aureus, Streptococcus pyogenes, Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *salmonella typhi,Candida albicans* and *Candida krusei*. The zones of inhibition (Table 4**)** of the isolated compound when compared to the reference drugs revealed that the isolated compound was more active than the reference drug ciprofloxacin against these organisms Methicillin resistant *Staphylococcus aureus* (35,0), *Staphylococcus aureus* (32,0), *Bacillus subtilis* (27,0), *Klebsiella pneumonia* (28, 0). Fluconazol had larger zones of inhibition than the isolated compound against *C. albicans* (26, 40). The ability of the isolated compound to inhibit the growth of fungal species also confirms the broad spectrum activity of the isolated compound. The results of the MICexpressed in µg/ml of the isolated compounds as shown in Table 4revealed that the compound inhibited the growth of Methicillin resistant *staphylococcus aureus* at 1.25 µg/ml while *staphylococcus aureus, Bacillus subtilis, Klebsiella pneumonia* had MIC of 2.5 µg/ml respectively. *Candida albicans* had MIC at 5µg/ml. This implies that the isolated compound is biologically active since it inhibited the growth of some of the organisms at much lower concentrations than ciprofloxacin and fluconazol which are standard drugs for the treatment of infections caused by these organisms. The result of the MBC/MFC for the isolated compound table 4expressed in micrograms per milliliter (µg/ml) equally revealed that Methicillin resistant *staphylococcus aureus* had MBC at 2.5µg/ml whereas *staphylococcus aureus* and *Klebsiella pneumonia* had MBC/MFC at 5 µg/ml. *Bacillus subtilis* and *Candida albicans* had MBC/MFC of 10µg/ml respectively. This implies that the isolated compound has a high activity and can therefore kill the organisms at much lower concentration; it also means that the isolated compound would be very effective in curing diseases caused by these microorganisms.

CONCLUSION

The result of this study revealed the antimicrobial potentials of the crude methanol extract and fractions of *Uapaca amplifolia.* The antimicrobial activity of the isolated compound F8 obtained offers significant potentials for the development of new antibiotics for the treatment of diseases

associated with the tests microorganisms. Results from this research validate the ethnomedicinal uses of the stem bark of *Uapaca amplifolia* for the treatment of several ailments by traditional medicine practitioners.

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