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Antimicrobial Activity of the Ethyl Acetate Extracts of *Chrysophyllum albidum* (African Star Apple) Cotyledons.

[*1]Babaiwa UF, [1]Ifijen DE, [2]Erharuyi O, [3]Eraga SO and [1]Akerele JO

[1]Department of Pharmaceutical Microbiology,

[2]Department of Pharmaceutical Chemistry,

[3]Department of Pharmaceutics and Pharmaceutical Technology,

Faculty of Pharmacy, University of Benin, PMB 1154, Benin City, 300001, Nigeria.

ABSTRACT

The challenge of increasing number of antibiotic-resistant nosocomial bacteria pathogens have led to a renewed focus on plants for possible discovery of lead molecule that could pave the way for new generation antimicrobial agent. This study evaluated the antimicrobial activity of the ethyl acetate extract of the cotyledons of *Chrysophyllum albidum*, with a view to exploring the antimicrobial properties of its chemical constituents. Powdered *C. albidum* cotyledons were screened for phytochemical constituents using standard methods and the extract obtained by maceration in ethyl acetate. The crude extract was evaluated for antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella aerogenes*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Candida albicans* and *Aspergillus niger* using standard agar-well diffusion method. Gas Chromatography-Mass Spectroscopy (GC-MS) was used to determine the chemical constituents of the extract. Alkaloids, phenolics, tannins, terpenoids and flavoniods were the major constituents present in the powdered cotyledons. A deep brown oily extract with a pleasant-sweet odour and a yield of 2.53% was obtained. The extract showed a high antimicrobial activity with inhibition zone diameter (IZD) range of 20.50 - 25.00 mm. Antimicrobial activity of the extract was bacteriostatic at minimum inhibitory concentration (MIC) value of 25.00 mg/mL and bactericidal at a concentration of 50.00 mg/mL (minimum bactericidal concentration (MBC)). Data obtained from GC-MS analysis showed presence of seventeen (17) components with 6 major spectral peaks; oleic acid (54.65%), n-hexadecanoic acid (13.27%), cis-9-hexadecenal (8.68%), betulin (7.23%), 2-pentanone (6.00%) and octamethyl (5.12%). The abundance of fatty acids in the cotyledons of *C. albidum* coupled with the observed inhibitory activities would present a potential candidate to be explored for the production of bioactive agents.

Keywords: Antimicrobial activity, *Chrysophyllum albidum*, ethyl acetate extract, GCMS

***Correspondence:** Upe Francisca Babaiwa upebabaiwa@uniben.edu +2348035467818

INTRODUCTION

Studies on herbal medicines used in folklore remedies have attracted the attention of many scientists in finding solutions to the problems of multiple resistance to existing antimicrobial agents. Investigations involving medicinal plants enjoy a choice of place in these studies because of the possible discovery of lead molecule that could pave the way for new generation of antimicrobial agents.

Chrysophyllum albidum is a dominant canopy tree of lowland mixed rainforest and sometimes in the riverine areas. It is widely distributed, spaning West Africa to the Sudan with an eastern limit in Kenya. It is often called the white star apple and distributed throughout the southern part of Nigeria where it is locally called "agbalumo" and "udara" in south-west and south-east, Nigeria respectively [1]. The fruit of *C. albidum* has immense economic potential as food as the fleshy fruit pulp is suitable for jams and potential source of iron and vitamins [1,2].

The roots, barks and leaves of *C. albidum* have been employed in folk medicine for the treatment of diseases. The bark is used for the treatment of yellow fever and malaria, while the leaf is used as an emollient and for the treatment of skin eruption, stomachache and diarrhea [1]. The cotyledons from the seeds are used as ointments in the treatment of dermatological infections in Western Nigeria and an excellent source of raw material for industries [3]. The extracts have also found use as liniments in the treatment of wounds. Its leaves and fruits have been reported to possess antioxidant properties *in-vitro* and *in-vivo* [4]. Studies suggest the cotyledons within the hard seed coat are useful for the treatment of vagina diseases [3].

Most of the documented antimicrobial activities of *C. albidum* seeds have been based either on their aqueous extraction or with organic solvents or fractions from partition between organic solvent [5-7]. A review of literature shows a lack of data regarding ethyl acetate extract of *C. albidum* cotyledon with respect to its antimicrobial activity, hence this study aims to determine the antimicrobial property and chemical constituents of *C. albidum* cotyledon using ethyl acetate as solvent for extraction.

MATERIALS AND METHODS

Materials

Ethyl acetate and dimethyl sulphoxide (DMSO) (JHD Science-Tech Co., China), ciprofloxacin powder (Sigma Aldrich, Germany), ketoconazole (Biochemika, India), Ripe fruits of *Chrysophyllum albidum* were purchased from a local market in Benin City in the month of April. The fruits were washed thoroughly and their seeds extracted. Extracted seed were washed, sun-dried for two (2) weeks and dehulled. The dehulled cotyledons were further dried for two days to a constant weight and pulverized using a kitchen blender (Kenwood BL460, England). The crude powdered cotyledon was stored in a desiccator under silica until use.

METHODS

Preliminary phytochemical analysis

Five grams (5.0 g) of the crude powdered *C. albidium* cotyledon was boiled with 75 mL of distilled water for 30 minutes. The solution was filtered hot and allowed to cool. The filtrate obtained was used to test for the presence of

alkaloids, tannins, saponins, anthraquinones, flavonoids and phenolic compounds in accordance with standard methods [8].

Preparation of crude extract

About 1.5 litres of ethyl acetate was used to macerate 500 g of the crude powdered *C. albidium* cotyledon for 72 hours. Intermittent stirring of the macerated mass was carried out with a glass rod stirrer at room temperature. After 72 hours the content in the maceration jar was filtered using a 125 mm pore filter paper (Whatman No. 42). Solvent was removed from the filtrate with a Stuart rotary evaporator operated at 40 °C and 250 rpm (Stuart RE400, UK). The resulting mass of extract was weighed before been kept in an air-tight bottle at 4 °C for further studies.

Standardization of test micro-organisms

Isolates obtained from the University of Benin Teaching Hospital, Benin City, (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella spp, Bacillus subtilis, Aspergillus niger* and *Candida albicans)* were sub-cultured on freshly prepared nutrient agar plates for bacteria and Sabouraud dextrose agar for fungi. Incubation of the plates was carried out for 24 hours at 37 °C for bacteria and for 72 hours at 25 °C for fungi respectively [9].

Portions of the streaked bacterial and fungal colonies were transferred into test tubes containing 8.0 ml of sterile nutrient broth and incubated for 12 hours at 37 °C and 72 hours at 25 °C, respectively. The growth of bacterial and fungal suspensions obtained were compared to that of freshly prepared barium sulphate opacity standard (0.5 mL of 1.0% barium chloride to 99.5 mL of 1.0% sulphuric acid (0.36 Normal). The turbidity was adjusted by adding more sterile nutrient broth to match 0.5 McFarland standards (10^8 CFU/mL).

Preparation of antibiotics and extract stock solutions

Using a 10% DMSO solution as diluent, stock solutions of ciprofloxacin, fluconazole and the extract were prepared with final concentrations of 0.5, 2.0 and 1000 mg/mL, respectively.

Test for antimicrobial activity of the extract

Using a modified agar well diffusion method, [10] antimicrobial susceptibility test was carried out. Mueller Hinton agar was prepared aseptically and poured into different Petri dishes, each containing 30 mL and allowed to set. The Petri dishes were dried in a hot air oven for about 10 minutes at 40 °C. The dried plates were then streaked with the test microorganism using a sterile swab stick. Four (4) wells were bored into each agar plate with a 10 mm sterile cork borer. Subsequently, two drops of molten agar was used in sealing the base of the wells, after removing the agar disks made with the cork borer. An equivalent of 200 mg (200 µL) of the extract was filled into each of two wells while the other two wells were separately filled with same volume of standard ciprofloxacin, equivalent to 0.1 mg or 100 µg of ciprofloxacin per well. Inoculated plates were incubated at 37 °C for 18 - 24 hours.

The same procedures were repeated for test fungi using Sabouraud agar and fluconazole as the standard and the inoculated plates incubated at 25 °C for 72 hours. Negative (10% DMSO solution) and positive (viability test for used organisms) controls were also conducted for

each experiment. Diameters of inhibition zone were measured in millimetres (mm) after incubation, as indices of the killing or inhibitory action of the test agents against a given microorganism.

Minimum inhibitory concentration (MIC) determination

The determination of the minimum inhibitory concentration (MIC) of the extract was carried out using the agar dilution method of Afolayan and Meyer [11]. A two fold serial dilution of the test extract stock solution (25.0, 50.0, 100, 200 and 400 mg/mL) and test drugs - ciprofloxacin/fluconazole (0.02, 0.04, 0.08, 0.16, 0.32 and 0.40 µg/mL) were carried out. Double strength nutrient or Sabouraud agar was prepared according to the Manufacturer instruction. Volumes ranging from 0.25 - 8.0 mL of the extract and 0.4 - 12.8 µL of ciprofloxacin/fluconazole with nutrient or Sabouraud agar was poured into 10 mL glass Petri dish and rocked gently, giving a concentration range of 25 - 400 mg/mL of the extract and 0.02 - 0.40 µg/mL of ciprofloxacin/fluconazole in the agar plates. Test bacteria or fungi inoculum was streaked with the aid of a sterile wire loop on each plate. Incubation of the inoculated plates was carried out at 37 °C for 18 - 24 hours for bacteria populations and at 25 °C for 72 hours for the fungi. After incubation, the plates were visually examined for growth in the inoculated spots. The lowest concentration of ciprofloxacin/fluconazole or the extract that inhibited growth was considered as the MIC.

Minimum fungicidal concentration (MFC) determination

The MFC was determined from the MIC plates by sequentially sub-culturing into 10 mL nutrient broth and then plated into 20 mL of double strength nutrient agar/Sabouraud agar that contained no extract. The plates were then incubated at 25 °C for 72 hours for the fungi [12]. After incubation, the plates were visually examined for growths and the lowest concentration of the extract that showed no growth was considered as the MFC in the fungal plates.

Gas chromatography - mass spectrometry analysis

GC-MS analysis of the extract was done using GCMS-QP2010 SE (Shimadzu, Japan) with capillary column specification of 5% phenylmethylsiloxane as stationary phase, 0.25 × 30 mm as internal diameter and a film thickness of 1.0 µm, in an Agilent 6890N gas chromatograph and Agilent Technologies 5973 Network Mass Selective Detector [13]. The carrier gas was helium with a constant flow rate of 3.22 mL/min and the inlet temperature set at 250 °C. The oven temperature was initially kept at 60 °C for 3.4 min then ramped at 12 °C/min to 240 °C. The temperature was gradually increased from 60 °C/min to 290 °C and held isothermally for 2 min. An amount of 1.0 µL of the sample solutions was injected in the split mode with split ratio 15:1. Mass spectra were obtained by electron ionization at 70 eV over the scan range m/z 1428. The compounds were identified by comparison of their mass spectra with those of NIST 05 L mass spectra library. The spectra match factor limit was set as 700 and any components with match factor less than 700 were not considered.

Statistical analysis

Data were reported as mean from triplicate determination for inhibition zone diameters (IZD) ± standard error. Data analysis and peak area measurement of raw GC-MS data were carried out using Agilent Chemstation and Pherobase software.

RESULTS

Results from the phytochemical analysis of the powdered seeds are presented in Table 1. The powdered cotyledons were found to contain alkaloids, phenolics, tannins, terpenoids and flavonoids.

Table 1: Phytochemical constituents of powdered *C. albidum* cotyledon

Phyto-constituents	**Occurrence**
Alkaloids	+
Carbohydrate	+
Flavonoids	+
Phenolics	+
Proteins	+
Reducing sugars	+
Tannins	+
Saponins	-
Amino acids	+
Phytosterol	+

(+) Present, (-) Absent

An oily deep brown coloured extract with a pleasant-sweet odour was obtained. There was a 2.5% yield from the extraction process. The extract showed good antimicrobial activity with IZD range of 20.5 - 25.0 mm. The extract inhibited the test organisms with varying concentrations range; minimum inhibitory concentrations (MICs) value of 25 mg/mL as expressed in Tables 2 and 3.

Table 2: Inhibition zone diameters of test extract and drugs against selected isolates

Test microorganisms	Ethyl acetate extract (mm)	Ciprofloxacin (mm)	Fluconazole (mm)
Staphylococcus aureus	25.10 ± 2.28	25.00 ± 0.94	NA
Escherichia coli	24.50 ± 0.76	34.00 ± 0.50	NA
Klebsiella spp	23.22 ± 0.13	22.00 ± 0.90	NA
Bacillus subtilis	20.50 ± 1.87	32.00 ± 0.50	NA
Pseudomonas aeruginosa	24.75 ± 1.89	32.00 ± 0.50	NA
Candida albican	0	NA	23.00 ± 0.6
Aspergillus niger	0	NA	23.00 ± 0.6

NA (Not applicable), Values ± Standard error

Table 3: Minimum inhibitory concentrations of the test extract and standard drugs against susceptible microorganisms

Test microorganisms	Ethyl acetate extract (mg/mL)	Ciprofloxacin (µg/mL)	Fluconazole (µg/mL)
	MIC	MIC	MIC
S. aureus	25.0 ± 0.011	0.02 ± 0.001	NA
E. coli	25.0 ± 0.011	0.02 ± 0.002	NA
Klebsiella spp	25.0 ± 0.021	0.02 ± 0.001	NA
B. subtilis	25.0 ± 0.022	0.02 ± 0.003	NA
P. aeruginosa	25.0 ± 0.013	0.02 ± 0.001	NA
C. albicans	> 400	0	0.32 ± 0.011

NA (Not applicable), Values ± Standard error

Data obtained from the GC-MS analysis of the extract showed that seventeen (17) components were detected with six (6) major peaks in the spectrum. These were; oleic acid (54.65%), n-hexadecanoic acid (13.27%), cis-9-hexadecenal (8.68%), betulin (7.23%), 2-pentanone (6.00%) and octamethyl (5.12%). Other chemical components in the extract accounted for a total of 5.05%, as shown in Figure 1 and Table 4.

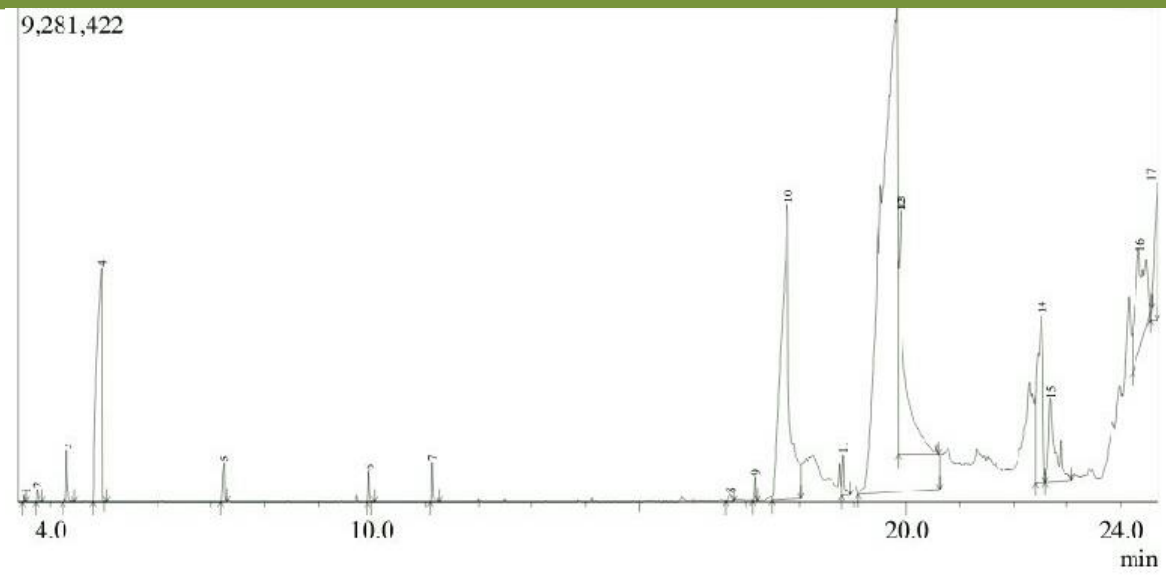

Figure 1: GC-MS spectra of ethyl acetate extract of *C. albidum* cotyledon

Table 4: Chemical constituents of ethyl acetate extract of *C. albidum* cotyledon

Peak number	Retention time (min)	Area (%)	Chemical compound
1	3.511	0.03	Propanoic acid
2	3.744	0.08	Propanoic acid
3	4.302	0.34	3-Penten-2-one
4	4.954	6.00	2-Pentanone
5	7.232	0.39	Ethanol
6	9.954	0.2	Acetamide
7	11.124	0.27	Formic acid
8	16.695	0.13	Eicosanoic acid
9	17.162	0.15	Hexadecanoic acid
10	17.765	13.27	n-Hexadecanoic acid
11	18.812	0.43	9-Octadecenoic acid (Z)
12	19.894	54.65	Oleic acid
13	19.896	8.68	cis-9-Hexadecenal
14	22.52	5.12	Octamethyl
15	22.691	2.86	2-Propen-1-amine
16	24.35	4.99	Betulin
17	24.692	2.42	Betulin

DISCUSSION

Extract obtained was oily in nature with a deep brown colour and a pleasant-sweet odour. The yield was 2.53% which was lesser than the literature value of ethanol and water extractive yields of 7.09% and 11.67% respectively [14]. The difference in yield could be attributed to variation in climate, plant species, soil condition and possibly processing techniques such as prolong exposure of harvested seeds to sunlight which is capable of impairing the yield considerably. It may also be due to factors such as the extraction process employed [15].

In general, crude extracts exhibiting inhibition zone diameters > 10.0 mm are considered to possess some level of antimicrobial activity [16]. Thus, it can be inferred that the ethyl acetate extract had a level of antimicrobial activity with IZD range of 20.5 – 25.0 mm. This is similar to the finding of Orijajogun *et al*. [17], who reported antimicrobial activity from the ethyl acetate extract of the exocarp fruits against *Escherichia coli*, *Staphylococcus spp* and *Klebsiella spp*. Additionally, the extract had a MIC of 25 mg/mL against all test microorganisms. This observation may be due to the abundance of fatty acids in the extract as previous biological assays have documented that antimicrobial lipids act as bacteriostatic (growth-inhibiting) or bactericidal (killing) agents depending on the drug concentration [18-20]. At a concentration of 0.02 µg of ciprofloxacin there was no bacteria growth in any of the plates seeded with micro-organism, which was due to the pure nature of this synthetic standard antimicrobial with confirmed efficacy compared to the extract which is still in its crude form and containing extraneous constituents that tend to dilute the active principle and reduce its activity.

Oleic acid (54.65 %) and n-hexadecanoic acid (13.27 %) which occurred in abundance in this extract belong to the fatty acids group. Fatty acid methyl esters have been reported to exhibit antifungal or antibacterial or both properties [21]. Antibacterial mode of action of these acids has been reported to target cell membrane, where they disrupts the electron transport chain and oxidative phosphorylation [22].

In addition, inhibition of enzyme activity, impairment of nutrient uptake, generation of peroxidation and auto-oxidation degradation products (cis-9-hexadecanal) and direct lysis of bacterial cells are other mechanism of causing bacteria death [23,24]. Their broad spectrum of activity, non-specific mode of action and safety makes them attractive as antibacterial agents for various applications in medicine, agriculture and food preservation [20]. Especially where the use of conventional antibiotics is undesirable due to the presence of multidrug resistant bacteria [25]. The potential for commercialization and biomedical exploitation of these antibacterial free fatty acids (FFAs) from natural product is of eminent importance as this could help curb the rising problem of multi drug resistant isolates in our clinics [20].

Betulin can easily be obtained from more than two hundred plants species, although the richest source of betulin is the betulaceae family, especially *Betula alba*, *B. pubescens*, *B. platyphylla* and *B. pendula* [26,27]. The activity of betulin acid has been linked to the induction of the intrinsic pathway of apoptosis also

known as programmed cell death. This is a sequence of organised reactions initiated by a damaged or stressed cell, leading to the death of the cell [28,29].

Furthermore, the observed antimicrobial activity of the study extract may not be due solely to the fatty acids present but in combination with the secondary metabolites present in the cotyledon. Alkaloids, phenolics, tannins, terpenoids and flavonoids have been found to have high antimicrobial activity against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumonia* [30].

It has been suggested that polyphenols act possibly by one or two mechanisms viz; binding to bacterial dihydrofolate reductase (DHFR) enzymes or inhibition of supercoiling activity of *E. coli* bacterial gyrase by binding to the ATP binding site of gyrase B or binding to bacterial DNA thereby inducing topoisomerase IV enzyme-mediated DNA cleavage and bacterial growth stasis [31]. Tannins are polymeric phenolic substances capable of precipitating gelatinous compounds from solution. Many human physiological activities, such as stimulation of phagocytic cells, host-mediated tumor activity and a wide range of anti-infective actions, have been assigned to tannins. Thus, their mode of antimicrobial action may be related to that of the quinones that not only have the ability to inactivate microbial adhesins but also enzymes and cell envelope transport proteins [32,33]. Previous studies have demonstrated that tannins are toxic to filamentous fungi, yeasts and bacteria. Tannins bound to bacteria cell walls induced bacterial stasis and protease activity [33,34]. Flavonoids are synthesized by plants in response to microbial infection and these compounds have been reported to be effective against many micro-organisms by their complexation with soluble proteins and cell wall components, resulting in microbial cell membrane disruption [35].

CONCLUSION

This study revealed that ethyl acetate extract of *Chyrsophyllum albidum* cotyledon have inhibitory effect at 25 mg/mL against *Stapyhlococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Bacillus subtilis* and *Klebsiella pyrogenes*. This could be attributed to the presence of a wide range of fatty acids such as n-hexadecanoic acid, cis-9-hexadecanal and oleic acid in the extract. Recognizing the challenges of antibiotic-resistant bacteria and taking advantage of the abundant supply of antimicrobial fatty acids in this extract, an excellent opportunity has been presented to further explore antimicrobial lipids as next-generation antibacterial agents for human health and medicine.

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