Phytochemical Screening And Biocidal Studies Of Stem Bark Chrysophyllum Albidum (Linn) And Straw Aristolochia Ringens.

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Abstract:
Introduction: Medicinal plants play a significant role in the health maintenance in underdeveloped countries, herbs and spices continue to serve as a new sources for herbal medicines.

Aims: The objective was to investigate phytochemical screening and biocidal activity on stem bark Chrysophyllum albidum (Linn) and straw Aristolochia ringens using chloroform.

Materials and Methods: Chloroform extracts of C. albidum and A. ringens were obtained and screened for phytochemical constituents. The colour intensity or the precipitate formation was used as analytical responses to different tests. The Antimicrobial activities of the crude extracts were evaluated against some microorganisms with broth microdilution test. The extracts were screened for in-vitro cytotoxic activity against liver tumour and breast tumour cells.

Results: The results of the phytochemical screening revealed the presence of alkaloids, saponins, steroids, anthraquinone and glycosides. C. albidum and A. ringens chloroform extracts showed high quantities of alkaloid and flavonoid respectively. While cardiac glycoside and anthraquinone were found with low quantity in C. albidum and A. ringens respectively.

C. albidum showed promising bacterial activity against Escherichia coli and cytotoxic activity to liver tumour cells. While A. ringens, showed excellent bacterial activity against Bacillus cereus and good fungal activity against Candida albicans. A. ringens also showed good cytotoxic to breast tumour and liver tumour cells. The observed antimicrobial and cytotoxic effects may be due to the antagonist or synergistic effect of the secondary metabolites identified in the chloroform extracts.

Conclusion: The secondary metabolites of C. albidum and A. ringens shows promising potential which support their consistent use in herbal medicines.

Keywords: Chrysophyllum albidum, Aristolochia ringens, Chloroform, Antimicrobial activity, cytotoxicity.

All co-authors agreed to have their names listed as authors.
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1. INTRODUCTION

Ever since ancient days, medicinal plants constitute one of the major sources to obtain a wide range of biological and pharmacological active compounds which have played a significant role in human society for treatment of wide array of ailments in various communities. Thus, extracts from herbs and spices have continue to play a very important role in all divisions of human population either directly as folk medicines or indirectly in the preparation of recent drugs used as antioxidant, antibacterial, antiparasitic, antifungal, cytotoxic, anti-Fusarium activities for food safety and consumption [1-7].

Chrysophyllum albidum (Linn) commonly known as white star apple is a Tropical forest fruit tree belongs to the Sapotaceae family with about 800 species [8]. C. albidum is native to West and Central Tropical Africa [9-11]. In Nigeria, it is found along forest savannah transitional and coaster savannah zones. The plant often grows to a height of 25-37 m in height with a mature girth varying from 1.5 to 2 m [12]. The tree has dark green, pale tawny with silver-white when mature, flowers appear on the leaf axis and fruit spherical in diameter, greenish-grey when unripe, turning orange-red when matured. Locally the plant is named ‘Agbalumo’, (Yoruba) ‘Udara’ (Igbo) according to the specific area. C. Albidum has been used traditional in herbal medicine to treat malaria and yellow fever, while the leaf is used as an emollient and for the treatment of skin eruption, stomach ache and diarrhea [13]. Studies have reported that seeds and roots extracts of C. albidium have demonstrated good antimicrobial, anti-inflammatory, anti-diarrheal and anti-hemorrhoidal properties [14].

Aristolochia ringens Vahl, one of the 500 aromatic species of Aristolochiaceae family, native to Brazil, Central America Mediterranean countries and the Caribbean [15]. The plant is commonly known by the Yoruba in the south western part of Nigeria as ‘okogun’. It is a rhizomatous aromatic climbing shrub. Aristolochia species are known to contain alkaloids and aristolochic acid [16]. Various Aristolochia species have been reported in herbal medicines since antiquity in obstetrics and in treatment of snakebite venom, festering wounds, and tumours, and they are still in use majorly in Chinese herbal medicine [17-19]. A. ringens is used to treat cholera, fever, bowel troubles, ulcers, leprosy, and poisonous bites [20,21]. The leaf, stem, and floral essential oil compositions of A. ringens have been previously reported [22-24]. The leaf oil has been dominated by sesquiterpenoids including (E)-caryophyllene (11.4%), trans-4(14),5-muuroladiene (13.0%), bicyclogermacrene (12.8%), spathulenol (8.0%), as well as the diterpenoid methyl copalate (10.3%). The study aimed at ascertaining the phytochemical constituents, antimicrobial and cytotoxic activities of chloroform extracts of C. albidum and A. ringens as an ingredient in the production of herbal medicine in Nigeria.

2. MATERIAL AND METHODS

2.1 Plant Collection

The stem bark of C. albidum (Linn) and straw A. ringens were purchased from a local market at Ibi Oro market, Lagos State, and authenticated at Botany Department, University of Lagos.

2.2 Plant Extraction

All the plant materials were washed thoroughly with distilled water and air-dried. Prior to crude extract, the plants were washed thoroughly with distilled water and air-dried for five days. Later, the plants were pulverized using an electric grinder and stored in a polythene bags until ready for use. 650 g of each pulverized plants of C. albidum and A. ringens respectively were soaked in 1.5 litres of chloroform for 24 hours. Thereafter, each crude extract were filtered using Whatman filter paper No 42 (125 mm). The filtrate were freeze dried, concentrated under reduced pressure of 300-500 mmHg at 50-60°C and preserved in refrigerator at 4°C for further use. The yield of the chloroform extract of C. albidum and A. ringens were determined and recorded as percentage of dry weight of the plants materials. All the chemicals used were of AR grade and were purchased from Olaolu Chemicals, Igbo Elerin, (Lagos).

2.3 Phytochemical screening

Phytochemical screening was performed on the extracts using standard procedures to identify chemical constituents.

2.3.1. Screening for Alkaloids

0.5 g of the extract was mixed with 5 ml of 1% HCl on a steam bath and filtered while hot. Distilled water was added to the residue and 1 ml of the filtrate was treated with a few drops of Wagner’s reagent. A reddish brown precipitate indicates the presence of alkaloids [25].

2.3.2. Screening for Flavonoids

2ml 10 % Sodium hydroxide was added to 5mL of distilled water containing 0.2 g of the chloroform extract. The appearance of a yellow colour indicates the presence of flavonoids [26].

2.3.3. Screening for Phenols

The chloroform extract (0.2 g) was diluted with 50% ethanol and added 3 drops of FeCl₃. Appearance of deep bluish green color indicates presence of phenols [26].

2.3.4. Screening for Tannins

0.2 g of the extract was dissolved in 5mL distilled water and filtered, few drops of 10% ferric chloride solution were then added to the filtrate. The appearance of a bluish black colour indicates the presence of tannins [26].

2.3.5. Screening for Anthraquinones

Crude extract (0.5 g) was mixed thoroughly with benzene (10 mL), and filtered. Followed by addition of few drops of 10% NH₃ solution shake and filtrated. The appearance of a pink, a reddish or violet color shows the presence of anthraquinones [25].

2.3.6. Screening for Cardiac Glycosides

Crude extract (0.5 g) dissolved in 2 mL glacial acetic acid containing few drops of 10% FeCl₃ solution followed by
addition of 2 mL of conc. sulfuric acid and filtered. Appearance of brown ring separation in between the mixture, indicates the presence of cardiac glycosides [26].

2.3.7. Screening for Phlobatannins

1 mL of (0.2 g) extract dissolved in 10 mL of water was added a few drops of 1% HCl and boiled. Appearance of red precipitation shows the presence of phlobatannins [26].

2.3.8. Screening for Terpenoids

Acetic anhydride (0.5 mL) was shaken thoroughly with 1 mL of (0.2 g) chloroform extract dissolved in 50% ethanol, followed by addition of few drops of conc. H₂SO₄. Appearance of bluish green precipitate shows the presence of Terpenoids [25].

2.3.9. Screening for Steroids

Crude extract (0.2 g) was mixed thoroughly with glacial acetic acid (2 mL) and the solution was heated, cooled, and then filtered. 0.5 mL of acetic anhydride was added to the residue, followed by the addition of few drops of conc. H₂SO₄. Appearance of violet to blue or bluish-green colour indicates the presence of steroids [25].

2.4 Quantitative determination of the phytochemical screening

2.4.1 Preparation of fat free sample

2 g of the sample were defatted with 100 mL of diethyl ether using a soxhlet apparatus for 2 h.

2.4.3. Determination of total alkaloids

200mL of 10% acetic acid dissolved in ethanol was added to (5 g) the crude extract in a 250mL beaker, covered and allowed for 4 h, and filtered, concentrate on a water bath until one quarter of the original volume was obtained. Aqueous ammonia solution was added dropwise to the mixture until all the crude extract was precipitated, washed, filtered and collected over aqueous ammonia solution. The solid alkaloid obtained was dried and weighed [27].

2.4.4. Flavonoid Determination of total flavonoid

10 g of the plant sample was extracted repeatedly with 100 mL of 80% aqueous methanol at room temperature. The whole solution was filtered through whatman filter paper No 42 (125 mm). The filtrate was later transferred into a crucible and evaporated into dryness over a water bath and weighed to a constant weight [28].

2.5. Antimicrobial Screening

The chloroform extract was screened for antimicrobial activity against Gram-positive bacteria, *Bacillus cereus*.

*Staphylococcus aureus*, *Staphylococcus epidermidis*; Gram-negative bacteria, *Pseudomonas aeruginosa*, *Serratia marcescens* and *Escherichia coli*. Minimum inhibitory concentrations (MIC) were determined using the microbroth dilution technique [29]. Dilutions of the solid extracts of *C. albidum* and *A. ringens* respectively were prepared in cation-adjusted Mueller Hinton broth (CAMHB) beginning with 50 μL of 1% w/w solutions of each crude extract in DMSO plus 50 μL CAMHB. The extracts were serially diluted (1:1) in CAMHB in 96-well plates. Organisms at a concentration of approximately 1.5 × 10⁶ colony forming units (CFU)/mL were added to each well. Plates were incubated at 37°C for 24 hr; the final minimum inhibitory concentration (MIC) was determined as the lowest concentration without turbidity. Gentamicin was used as a positive antibiotic control; DMSO was used as a negative control. Antifungal activity was determined as described above using *Candida albicans* in yeast-mold (YM) broth and infected leaves of tomato with approximately 7.5 × 10⁷ CFU/mL. Antifungal activity against *Aspergillus niger* and *Botrytis cinerea* were determined as above using potato dextrose broth inoculated with *A. niger* hyphal culture and grey mould horticulture respectively diluted to a McFarland turbidity of 1.0. Amphotericin B was the positive control.

2.6. Cytotoxicity Screening

Hepatocarcinoma cell line of human being marked (HepG2, CRL-11997™) cells grown in complete medium marked (DMEM: F12 made up of L-glutamine and sodium bicarbonate, 10% of FBS, with 1% penicillin/streptomycin) were kept warming at 37°C in a 5% CO₂ of the environment. Once about 90% of confluent, the cells were risen with phosphate buffered saline (PBS), containing about 0.25% (w/v) of Trypsin/EDTA, estimated and suspended in fresh complete media. 5 × 10⁵ cells/well (100 μL) seeded in a 96- well plates and warmed for 24 h to attach. Cells were then treated with the chloroform extracts at a concentration of 10 and 50 μg per mL in 200 μL medium for 72 hours. Thereafter, the medium was gently removed, and DMEM:F12 media with MITT (5 mg/mL in PBS) were added to the cells and warmed for 1 hr. The MITT medium was gently replaced with DMSO (200 μL/well), plate was then mixed carefully to allow the dissolution of formazan crystals. Absorbance was measured at 550 nanometer. All extracts were tested in triplicate.

MCF-7 (human breast tumor marked ATCC No. HTB-22) cells with a size of RPMI 1640 supported with 10% Fetal bovine serum (FBS), 30 mM HEPES, sodium bicarbonate, 100,000 units penicillin/streptomycin (10 mg/L) at pH=7.35 were prepared. MCF-7 cells plated in a 96-well cell, culture plates at a concentration of 1.2 × 10⁴ cells/well at a volume of 100 μL in each well were warmed at 37°C of 5% CO₂ for 2 days. After 2 days, the cells developed to about 80% growth. The supernatant solution was gently removed and resuspended with 100 μL growth medium containing 1 μL of the chloroform extract (1% in DMSO), to give a final conc. of 100 μg per mL (100 ppm). The plate was warmed at 37°C and 5% CO₂ for 2 days. After 2 days, the liquid was gently removed from the well. The absorbance were measured using spectrophotometric technique at 570 nm (using a Molecular Devices SpectraMax Plus 384 microplate reader) from each well at 100 μL of the MITT solution containing a pre-read absorbance. Formazan crystals were precipitated for a period of 4 h at 37°C and 5% CO₂. After 4h, the formed Formazan crystals were dissolved in a DMSO solution to give a purple crystal. Growing medium, DMSO, and tingenone (100 μg/mL) were used as negative and positive control compounds,
respectively. Eight replicate solutions were added to wells. The percent kill ratios (% kill\_compound / % kill\_control) were determined and average absorbance, standard deviations were also calculated.

3. RESULTS AND DISCUSSION

The results of chloroform extracts of *C. albidum* and *A. ringens* were shown in (Table 1). Chloroform extracts values were higher in *C. albidum* indicating the possibility of considerable amount of secondary metabolites in the leaves of *C. albidum* compared to the stem of *A. ringens* respectively. The phytochemical screening of *C. albidum* revealed the presence of alkaloid, flavonoid, cardiac glycoside and steroid while the extracts of *A. ringens* revealed the presence of alkaloid, flavonoid, steroid and anthraquinone as the only secondary metabolites (Table 2). Quantitative assessment of the percentage chemical constituents of the chloroform extracts were summarized in (Table 3). Both extracts of *C. albidum* and *A. ringens* revealed the presence of Alkaloid and Flavonoid respectively with *C. albidum* having higher quantities, while cardiac glycoside were found only in *C. albidum* with low quantity. Polar phytochemicals such as Phenol and Tannins were absent in both crude extracts. Antimicrobial screening (Table 4) revealed that the stem bark extract of *C. albidum* exhibited antibacterial activity against *Escherichia coli* (MIC = 19.5 μg/mL) while the straw of *A. ringens* inhibited *B. cereus* (MIC = 19.5 μg/mL) but both *C. albidum* and *A. ringens* showed marginal activity against any of the tested fungi (MIC = 625 μg/mL). The results of cytotoxicity for the chloroform extracts of *C. albidum* and *A. ringens* revealed that *A. ringens* is cytotoxic to two different cell lines while *C. albidum,* were selectively cytotoxic to liver tumor cells, but not to breast tumor cells. This indicated that Nigerian *A. ringens* showed notable in-vitro cytotoxicity and this could be attributed the secondary metabolites present in these medicinal plants.

Phytochemical screening of the chloroform extracts of *C. albidum* and *A. ringens* revealed some differences in their constituents. The results support the idea that the *C. albidum* and *A. ringens* possesses active medicinal agents as well as revealing their physiological activities [25]. The antimicrobial actions of *C. albidum* and *A. ringens* are primarily due to the presence of active components in a concerted synergistic or antagonistic activities. Studies had revealed that mixtures of these chemical constituents showed a broad spectrum of biological effects and pharmacological efficacy [30,31]. The antimicrobial activities could likely be attributed to the presence of alkaloid, flavonoids, cardiac glycosides, steroid and anthraquinone present in the chloroform extracts. This study provides the basis for their pharmacological use as a cure for some human ailments and diseases. Cardiac glycosides have been found to be effective in congestive heart failure [32]. The presence of alkaloids may be responsible for their antimalaria efficacy. Possession of analgesic properties of these medicinal plants confirmed their use in treatment of stomach disorder, a view supported by [33]. Flavonoids are potent water soluble antioxidants and free radical scavengers’ agents which prevent oxidative cell damage and have strong anti cancer activity [34]. Studies had shown that flavonoid in intestinal tracts lower the risk of heart failure [33,35]. The presence of the above metabolites in the studied medicinal plants confirmed their use for the treatment of various diseases conditions to their antimicrobial, anti-inflammatory, and anti-carcinogenic effect. However, further research work is ongoing towards bio-guided column chromatography and in-vivo studies of these plants.

<table>
<thead>
<tr>
<th>Plant Extract</th>
<th>Extract Texture</th>
<th>Extract Yield</th>
<th>Percentages Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albidum</em></td>
<td>Dark Green</td>
<td>Semi Sticky</td>
<td>120.00 g</td>
</tr>
<tr>
<td><em>A. ringens</em></td>
<td>Dark Brown</td>
<td>Sticky Semi Solid Powder</td>
<td>84.00 g</td>
</tr>
</tbody>
</table>

- +++ = Test strongly positive ; + + = Positive; + = Weakly positive; - = No effect

<table>
<thead>
<tr>
<th>Secondary metabolites</th>
<th><em>C. albidum</em> mg/100g</th>
<th><em>A. ringens</em> mg/100g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tannins</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>120.00 g</td>
<td>7.0</td>
</tr>
<tr>
<td>Cardiac glycoside</td>
<td>5.0 ± 0.50</td>
<td>4.5 ± 0.70</td>
</tr>
<tr>
<td>Steroid</td>
<td>26.0 ± 0.80</td>
<td>13.5 ± 0.30</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>27.10 ± 0.80</td>
<td>-</td>
</tr>
<tr>
<td>Anthraquinone</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2. Qualitative analysis of secondary metabolites found in *C. albidum* and *A. ringens*
Table 4. Antimicrobial activity (MIC, μg/mL) of C.albidum and A. ringens

<table>
<thead>
<tr>
<th>Sample</th>
<th>C. albidum</th>
<th>A. ringens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. aerug</td>
<td>312.5</td>
<td>625</td>
</tr>
<tr>
<td>E. coli</td>
<td>&lt; 19.5</td>
<td>&gt; 2500</td>
</tr>
<tr>
<td>S. aureus</td>
<td>2500</td>
<td>&gt; 2500</td>
</tr>
<tr>
<td>B. cereus</td>
<td>312.5</td>
<td>&lt; 19.5</td>
</tr>
<tr>
<td>S. epi</td>
<td>&gt; 2500</td>
<td>&gt; 2500</td>
</tr>
<tr>
<td>S. mar</td>
<td>&gt; 2500</td>
<td>1250</td>
</tr>
<tr>
<td>Fungi</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. niger</td>
<td>625</td>
<td>625</td>
</tr>
<tr>
<td>Candida</td>
<td>625</td>
<td>156</td>
</tr>
<tr>
<td>Botrytis</td>
<td>625</td>
<td>625</td>
</tr>
</tbody>
</table>

4. Conclusion: The results of the study support the idea that herbal medicines is still relent as health care substitute in developing countries and provide new chemotherapeutic agents for development.

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AUTHORS’ CONTRIBUTIONS
Owolabi M.S conceived and designed the experiments; Omowonuola A.A, Avoese N.A and Jaiyeola Elizabeth, performed the experiments on phytochemical screening, Lawal O.A and Ogunwande I.A contributed reagents/analysis tools; Setzer W.N designed the experiment on antimicrobial and cytotoxicity; Setzer W.N and Owolabi M.S analyzed the data and wrote the paper.

COMPETING INTERESTS
The author(s) declare no conflict of interest.

References