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Phytochemical Screening and Antimicrobial Analysis of *Hymenocardia acida*

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Abstract The leaves, stem bark and root bark of *Hymenocardia acida* were subjected to phytochemical and anti-microbial screening. Extraction was carried out by continuous Soxhlet extraction using petroleum spirit, chloroform and methanol as solvents. The phytochemical screening confirmed the presence of terpenes, tannins, sugars, steroids, saponins, carbohydrates, flavonoids and anthraquinones in the three plant parts. The antimicrobial screening of petroleum, chloroform, and methanol extracts of the leaves of *Hymenocardia acida* did not show any activity on *Klebsiella pneumoniae*, *Salmonella specie*s and *Candida albicans* but showed inhibition against *Streptococcus pyogenes*, *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The methanol extract of the root and stem bark inhibited all the test organisms but the root extract showed more sensitivity on the test organisms considering its minimum inhibitory concentration (MIC) on *Escherichia coli* (8.0×10^2 mg/mL) and for *Staphylococcus aureus* (4.0×10^2 mg/mL), while the MIC of stem bark extract for *Escherichia coli* is 10.0×10^2 mg/mL and 6.0×10^2 mg/mL for *Staphylococcus aureus*. None of the leaves extract exhibited bactericidal or fungicidal effect on test organisms. However, the methanol extract of the root and stem bark exhibited bactericidal and fungicidal effects on *streptococcus pyogenes*, *Staphylococcus aureus* and on the fungus, *Candida albicans* at minimum concentration of 1.0×10^3 mg/mL respectively. The methanol extract of the root appears more potent than the stem and leaves as portrayed by the MIC values. Chromatographic separation of the methanolic extract of the root yielded some components which were characterized by GC-MS spectral technique and after correlation with the search libraries (C:/Database/NISTO2.L) were confirmed to be Beta sitosterol, gamma sitosterol, stigmasterol, lupeol, Friendan-3-one and ethyl iso-allocholate.

Keywords Phytochemical screening, antibacterial, analysis, *Hymenocardia acida*

1. Introduction

The plant kingdom holds many species of plant that contain many substances of medicinal value which are yet to be discovered. Large number of plants are constantly been screened for medicinal value [1] and only a few out of diverse plant species present in Nigeria have been subjected to scientific evaluation of their potential chemotherapeutic value. The pendulum of the search for plant-sourced-drugs keeps swinging from decade to decade. At one time, isolation of the active ingredient (s) was emphasized and at another, the use of a whole plant sample was advocated. Even in some occasions not a single plant is used but a combination of some plants is

utilized to achieve the desired cure. In a situation like this, no single plant can be credited with the curative ability if the patient is finally relieved [2]. The use of herbs for the treatment of ailments has been an old practice. It is the fact that products and concoction/decoctions are readily available for sale in most Nigerian markets today, either in powder or liquid forms. The increasing presence of these herbal preparations in markets may be due to the fact that most rural dwellers in Nigeria are finding it more useful and cheaper so they are competing favourably with the orthodox health care delivery system.

In the time past, our forefathers depended on herbs for their medications. Plants have been the major source of medicine for the survival of children right from conception to adulthood. The traditional healers specialized in some specific areas such as: eye diseases, psychiatry, orthopaedic, midwifery, etc. in which herbs were all used for treatment [3]. Many medicinal plants have remained unscreened. This has posed a great challenge considering the little resources available to the researchers. Many approaches have been adopted to develop drugs from plants. Among these methods is the phytochemical screening, which is often accompanied by biological screening. As a result of modern techniques in isolation and pharmacological testing procedures, new plants that are screened usually find their way into orthodox medicine as purified substances rather than being in form of galenical preparations [1]. It is a common practice among Chemists that any unlabelled bottle in the laboratory should be discarded. In this wise a true chemist should dissociate himself/herself from any uncharacterized drugs no matter how effective the drug may be. Also, extraction and isolation of bioactive agents from plants is one of the most intensive areas of natural products research and this field is far from been exhausted [2]. It has been reported that only about 10 % of all plants found in the world have been investigated in detail for possible presence of bioactive agents [4], hence the need to continue investigating virgin plants for medicinal purposes, since this enables structure related activity to be carried out thereby leading to the possible synthesis of more potent drugs with reduced toxicity.

2. Materials and Methods

Materials

Molisch Reagent, Tetraoxosulphate (VI) Acid, Fehling's Solutions I and II, 5 % Ferric Chloride, Hydrochloric Acid, 10% Sodium Hydroxide, Dragendoffs Reagent, Wagner's Reagent, Mayer's Reagent, Anhydrous Sodium Sulphate, Acetic Anhydride. Petroleum Ether (60 - 80) ^{o}C Chloroform, Methanol, Ethyl Acetate, Chromatographic Tank, Micro Slides, Ultraviolet Lamp, Capillary Tubes for Sample Application, Silica Gel as Stationary Phase, Column, Bottle for Collection of Eluent, Microbiological Media (Nutrient broth): Nutrient agar and Sabourand dextrose agar, Test Organisms: *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Salmonella sp Streptococcus pyogenes, Escherichia coli*, *Candida albicans*, Apparatus Syringe and Needles 2ml and 5mL, Petri dishes, Test Tubes, Sterile Pipettes, Incubator, Autoclave, Disinfectant, Sample Bottle, Soxhlet Extractor, Timble, Rotary Evaporator, Round Bottom Flask, Churchill Chiller, Heating Mantle [5].

Methods

The root, stem bark and leaves of *Hymenocardia acida* were collected from Makurdi, Benue State, Nigeria in September 2007. They were identified by Mr. Waya, J. I. of the Department of Biological Sciences, Faculty of Science, Benue State University Makurdi. The plant parts were air-dried, pulverized using wooden pestle and mortar, stored in airtight polythene bags and kept away from moisture until they were needed for extraction [6].

The pulverized parts of *Hymenocardia acida* (300g) were weighed and packed into Soxhlet extractor using thimbles, taking one sample at a time. The solvent and anti-bumping chips were put into a round bottom flask which was heated on a mantle. The leaves part was defatted using petroleum ether (60 - 80) ^{o}C, followed by chloroform and exhaustively extracted using methanol to yield various crude extracts. In the case of the root and stem bark, methanol only was used to extract them.

Concentration of the extracts was done at 40 oC using rotary evaporator to give concentrated extracts which were further dried in the air. The constant weights of the extracts were obtained and recorded. The crude extracts were later subjected to bioassay analysis.

Phytochemical Screening

In the phytochemical screening of the various plant parts, the pulverized plant materials were used. The plant metabolites tested for include alkaloids, anthraquinones, carbohydrates, flavonoids, resins, saponins, sterols, sugars, tannins and terpenes.

Test for Alkaloids

Extraction of the alkaloids from the plant materials: The pulverized parts of the plant (1g) were respectively put into a 100mLbeaker and moistened with Conc-ammonia solution. This was left for 15 minutes to enable the ammonia solution penetrate the plant sample. This was followed by the addition of a 1:1 mixture of chloroform and ethanol. The quantity of this mixture added was sufficient to soak and suspend the pulverized material and this was allowed to stand for thirty minutes with occasional stirring using glass rod. At the expiration of this time, the mixture was filtered through a plug of cotton wool. The material was washed twice with 5cm3 of chloroform each time and these washings were combined.

The resulting filtrates were concentrated using a water bath and the respective residues were then dissolved in chloroform. They were transferred into separating funnels and shaken vigorously with dilute sulphuric acid. The systems were allowed to settle and only the aqueous layers were collected while the chloroform layers were discarded. The aqueous layers were again made alkaline using concentrated ammonia solution. The new systems were treated with 10cm3 of chloroform shaken and allowed to stand to separate. The chloroform layers were collected and the process repeated using another 10cm3 of chloroform. The two fractions were combined and concentrated.

The final residues obtained were dissolved in 5mLof 2% hydrochloric acid. The solutions were then divided into three portions,

- To the first portion in test tube, 2 drops of Dragendorff's reagent were added and a reddish-brown precipitate was observed.
- To the second portion, 2 drops of Wagner's reagent were addedand a brown precipitate was observed.
- To the third portion, 2 drops of Mayer's reagent were added and a cream precipitate was obtained.

Test for Flavonoids

Ammoniacal Silver Nitrate Test

1 g of the plant material was dissolved in 5 mL of distilled water and four drops of ammoniacal silver nitrate solution was added. Brown coloured solution with brown precipitate was observed before heating and it turned brownish black after heating.

Test for Saponins

The plant material (Ig) was shaken with 10ml distilled water and allowed to stand for 45 minutes. sPersistent honey comb froth was observed.

Test for Resins

The plant materials (Ig) were dissolved in acetic anhydride (1ml) and one drop of concentrated sulphuric acid was added. No colour change was observed.

Test for Carbohydrates

General test for Carbohydrates (Molisch's Test)

Each plant material (1 g) was extracted with distilled water (5mL) by heating in a water bath then filtered. Molisch's reagent (two drops) were added to 2 mL of the filtrate in a test tube followed by few drops of sulphuric acid down the side of the test tube. A purple coloure dring at the interface was observed.

Test for Sugars (Braford's Test)
To 1 mL of aqueous solutions of the plant samples in separate test-tubes, 1 mL of Braford's reagent was added and heated in a water-bath. Red precipitate was observed.

Test for free Reducing Sugar (Fehling's Test)
The respective aqueous solutions (2 mL) of the plant materials were shaken separately in a test tube and heated. Equal mixture (5mL) of Fehling's solutions I and II were added stepwise and boiled for two minutes. Brick red precipitate was observed.

Test for Tannins
5 g of the respective plant materials were shaken with 10mL of distilled water and filtered. A solution of 5% ferric chloride (3 drops) was added to 3mL of each extract in a test tube. A greenish black coloration was obtained.

Test for Steroids and Triterpenes
Two drops of ethanol were added to 3mL of each extract in a test tube, followed by the addition of chloroform and dehydrated with anhydrous sodium sulphate crystals then divided into 2 portions.

Lieberman - Buchard Test
Acetic anhydride (3 drops) was added to one test tube (above), and then concentrated sulphuric acid was added to the side of each test rube. Reddish brown ring at the interface was observed.

Salkowskii's Tests
To the second portion of each extract, concentrated sulphuric acid was added to the slanted test tube. A reddish colour at the interface was observed.

Test for Anthraquinones
Each plant material (1 g) was put in a clean dry test tube and 5 mL of chloroform added to it and shaken for six minutes. Each system was filtered and-the filtrate was treated with 10% ammonia solution. A bright pink colour was observed.

Antimicrobial Screening
Media Preparation
The agar (1.3g/100 mL) (Sabourand dextrose agar and Nutrient agar) were respectively dissolved in distilled water and fully solubilized by heating. Aliquot of 20 mL of the solution were dispensed into sterile petri dishes. They were sterilized in an autoclave at 121 oC and at a pressure of 15 psi for fifteen minutes. This was allowed to cool and homogenized. The prepared plates were kept overnight in a refrigerator.

Preparation of Test Organisms
Seven (7) different microbes were used in this work and all were purely fresh isolates. The pure isolates namely: *Streptococcus pyogenes, Klebsiella pneumoniae, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella specie and Candida albicans* were collected from the Microbiology Department of National Research Institute for Chemical Technology (NARICT), Zaria. They were sub-cultured on sterile nutrient broth and incubated at 37^{o}C for 24 hours for bacteria. While *Candida albicans* was sub-cultured on the slant agar of

Sabourand dextrose agar arid incubated at 25 ^{o}C for forty-eight hours. They were harvested into sterile nutrient broth and disaggregated using sterile glass beads.

Preparation of Plant Extract Solution and Paper Discs

Stock solutions of the plant extracts were prepared by dissolving 1.0g of each plant extract in dimethyl sulphoxide (DMSO) and for each extract, 1000, 800, 600, 400, and 200mg/mL were prepared by serial dilution. One-millimeter (1 mL) aliquot of each of these solutions was transferred into a sterile glass evaporating dish followed by the addition of sufficient number of sterile filter paper discs to completely absorb the extract.

The paper discs impregnated with the respective concentrations of the extract were then dried in an oven at 40 ^{o}C.

Determination of Antimicrobial Activity of the Extract from Leaf, Stem bark and Root of *Hymenocandia acida.* Using Agar Diffusion Method.

Standardized suspension (0.2 mL) of the organism were mixed with the molten cooled agar media and poured on sterile petridishes and allowed to cool and solidify. The impregnated paper discs were then placed aseptically and pressed firmly on the surface of inoculated agar plate for the extract to diffuse into the agar media. Control experiment was set up using sterile paper discs soaked in DMSO. All the plates were incubated aerobically at 30^{o}C for 24 hours for bacteria while the plate containing the *Candida albicans* was incubated at a temperature of 22 oC for forty-eight hours. At the end of the incubation period, the plates were examined for zones of inhibition. These zones were measured using a pair of dividers and a transparent ruler and recorded.

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentrations of the crude extracts (pet. ether, chloroform and methanol extracts) were determined against the organisms. Nutrient broth (1.3g/100 mL) media was prepared according to the manufacturer's specification. The broth was cooled to 45^{o}C and 2 mL of it was dispensed into respective sterile test tubes near a gas flame in order to prevent contamination.

Ten (10) separate test tubes were used for each test organism. These were cotton plugged, ready to be used, while another ten (10) set of test tubes containing the nutrient broth were equally cotton plugged to serve as a control for each set of test organism and were labelled accordingly. Serial dilution of the respective extracts with the broth was done for each set of the test tubes to obtain various concentrations.

Using a syringe, 0.2 mL of the bacterial and fungal suspensions was transferred into a set of test tubes respectively with the exception of the sets labelled as control. The tubes were finally incubated at 37^{o}C for 24 hours for bacteria and 22^{o}C for *fungi* after which they were observed for turbidity of growth or complete death of the test organism leading to clear solution.

The tube with the lowest concentration of the extract showing clear solution or death of organism represents minimum inhibitory concentration (MIC) [7]

Determination of minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of the crude extracts

These tests were carried out in order to determine whether the organisms could be killed or could only be inhibited by the extracts. Nutrient agar plates were prepared for the bacteria, while Sabourand dextrose agar was prepared for fungi according to the manufacturer's specifications. The contents of the specific tubes designated as the MIC obtained for the extracts and the tube preceding the MIC tube were respectively sub-cultured onto appropriate quadrants of the agar plate by dipping a sterile wire loop into each- test tube and streaking the loop on the labelled quadrants. The plates were then incubated at 37 ^{o}C for 24 hours for bacteria and 22 ^{o}C for 48 hours for the fungi after which they were all observed for growth or otherwise.

The minimum bactericidal concentration and minimum fungicidal concentration were the quadrants with the lowest concentration of the extract without growth and the result obtained are shown in tables 7-18.

Thin Layer Chromatography (TLC)

The methanol extract of the root which showed the highest sensitivity to the test organisms was partitioned into ethyl acetate and methanol to reduce complexity. This was done by first dissolving the methanol extracts in distilled water then it was transferred into 250 mL separator/funnel and it was shaken with 100 mL ethyl acetate in a slanted manner. It was allowed to settle and the organic layer was collected. This was repeated two more times on the aqueous layer. The organic layers were combined and concentrated using rotary evaporator. The same process was carried out with methanol to extract back the remaining components from the aqueous medium. Both methanol and ethyl acetate extracts were allowed to dry and TLC was performed on them to ascertain the number of components present in them. The TLC was done on commercially pre-coated silica gel plate prepared by MERCK [8].

The plant extracts were respectively dissolved in minimal amount of methanol and ethyl acetate respectively and were spotted on the base of the plate and developed using 100 % ethyl acetate, 100 % chloroform and mixture of chloroform and ethyl acetate (2:1) respectively. The plates were then observed under the UV-lamp at 254 nm for the components thus separated.

Column Chromatography

At the end of TLC, the ethyl acetate extract of the root bark was more promising thus; it was subjected to column chromatography because the roots part was the most active in the antimicrobial screening process. Silica gel column was used and it was first activated in an oven for three hours at the temperature 110 ^{o}C, then allowed to cool and was packed on the column. The extract was dissolved in a minimum amount of ethyl acetate and solidified with the silica gel and was loaded on top of the silica gel in the column. The eluting solvent chloroform ethyl acetate 2:1 was introduced at the top [8-12].

Gradient elution was used and eluent collected at 2 mL interval. Some of the fractions that showed the same number of components were combined and were further purified in another column using the same procedure to obtain J2 and J4. These components were separated out as waxy white solid and white crystals.

Spectroscopic Measurement

The fractions so separated (J2 and J4) were analyzed using Agilent Gas Chromatography (6890N Model) coupled to 5973 Mass selective detector, in $CDCI_3$ as solvent.

The results obtained were compared with an in-built Main library (C:/Database/NIST02) and this library enabled us to confirm the presence of suspected phytochemicals [13].

3. Results and Discussion

Results

Continuous extraction was used to extract respectively 300g of pulverized root, stem bark and leaves of *Hymenocardia acida* plant materials using soxhlet extractor. Petroleum ether (60-80^{o}C) was initially used in-defatting the leaf part followed by chloroform and finally methanol. The root and stem bark were respectively extracted using only methanol. Each crude extract was then concentrated *in vacuo* at 40^{o}C using rotary evaporator and the resultant crude extracts were air-dried until constant weights were obtained.

Methanol extracted the largest amount of material from the plant, followed by pet-ether and finally chloroform. The extractive values are given in Table 1.

Table 1: Amount of Extracts from 300 g Pulverized Leaves of *Hymenocardia acida*

Solvent medium	Weight of extract (g)	Extractive values (%)
Petroleum ether	9.40	3.13
Chloroform	4.65	1.55
Methanol	14.94	4.98

Table 2: Amount of Extracts from 300g Pulverized Root and Stem Bark of *Hymenocardia acida*

Sample	Root	Stem bark
Solvent	Methanol	Methanol
Weight of extract (g)	40,60	36.32
Extractive Values. (%)	13.5	12.1

Phytochemical Screening

The phytochemical screening of root, stem bark and leaves of *Hymenocardia acida* confirmed the presence of carbohydrates, free reducing sugar, saponins, alkaloids, steroids, tannins, flavonoids, terpenes and anthraquinones. However, resins were absent.

Table 3: Results of Phytochemical Screening of the Root, Stem bark, and Leaves of the *Hymenocardia acida*

Constituents	Root	Stem bark	Leaves
Alkaloids	+	+	+
Anthraquinones	+	+	+
Flavonoids	+	+	+
Carbohydrates	+	+	+
Resins	-	-	-
Saponins	+	+	+
Steroids	-	+	+
Sugars	+	+	+
Tannins	+	+	+
Terpenes	+	+	+

Key: + Present - Absent

Table 4: Results of Test for Alkaloids in the Root, Stem bark and Leaves of the Plant

Test	Observation	Inference
Dragendoffs Reagent Test	Reddish Brown Precipitate	Alkaloid Present
Mayer's Reagent Test	Cream Precipitate	Alkaloid Present
Wagner's Reagent Test	Brown Precipitate	Alkaloid Present

Table 5: Results of Test for Carbohydrate and Sugar in the Plant Materials

Test	Observation	Inference
Molisch's Test	Purple ColouredRing at the Interface	Carbohydrate Present
Barfoed's Test	Red Precipitate	Sugar Present
Fehling's Test	Brick Red Precipitate	Free Reducing Sugar Present

Table 6: Results of Test for Steroids and Triterpenes

Test	Observation	Inference
Lieberman-Buchard	Reddish Brown Ring at the Interface	Steroids and Terpene Present
Salkowskii's Test	Reddish Colour at the Interface	Unsaturated Steroids Present

The presence of alkaloids, saponins, tannins and steroids in plant materials call for an immense research. These metabolites are of various pharmacological importance. Many triterpenes and their aglycones have been reported to have varied uses as anti-ulceroginic, anti-inflammatory, fibrinolytic, anti-pyretic, analgesic and anti-dematous in action [2, 14].

Flavonoids provide many health-promoting benefits. They act as antihistamine, which is useful in reducing allergy symptom and help to reduce inflammations associated with various forms of arthritis [15]. Tannins have been used in medicine to aid the healing of wounds and burns. They equally have some protective value against toxins. They have been claimed to have anti-viral and anti-tumour properties [16].

The alkaloids like quinine and morphine are used for malaria treatment. Morphine has also been reported to be of use as an analgesic. The steroids like the sitosteroid has been reported to lower the body cholesterol and thereby prevent the oxidative damage through its antioxidant activity.

Antimicrobial

The petroleum ether, chloroform and methanol extracts of the leaves and methanol extracts of the root and stem bark were tested against seven microorganisms namely: *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Salmonella specie, Streptococcus pyogenes, and Candida albicans.* The results obtained are in Tables 7-18.

Table 7: Diameter of Zone of Inhibition of Pet-Ether Extract of the Leaves of *Hymenocardia acida*

Test organism	**Concentrations in mg/mL**				
	1.0 x 10^3	**8.0 x10^2**	**6.0 x10^2**	**4.0 x10^2**	**2.0 X10^2**
Streptococcus pyogens	11.00	8.00	6.00	+	+
Klebsiella pneumoniae	+	+	+	+	+
Escherichia coll	12.00	10.00	7.00	+	+
Staphylococcus aureus	11.00	9.00	6.00	+	+
Pseudomonas aeruginosa	8.00	6.00	+	+	+
Salmonella sp	+	+	+	+	+
Candida albicans	+	+	+	+	+

Key: + Growth (Not inhibited)

Table 8: Diameter of Zones of Inhibition of Chloroform Extract of the Leaves of *Hymenocardia acida*

Test organism	**Concentrations in mg/mL**				
	1.0 x 10^3	**8.0 x10^2**	**6.0 x10^2**	**4.0 x10^2**	**2.0 X10^2**
Streptococcus pyogens	12.00	9.00	7.00	+	+
Klebsiella pneumoniae	+	+	+	+	+
Escherichia coli	11.00	9.00	8.00	+	+
Staphylococcus aureus	12.00	10.00	8.00	6.00	+
Pseudomonas aeruginosa	9.00	7.00	+	+	+
Salmonella species	+	+	+	+	+
Candida albicans	+	+	+	+	+

Table 9: Diameter of Zones of Inhibition of Methanol Extract of the Leaves of *Hymenocardia acida*

Test organism	**Concentrations in mg/mL**				
	1.0 x 10^3	**8.0 x10^2**	**6.0 x10^2**	**4.0 x10^2**	**2.0 X10^2**
Streptococcus pyogens	14.00	11.00	8.00	6.00	+
Klebsiella pneumoniae	+	+	+	+	+
Staphylococcus aureus	14.00	12.00	9.00	7.00	+
Escherichia coli	13.00	11.00	7.00	+	+
Pseudomonas aeruginosa	10.00	8.00	+	+	+
Salmonella species	+	+	+	+	+

Candida albicans	+	+	+	+	+

The three respective extracts of petroleum, chloroform and methanol of the leaves did not show any sensitivity to *Klebsiella pneumoniae, Salmonella* species and *Candida albicans.* However, the extracts showed inhibition against *Streptococcus pyogenes, Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa*. The methanol extract showed the highest zone of inhibition against these organisms followed by the chloroform extract and then the petroleum ether extract.

Table 10: Diameter of Zones of Inhibition of Methanol Extracts of the Root and Stem Bark

Plant part	Conc. Mg/mi	Zones of Inhibition in mm						
		Streptococcus pyogens	***Klebsiella pneumonae***	***Escherichia coli***	***Staphylococcus aureus***	***Pseudomonas aeruginosa***	***Salmonella species***	***Candida albicans***
Root Bark	1000	15.00	9.00	14.00	15.00	10.00	8.00	10.00
	800	13.00	6.00	12.00	12.00	8.00	+	+
	600	10.00	+	9.00	11.00	6.00	+	+
	400	7.00	+	6.00	7.00	+	+	+
	200	+	+	+	+	+	+	+
Stem Bark	1000	13.00	8.00	12.00	14.00	11.00	7.00	8.00
	800	11.00	+	11.00	12.00	9.00	+	+
	600	8.00	+	7.00	10.00	7.00	+	+
	400	6.00	+	+	6.00	+	+	+
	200	+	+	+	+	+	+	+

These results in table 4 -1 show that the methanol extract of the root is more active on the test organisms than of the stem bark.

Table 11: Minimum Inhibitory Concentration (MIC) of Pet-ether, Chloroform and Methanol Extracts of the Leaves of *Hymenocardia acida*

Test organisms	Concentrations Representing (MIC)/mg/mL x 10^2)		
	Petroleum Ether	**Chloroform**	**Methanol**
Streptococcus pyogens	6.0	6.0	4.0
Klebsiella pneumoniae	-	-	-
Escherichia coli	6.0	6.0	6.0
Staphylococcus aureus	6.0	4.0	4.0
Pseudomonas aerugimosa	8.0	8.0	8.0
Salmonella species	-	-	-
Candida albicans	-	-	-

The minimum inhibitory concentration (MIC) represents the lowest concentration (or highest dilution) of the test samples to which an organism shows susceptibility. The result showed that crude petroleum ether, chloroform and methanol extract of the leave all inhibited the *Pseudomonas aeruginosa* and *Escherichia coli* at the lowest concentration of 8.0 x 10^2 and 6.0 x 10^2 respectively. It was also observed from the resultthat the crude extracts showed more activity on the *Staphylococcusaureus* and *Streptococcus pyogenes* where the MIC of the lowest concentration is seen.

Table 12: MIC of Methanol Extracts of the Root and Stem Bark of *Hymenocardia acida*

Test Organisms	Root x 10^2mg/mL	Stem bark x 10^2 mg/mL
Streptococcus pyogens	4.0	4.0
Klebsiella pneumoniae	8.0	10.0
Escherichia coli	4.0	6.0
Staphylococcus aureus	4.0	4.0
Pseudomonas aeruginosa	6.0	6.0
Salmonella species	10.0	10.0
Candida albicans	10.0	10.0

The MIC of methanol extract of the root and that of the stem bark are similar but that of the root showed more sensitivity on the test organisms considering its MIC on *Klebsiella pneumoniae* (8.0 x 10^2 mg/mL) and *Escherichia coli* (4.0x10^2 mg/mL) while the MIC of the stem bark extracts for *Klebsiella pneumoniae* is 10.0x10^2 and 6.0 x 10^2 mg/mL for *Escherichia coli*

When the various inhibitory concentrations were determined i.e. the lowest concentration at which the organism will be inhibited from further growth, the minimum Bactericidal concentration (MBC) and the minimum fungicidal concentration (MFC) were determined i.e. the minimum concentration of the test sample (extract) at which the organism will completely be exterminated.

Table 13: Minimum Bactericidal Concentration (MBC and MFC) of the Petroleum ether, Chloroform and Methanol Extracts of the Leaves of *Hymenocardia acida*

Test organisms	**Concentrations in mg/mL**				
	1.0x10^3	**8.0 x10^2**	**6.0 x10^2**	**4.0 x10^2**	**Centre**
Streptococcus pyogens	++	++	++	NA	++
Klebsiella pneumoniae	NA	NA	NA	NA	++
Escherichia coli	++	++	++	NA	++
Staphylococcus aureus	++	++	++	NA	++
Pseudomonas aeruginosa	++	++	NA	NA	++
Salmonella species	NA	NA	NA	NA	++
Candida albicans	NA	NA	NA	NA	++

KEY: ++ = *Growth observed.*

NA = Not applicable, i.e. the organisms were not inhibited at these concentrations.

None of the leaf extracts exhibited bactericidal or fungicidal effect on the test organisms. However, inhibitory effects on some of the test organisms were observed. This is an indication that the leaves of *Hymenocardia acida (*Tul*)* contained low- concentration of the active components, therefore could not completely exterminate the test organisms. This implies that the use of the leaves of *Hymonecardia acida* (alone) traditionally to cure any infection or diseases caused by any of these test organisms may not be very effective.

Table 14: Minimum Bactericidal Concentration (MBC) of Methanol Extract of Root Bark of *Hymenocardia acida*

Test Organisms	**Concentrations in mg/mL**				
	2.0x10^3	**1.0x10^3**	**8.0x10^2**	**6.0 x10^2**	**Control**
Streptococcus pyogens	- -	-x	++	++	++
Klebsiella pneumoniae	++	++	++	++	++
Escherichia coli	++	++	++	++	++
Staphylococcus aureus	- -	-x	++	++	++
Pseudomonas aeruginosa	++	++	++	++	++
Salmonella species	++	++	++	++	++

Table 15: MBC of Methanol Extract of the Stem Bark of *Hymenbcardia acida*

Test Organisms	**Concentrations in mg/ml**				
	2.0x10^3	**1.0x10^3**	**8.0 x10^2**	**6.0 x10^2**	**Control**
Streptococcus pyogens	- -	-x	++	++	++
Klebsiella pneumoniae	++	++	++	++	++
Escherichia coli	++	++	++	++	++
Staphylococcus aureus	- -	-x	++	++	++
Pseudomonas aeruginosa	++	++	++	++	++
Salmonella species	++	++	++	++	++

Key: ++ = Growth observed - - = No growth observed - x = MBC

Table 16: Minimum Fungicidal Concentration of the Methanol Extract of Root Bark of *Hymenocardia acida*

Test organism	Concentrations of the Extract in mg/mL					
	2.0 x 10^3	**1.0x10^3**	**8.0 x 10^3**	**6.0 x 10^2**	**4.0 X10^2**	**Control**
Candida albicans	--	**-xx**	++	++	++	++

Table 17: Minimum Fungicidal Concentration (MFC) of the Methanol Extract of the Stem Bark of *Hymenocardia acida*

Test organism	Concentrations of the Extract in mg/mL					
	2.0 x 10^3	**1.0x10^3**	**8.0 x 10^3**	**6.0 x 10^2**	**4.0 X10^2**	**Control**
Candida albicans	--	-xx	++	++	++	++

Key: -xx = MFC

Table 18: Summary of the MBC and MFC of the Methanol Extract of Root and Stem Bark Concentrations of *Hymenocardia acida* representing MBC and MFC mg/mL

Test Organisms	Root x 10^3	Stem Bark x 10^3
Streptococcus pyogens	1.0	1.0
Klebsiella pneumoniae	Na	Na
Escherichia coli	Na	Na
Staphylococcus aureus	1.0	1.0
Pseudomonas aerugimosa	Na	Na
Salmonella species	Na	Na
Candida albicans	1.0	1.0

Key: Na = Not Applicable

The methanolic extracts of the root and stem bark exhibited bactericidal and fungicidal properties on only two species of bacteria which are *Streptococcus pyogenes* and *Staphylococcus aureus* and on the fungus *Candida albicans* at a minimum concentration of 1.0 x 103 mg/mL respectively.

This justifies the use of *Hymenocardia acida*root part in treatment of pneumonia and cold as the *Streptococcus pyogenes* is the causative organism of most upper respiratory track.

Spectral Result

The result of the spectral analysis of J2 using GC-MS machine after correlation with the search libraries (C:/Database/NIST02.L) confirmed that, it is a combination of Beta-sitosterol, Gamma-sitosterol and Stigimasterol. The result of J4 confirmed that it is a combination of Lupeol, friedran 3-one and ethyl Iso-allocholate.

Compound 1Beta sitosterol

White waxy solid, MP 160-164^{o}C, TLC Rf value 0.48 in ethyl acetate/chloroform (1:1), m+: m/z 414). Molecular formula $C_{29}H_{50}O$ Retention time 46.73 min., Qual. 99%, % area 24.78.

Compound 2 Gamma sitosterol

White waxy solid, MP 160-164^{o}C, TLC Rf value 0.48 in ethyl acetate/chloroform (1:1), m+: m/z 414). Molecular formula $C_{29}H_{50}O$ Retention time 46.73 min., Qual. 99%, % area 24.78.

Compound 3 Stigmasterol

White waxy solid, MP 160-164^{o}C, TLC Rf value 0.48, in ethyl acetate/chloroform (1:1), M+: m/z 412, Molecular formula $C_{29}H_{48}O$, Retention time 46.68, Qual, 98% and % area 42.39.

Compound 4 Lupeol

White crystals, MP 210-211^{o}C, TLC Rf value 0.64 in ethyl acetate (Chloroform (1:1), M+: m/z 426, Molecular formula $C_{30}H_{50}O$, Retention time 45.26min, Qual. 98% and % area 35.56.

Compound 5 Friedelan-3-one

White crystals, MP 210-211^{o}C, TLC Rf value 0.64 in ethyl acetate/chloroform (1:1), M+: m/z 426, Molecular formula $C_{30}H_{50}O$, Retention time 48.17min, Qual. 96% and % area 42.74.

Compound 6 Ethyl Iso-Allocholate

White crystals, MP, 210-211^{o}C, TLC Rf value 0.64 in ethyl acetate/chloroform (1:1), M+: m/z 432, Molecular formula $C_{26}H_{40}O_5$, Retention time 35.27, Qual. 72%, and % area 17.42.

4. Conclusion

Phytochemical screening of the plant material revealed the presence of alkaloids, tannins, steroids, carbohydrates, sugar, flavonoids, anthraquinone and saponins. Antimicrobial screening of the crude extracts was carried out *in vitro* on some micro-organisms. Petroleum ether, chloroform and methanol extracts of the leaves inhibited the growth of only four of the test organisms namely *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli* and *Pseudomonas aeruginosa,* but could not completely kill any of these organisms. The methanol extract of the root and stem bark inhibited the growth of five of the test organisms alike at these lowest inhibitory concentrations (MIC): *Streptococcus pyogenes* ($4.0x10^2$mg/mL), *Pseudomonas aeruginosa* ($6.0x10^2$mg/ml), *Salmonella specie* ($10.0x10^2$mg/Ll), *Staphylococcus aureus* ($4.0x10^2$mg/mL) and *Candida albicans* ($10.0x10^2$mg/mL). The extracts showed some differences in their activities against *Escherichia coli* and *Klebsiella pneumoniae.* The MICs of the root extract for *Escherichia coli* and *Klebsiella pneumoniae* are ($4.0x10^2$mg/mL) and ($8.0x10^2$mg/mL) respectively, while the MIC of the stem bark extract for *Escherichia coli* and *Klebsiella pneumoniae are* ($6.0x10^2$mg/mL) and $10.0x10^2$mg/ml) respectively. These differences in MICs showed that the root bark extract is more active than the stem bark. The root bark and stem bark extracts were able to kill three of the test organisms which are *Staphylococcus aureus, Streptococcus pyogenes* and *Candida albicans* all at the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of $1.0x10^3$ mg/mL respectively. The methanol extract of the root bark was separated and purified by column chromatography and the spectral analyses of the two isolated compounds using GC-MS showed that the isolated components are combination of stigmasterol and sitosterol (Beta and Gamma sitosterol), Friedan-3-one, Lupeol and Ethyl Iso-allocholate.Beta sitosterol is mainly known and used for *its cholesterol* lowering property, anti-cancer (prostate cancer), anti-inflammatory, anti-ulcer, anti-diabetic and anti-viral properties and in prevention and in treatment of rheumatoid arthritis. These results really justify the use of this plant in ethnomedicinal practice in Nigeria especially in the treatment of diarrhoea and dysentery caused by *Escherichia coli*, skin diseases and caused by *Staphylococcus aureus* and *Klebsiella*, *pneumoniae*, cough and fever caused by *Streptococcus pyogenes* and bronchopneumonia where *Klebsiella pneumoniae* is implicated. Further work should be carried out on this plant to isolate more useful active components and the pharmacological studies on the isolated components should be carried out to encourage syntheses of appropriate drugs.

References

[1]. Trease and Evans, W.C. (1980): Trease and Evans' Pharmacognosy pp185-187; 340, 246.

[2]. Ndukwe, I.G., Achimugu, M.O. and Amako, N.F. (2005): Phytochemical and Antimicrobial Screening ofCrude Extracts from Stem Bark of *Irvingia gabonensis.* J. Pests, Disease and Vector Management; Vol. 6: 391-397.

[3]. Owonubi, M.O. (1999): Pharmacognostical and Pharmacological Investigation of *Bilghia Sapida* Koenig. A Ph.D Thesis of ABU Zaria, Nigeria. Pp. 1,4,6,8.

[4]. Sofowora A. (1993): Medicinal Plant and Traditional Medicine of Africa. John Wiley and Sons Ltd. Chinchester. Pp. 145, 148, 135-53.

[5]. Harbone, J.B, (1973): Phytochemical Methods. A Guide to Modern Techniques of Plant Analysis. Chapman and Hall Ltd. pp. 183, 4-5, 6.7.

[6]. Finar, I.L. (1975): Organic Chemistry Vol. 2. Stereochemistry and the Chemistry of Natural Products. Longman Singapore Publisher Ltd. 5th Edition pp. 348-349.
[7]. Miller, A. J. (1973): Naturally Occurring Substances that can Induce Tumors. Toxicants Occurring Naturally in Foods. Committee of Food Protection, Food and Nutrition Board. Washington D.C. pp. 535-536, 2nd Edition.
[8]. Burkill, H.M. (1994): The useful Plants of West Tropical Africa. Pp. 360-385.
[9]. Daiziel, J.M. (1939): The useful Plants of West Tropical Africa. The Crown Agents for the Colonies London: pp. 243-248.
[10]. http://www.worldagroforestrycentre 12 March 2007
[11]. Tona L., Kambu K., Mesia K., Cimanga K., Aspers S., De Bruyne T., Pieters L. and Totle J. (1999): Biological Screening of Traditional Preparations from some Medicinal Plants used as Anti-diarrhoea in Kinshasa, Congo. Phytomedicine 601:59-66.
[12]. Ibrahim, H. and Sani, F.S. (2006): Photochemical and Anti-sickling Studies of *Hymenocardia acida* Tul. *The East and Central African Journal of Pharmaceutical Science* Vol. 9(2) pp. 58-59.
[13]. Adjanahoun, E., Ahy, M.R.A, Ake-Asi L., Elewude, J.A. Fadogu, S.O., Gbile, Z.O., Goudde E., Johnson, C.L.A., Keita A., Morakinyo O., Ojewole, J.A.O. OIatunyi, A.O. and Sofowora E.A. (1991): Traditional Medicine and Pharmacological Contribution to Ethnobotanical Floristic Studies in Western Nigeria. Organization of African Unity, Science Technical and Research Commission Lagos, Nigeria, p. 141.
[14]. Igoli, J.O., Igoli, N.P. and Igwe, I.C. (2003): Traditional Medicine Practice amongst the Igede People of Nigeria. *J. Herbs, Spices and Med. Plants* 10(4): 1-10.
[15]. Muanza, D.N., Eular, K.L., Williams L. and Newman, D.J. (1995): Screening for Anti-tumor and Anti-HIV Activities of Nine Medicinal Plants from Zaire; *International Journal of Pharmacognosy*. Vol. 33(2): 98-106.
[16]. Muanza, D.N., Eular, K.L., Williams L. and Newman, D.J. (1994): Anti-bacterial and Anti-fungal Activities of Nine Medicinal Plants from Zaire. *Int. J. Pharmacog*. 32(4): 337-345.
[17]. Jones D., Board, R.G., Sussman, M. (1990): "Staphylococci" Society for Applied Microbiology Symposium, Series No. 19. Blackisell Scientific Oxford.