PHYTOCHEMICAL AND ANTIBACTERIAL SCREENING OF PETROLEUM ETHER AND ETANOL EXTRACTS OF SIDA CORDIFOLIA LEAVES

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Abstract
The plant Sida cordifolia is used in traditional medicine by Sokoto people to treat microbial infections and stomach upset. This research is aimed at isolation of pure compounds and testing them on some selected bacterial strains. The powdered plant leaves was extracted by maceration for 48 hours using petroleum ether and ethanol. The qualitative phytochemical screening of alkaloids, tannins, steroids, saponins, cardiac glycosides, flavonoids and anthraquinones were carried out using standard protocols. The extracts were screened for antibacterial activity against Staphylococcus aureus, Bacillus subtilis, Escherichia coli and Pseudomonas aeruginosa using cup plate method. Column chromatography was carried out on the ethanol extract using gradient elution method. Further purification of column fractions was carried out using preparative TLC. Stigmastanol was isolated and characterized using NMR and LCMS. The percentage yield of the extracts in petroleum ether and ethanol were 2.87 % and 7.18% respectively. The phytochemical screening of the ethanol extract revealed the presence of alkaloids, tannins, saponins, cardiac glycosides, steroids and flavonoids. The ethanol extract exhibited good antibacterial activity on all the test organisms with zone of inhibition ranging between 10 mm to 16 mm at concentration of 5 mg/mL, 10 mg/mL, 15 mg/mL and 20 mg/mL. The petroleum ether fraction did not show activity on the test organisms. The MIC and MBC results indicated that ethanol extract is both bacteriostatic and bactericidal on the test organisms at concentration of 6.25 mg/mL. It can be concluded from the results obtained from this research that the ethanol extract of Sida cordifolia had antibacterial activity and has provided preliminary scientific evidence for the use of the plant leaves extract for the treatment of bacterial infections in traditional medicine.

Key words: Sida cordifolia, Phytochemical, Antibacterial and Stigmastanol

Introduction
Nigeria is blessed with varieties of trees and shrubs with medicinal potentials. The leaves, stems, bark, roots and fruits of these plants are medicinally important. Nature has been the source of medicinal agents for thousands of years. Many drugs have been developed from natural sources and were based on their traditional use [1]. About 25% of prescribed drugs in the world are of plants origin [2]. Herbal medicines are known to serve the health needs of about 80% of the world population and millions of people in the vast rural areas of developing countries rely on traditional or herbal medicines for their primary health care [1]. Plants have long formed the basis of traditional medicines systems. Pharmaceutical industries have produced a number of new antibiotics in the past decades; resistance to these drugs by microorganisms has increased [3]. Bacteria have the genetic ability to transmit and acquire resistance to drugs which are utilized as therapeutic agents. The affordability, reliability, availability and low toxicity of medicinal plants in therapeutic use has made them popular and acceptable by all religions for usage in health care all over the world [3]. Plants are indeed the first alternative materials used as remedy against many diseases [3]. The aqueous leaves extract of Sida cordifolia is used in Sokoto to treat stomach upset and bacterial infections (oral communication). This research is worth doing since it has the potential of discovering new antibacterial agents that can be used to alleviate human sufferings related to bacterial infections.

Material and Methods
Collection of Plant Sample
The leaves of Sida cordifolia was collected in December, 2014 from Kwakwalawa village in Sokoto
State-Nigeria. The plant was identified at the herbarium of Botany Unit, Department of Biological Sciences Usman Danfodiyo University Sokoto, Nigeria by Malam Abdulaziz Salihu. The voucher specimen with number (UDUH/ANS/0030) was deposited at the herbarium for reference purposes.

Sample Treatment
The leaves were washed with water to remove the dust and dried under shade for three days. The dried leaves of were ground using mortar and pestle then sieved to obtain fine powder. The powdered samples were stored in an air tight water free polythene bag and used for the analysis.

Extraction of Leaves
The powdered leaves (273 g) were defatted by maceration using 1000 cm$^3$ of Petroleum ether. The marc was allowed to dry and then extracted with 500 cm$^3$ of ethanol. In each case the leaves were left in contact with the solvent for 48 hours with intermittent stirring to ensure maximum extraction [5]. The extracts were filtered using Whatman filter No.1. The filtrates were concentrated using air circulated oven at temperature between 80-100 $^\circ$C.

Qualitative Phytochemical Screening of the Extracts
The tests for flavonoids, tannins, saponins, steroids/triterpenoids, alkaloids, cardiac glycosides and anthraquinones were carried according to the methods described by [5; 6 and 7].

Chromatographic Studies
Thin Layer Chromatography:
The TLC was carried out according to the method described by [4].

Column Chromatography
The ethanol extract was column chromatographed as described by [8]. Compound A was isolated.

Spectroscopic Studies
The compound isolated was characterized by means of $^1$HNMR, $^1$CNMR and LC-MS.

Antibacterial Studies
Test Organisms
The test organisms were obtained from the Department of Pharmaceuticals and Pharmaceutical microbiology, Faculty of Pharmaceutical Sciences, Usman Danfodiyo University, Sokoto-Nigeria. The microorganisms were standard laboratory strains of Staphylococcus aureus (gram +ve), Bacillus subtilis (gram +ve), Escherichia coli (gram –ve) and Pseudomonas aeruginosa (gram –ve).

Susceptibility Test
The antibacterial susceptibility test was conducted using the cup plate method described by Garrod [9]. The zone of inhibition was observed and recorded in millimeters using a metric rule.

Minimum Inhibitory Concentration (MIC)
This was carried out as described by [10]. The lowest concentration where no turbidity was observed was determine and noted as the Minimum Inhibitory Concentration (MIC).

Minimum Bactericidal Concentration (MBC)
The minimum bactericidal concentration was determined from nutrient broth dilution test obtained from the MIC tubes as described by [10]. The lowest concentration of the extract that showed no growth was noted and recorded as the minimum bactericidal concentration.

Results and Discussion
Extraction and Qualitative Phytochemical Screening
The percentage yields of petroleum ether extract and ethanol extract 2.87 and 7.18 respectively. From the result, the ethanol had the highest percentage yield. This may be due to its polarity, as polar compounds can only be extracted by polar solvent [11]. The result of the extraction revealed that Sida cordifolia leaves consist of non-polar and polar compounds. The phytochemical screening of the ethanol extract revealed the presence of alkaloids, tannins, saponins, cardiac glycosides, steroids and flavonoids (Table 1).

Table 1: Qualitative Phytochemical Screening of Sida cordifolia leaves Extracts

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Petroleum Ether</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Mayer’s test</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Wagner’s test</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Dragendorff’s</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Flavanoids</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Sodium hydroxide test</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Iron (III) chloride test</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Shindola’s test</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Iron (III) chloride test</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Lead acetate</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Anthraquinones</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Borntrager’s test</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Cardiac glycosides</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Keller-kiliani’s test</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Frothing test</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Key: (+): detected (-): not detected
Antibacterial Activity
The ethanol extract exhibited good antibacterial activity on all the test organisms with zones of inhibition ranging between 10 mm to 16 mm. The ethanol extract showed higher activity on gram positive bacteria than on gram negative bacteria (Table 2). This may be due to the difference in the cell wall composition of the organisms. The isolated compound also showed a good antibacterial activity on the test organisms with zone of inhibition that ranged between 10 mm to 27 mm in diameter (Table 2). The antibacterial activity of the isolated can be compared with Ciprofloxacin (Standard Antibiotic Table 2). According to [10], the diameter of zones of inhibition must be ≥ 10 mm for an extract to be considered as active on microorganisms. The antibacterial activity demonstrated by the ethanol extract may be due to the presence of tannins, saponins and alkaloids (Table 1). The antibacterial activities of these phytochemicals have been reported by [10&11].

Table 2: Antibacterial Activity of Ethanol Fraction of Sida cordifolia

<table>
<thead>
<tr>
<th>Conc. Mg/mL</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>Cip.</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td>13.5</td>
<td>14.0</td>
<td>14.5</td>
<td>16.0</td>
<td>29.0</td>
<td>27.0</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>12.5</td>
<td>13.0</td>
<td>14.0</td>
<td>16.0</td>
<td>29.0</td>
<td>27.0</td>
</tr>
<tr>
<td>E. coli</td>
<td>10.0</td>
<td>12.5</td>
<td>13.0</td>
<td>16.0</td>
<td>29.0</td>
<td>27.0</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>12.5</td>
<td>14.0</td>
<td>14.5</td>
<td>16.0</td>
<td>30.0</td>
<td>27.0</td>
</tr>
</tbody>
</table>

The MIC and MBC of the ethanol extract (Table 3), produced MIC of 1.563 mg/mL on S. aureus, 0.098 mg/mL on B. subtilis, 6.250 mg/mL on E. coli and 6.250 mg/mL on P. aeruginosa. Also, the results of the MBC tests revealed MBC of 1.563 mg/mL on S. aureus, 0.098 mg/mL on B. subtilis, 6.250 mg/mL on E. coli and 6.250 mg/mL on P. aeruginosa (Table 3). From these results, it can be deduced that the ethanol extract is both bacteriostatic and bactericidal on all the test organisms at 6.250 mg/mL.

Table 3: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Ethanol extract of Sida cordifolia

<table>
<thead>
<tr>
<th>Test Organisms</th>
<th>MIC (mg/mL)</th>
<th>MBC (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td>1.563</td>
<td>1.563</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>0.098</td>
<td>0.098</td>
</tr>
<tr>
<td>E. coli</td>
<td>6.250</td>
<td>6.250</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>6.250</td>
<td>6.250</td>
</tr>
</tbody>
</table>

Chromatography of Ethanol Extract
The column chromatography of the ethanol extract afforded one compound (A) with mass of 0.081 g and Rf value of 0.85. The purity of the compound was assessed on the basis of TLC. A homogeneous single spot in different solvent system confirm its purity. The compound appeared purple when developed and sprayed with 10% H2SO4 with subsequent heating at 105 °C on thin layer plate. The compound is highly soluble in chloroform and ethyl acetate. The compound gave positive reaction with Salkowski’s and Keller-kiliani’s tests. This confirmed the presence of steroidal nucleus.

Structure Elucidation of Compound A
LC-MS
The isolated compound was identified on the basis of LC-MS spectral libraries which were searched for matches. The mass spectra of the compound showed molecular ion peak [M]+ at m/z 411 which represent an intact molecule and also give the molecular weight of the compound. Therefore using the [M+1] rule, molecular weight of the compound is m/z 412. Other fragment ion peaks occur at m/z 411, 410, 409, 248 and 112. The fragmentation pattern of compound A is shown in scheme 1. From the spectrum, the m/z 410 and 409 occurred due to loss of 1H and 2H from m/z 411 respectively. The m/z 248 and m/z 112 occurred as a result of loss in mass of 164 (C11H16O) and 300 from m/z 412 respectively. This occurred as a result of decomposition that occurred in the molecule. This also suggests the presence of bulky side chain in the compound which is characteristic of steroids [4]. These data are characteristic and consistent with Stigmasterol (Scheme 1)
Scheme 1: Fragmentation Pattern of Compound A (Stigmasterol)

**1H NMR and 13C NMR of Compound A**

The 1H NMR (Table 4) showed methyl (CH₃) signals between δ_H (ppm) 0.8 to 0.97. This suggests that there are methyl groups present in the compound. Also the presence of signals between δ_H (ppm) 1.22 to 1.42 showed the presence of methylene (CH₂) protons and signals between δ_H (ppm) 1.67 to 4.29 indicated the presence of methine proton (CH). The presence of down field signals at δ_H (ppm) 5.30 and 5.40 suggest the presence of olefinic proton and the appearance of a signal at δ_H (ppm) 2.0 indicates the presence of a hydroxyl group. In the 13C NMR (Table 4) appearance of signal at δ_c (ppm) 71.98 gave vital information about the primary functional group in the compound. This carbon (C-3) appears in the region usually occupied by alcoholic groups. The carbon is coupled with hydrogen and this confirms the presence of alcoholic function in the compound. The presence of signals between δ_c (ppm) 119.30 to 132.61 confirms the presence of carbon that holds the olefinic proton. The signals observed between δ_c (ppm) 13.94 to 30.75 are due to the presence of (CH₃) with only hydrogen or R at both α and β carbons and signals between δ_c (ppm) 20.17 to 38.89 are due to (CH₂) with only hydrogen or R at both α and β carbons. Also signals between δ_c (ppm) 30.53 to 68.78 confirm the presence of a (CH) or (-C-) with only hydrogen or R at both α and β carbons. The 1H NMR and 13C NMR data matched very well with published data on stigmasterol [12 &13]. These data together with the LC-MS are characteristic of stigmasterol.
Conclusion
The ethanol extract of *Sida cordifolia* leaves contain alkaloids, saponins, tannins, flavonoids, cardiac glycosides and steroids. The isolated compound from the plant showed strong antibacterial activity against *Staphylococcus aureus* (gram +ve), *Bacillus subtilis* (gram +ve), *Escherichia coli* (gram -ve) and *Pseudomonas aeruginosa* (gram -ve). This is the first time the presence of stigmasterol is reported in the plant.

References
13. from Odontonema strictum (Acanthaceae), Journal of Innovation in Pharmaceutical and Biological Science, 2(1), 88-95.