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Research Article

ANTIBACTERIAL AND PHYTOCHEMICAL EVALUATION OF *HARUNGANA MADAGASCARIENSIS* L (HYPERICACEAE) SEEDS

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ABSTRACT

Clinical cases of resistance to orthodox drugs are on the rise. Medicinal plants are a source of lead compounds in drug discovery. *Harungana madagascariensis* is known for its ethno medicinal uses in the treatment of dysentery and related bacterial infections. In view of this, investigating its antibacterial drug lead potential is an imperative .The crude methanol extract of the fresh seeds was fractionated with n-hexane, chloroform, ethyl acetate and acetone successively. Agar dilution method was used for the antibacterial evaluation with clinical and ATCC strains of *.E. coli and S.aureus* as test microorganisms. Phytochemical methods were by using standard phytochemical screening reagents.The Chloroform fraction was the most active against the test pathogenic organisms with observed minimum inhibitory concentration MIC trend: chloroform (0.03125 mg/ml) > ethyl acetate (0.50000 mg/ml). Anthraquinones were found only in the most active chloroform fraction with some amount of flavonoids aglycones, and triterpenoids. This study showed that anthraquinones and/or flavonoids aglycones could be responsible for the antibacterial activity with possible synergistic effect from flavonoids and terpenoids. This further confirmed its ethnomedicinal uses.

Keywords: *H. Madagascariensis;* antibacterial, and drug discovery

INTRODUCTION

For the millions of rural populations in the developing world, infectious diseases continue to be the major cause of mortality, with an estimated 1 billion episodes of illness and some 5 million or more deaths in children under 5 years[1]. Among this low income population, preparations from herbs and plants remain the most common forms of treatment for infectious diseases. *Harungana madagascariensis* (L.) is a species of flowering plants in the family Hypericaceae and the sole member of the genus *Harungana*. It is found in tropical Africa[2]. Preparations from different parts of *H. Madagascariensis* locally called "elepo" in Yoruba *have* been used in African folk medicine for the treatment of a wide spectrum of human and livestock diseases. The red juice from the leaves and stem bark are used as anti-haemorrhage during child birth, boiled water decoction of the root is used as an antidote in cases of liver and kidney poisonings while the unopened buds are used in the treatment of skin diseases, anaemia, parasitic infestations, and puerperal and wound infections, as well as a spasmolytic agent[3-7]. Scientific validation of the reported medicinal utilities of the leaves, root and stem bark have been reported[3-8]. However, no scientific validation of the medicinal effect of the seeds has been documented. This study, is a preliminary phytochemical and antibacterial investigation of the seeds of *Harungana madagascariensis* seeds with the view of exploring its drug lead potentials in the treatment of infectious diseases caused by bacteria

MATERIALS AND METHODS

Sample Collection, Identification and Preparation

Fresh seeds of *Harungana madagascariensis* used for this study were collected from the medicinal plant garden of the University of Port Harcourt in Rivers State in the month of August, 2011 and authenticated by Mr S.E Albert of the Department of Plant Science and Biotechnology, University of Port Harcourt, Rivers state. The fresh seeds were pulverized using a grinder and extracted immediately using absolute methanol by cold maceration.

Reagents and apparatus

In the course of the research work, the following analytical grade reagents were used; methanol, ethyl acetate, chloroform, n-hexane, ethanol, acetone, ferric chloride, hydrochloric acid, distilled water, Dragendorff's reagent, Mayer's reagent, Hager's reagent, Molisch's reagent, lead acetate solution, sodium hydroxide, Fehlings solution, A and B, ammonia solution, conc sulphuric acid, acetic anhydride, magnesium fillings, nutrient agar, levofloxacin USP (99 %). The apparatus and instruments used includes mortar and pestle, chromatographic tank, glass funnels, weighing balance, beakers, pipettes, separating funnels, Whatman's No. 1 filter paper, test tubes, water bath, pre-coated silica gel HF_{254}TLC plates, micro capillary tubes, inoculating loop, sterile Petri dishes, bunsen burner, autoclave, marker, lighter, paper strip.

Microorganisms

A representative organism of gram-positive bacteria *(Staphylococcus aureus*) including both its clinical isolates and type culture ATCC 25923 and one gram negative bacteria *Escherichia coli* including both its clinical isolate and type culture ATCC 35219 were used.

Extraction of Plant Materials

500 g of the fresh plant material was pulverised in a mortar and extracted exhaustively by cold maceration in absolute methanol for 72 hours and filtered to obtain the methanol filtrate. The marc was exhaustively macerated with more portions of the methanol until a colourless extract was obtained. The methanol filtrates were pooled together and concentrated by evaporation to one tenth of the volume to obtain the crude methanol extract. The concentrated methanol extract was partitioned in succession with: 30 ml x 3 n-hexane , 30 ml x 4 chloroform, and 20 ml x 5 ethyl acetate to obtain the n-Hexane faction (HSF), chloroform soluble fraction (CSF),ethyl acetate soluble fraction (ESF) and a viscous aqueous layer ASF.

Phytochemical Methods

Preliminary phytochemical tests were carried out on the extract and fractions to screen for the presence or absence of

saponins, tannins, flavonoids carbohydrates, reducing sugars, alkaloids, anthraquinone, triterpenes and steroids [9].

Antimicrobial susceptibility Test

The Agar dilution method was used[10]. 0.4 ml of a 40 mg/ml stock solution of the crude methanol extract was aseptically transferred into a bijou bottle containing 9 ml Mueller Hinton agar to give a final test concentration of 4.25 mg/ml, this was poured into a sterile petri dish allowed to set, and incubated at 37^0C for 24 hours, after streaking the organisms in their respective quadrants. For the fractions, I ml of each of a10 mg/ml stock solution was aseptically transferred into the respective bijou bottles containing 9 ml Agar to yield a final test concentration of I mg/ml of each of the fraction. This was then poured aseptically into their respective sterile Petri dishes and the plates were rotated gently to ensure even distribution of the test extract and controls, they were then allowed to set. To the petri dishes which were previously divided into four quadrants a loop full of the respective organisms was applied by streaking on their respective marked quadrant. The streaked plates were then incubated at 37^0c for 48 hours to determine the susceptibility of the organisms to the test extracts/fractions. For the standard drug levofloxacin, 1 ml of a 0.8 mg/ml stock solution was aseptically transferred into a bijou bottles containing 9 ml Agar to yield a final test concentration of 0.08 mg/ml .This was then divided into four quadrants as for the test phytodrugs, streaked with the respective test organisms, and incubated at 37^0C for 48 hours. A growth control was also prepared using only 9 ml agar divided into four quadrants and streaked with respective organism in each quadrant and incubated at 37^0C for 24 hrs. For the sterility control, 1ml of dimethyl sulphoxide was aseptically transferred into a bijou bottle containing 9 ml agar, mixed and poured on a petri dish mark sterility control and incubated at 37^oc for 24 hrs without streaking the test organisms. A negative control was also prepared as for the sterility control but this was streaked with the respective organisms as in the test phytodrugs and incubated at 37^0C for 48 hrs.

Minimum Inhibitory Concentration

After the antimicrobial susceptibility test, a 1 in 10 serial dilution was prepared from the 100 mg/ml stock of the most active fractions (ESF and CSF) to yield a concentration of 10 mg/ml. From the 10 mg/ml concentration a five- fold 1 in 2 serial dilution was performed to yield respective concentrations of 5.0000, 2.5000, 1.2500, 0.6250 and 0.3125 mg/ml. 1ml of each of the serial dilutions was then aseptically mixed with 9 ml agar respectively and transferred aseptically into sterile petri dishes which had already been divided into four quadrant. This is to give a final test concentration of 0.5000, 0.2500, 0.1250, 0.0625 and 0.03125 mg/ml respectively. The agar was allowed to set and the respective organisms were streaked in their respective quadrants using the inoculating loop. The streaked plates were then incubated at 37^0C for 24 hours to determine the minimal inhibitory concentrations.

Table 1: Result of phytochemical screening

	Screened Phytochemical	ESF	CSF	HSF	ASF	CME
1	Saponins: Froth test	-	-	-	-	-
2	Phenolics/tannins:					
	i) $FeCl_3$ test	+	-	-	+	+
	ii) Phlobatannins test	–	–	–	+	–
	iii) Chlorogenic acid test	-	-	-	-	-
3	Flavonoids: i) Shinoda test	+	+	-	+	+
	ii) NaOH test	+	+	-	+	+
	iii)Lead acetate test	+	+	-	+	+
4	Alkaloids: i) Hagers test	-	-	-	-	-
	ii) Mayer's test	-	-	-	-	-
	iii)Dragendorff's test	-	-	-	-	-
5	Anthraquinones:(Borntragers test)	-	+	-	-	+
6	Carbohydrates:i) Molisch's test	-	-	-	+	+
	ii) Fehlings test	+	+	+	+	+
7	Terpenoids and steroid					
	i) Liebermann-Buchard test	-	+	+	-	+
	ii) Salkowski test	-	+	+	-	+

+ = Present; - = Absent; ESF = Ethyl acetate Soluble Fraction; CSF = Chloroform Soluble Fraction; HSF = n- Hexane Fraction; ASF = Aqueous Fraction; CME = Crude Methanol Extract

Table 2: Antimicrobial susceptibility screening results

Test organisms	ESF (1mg/ml)	CSF (1mg/ml)	HSF (1mg/ml)	ASF (1mg/ml)	Levofloxacin 0.08mg/ml	CME (4mg/ml)	Sterility	Growth control
S.aureus ATCC	-	-	-*	-*	-	+	-	+
S.aureus clinical	-	-	-*	-*	-	+	-	+
E.coli ATCC	-	-	-*	-*	-	-*	-	+
E.coli clinical	-	-	-*	-*	-	-*	-	+

+ = visible growth after 24 hrs incubation; - = no visible growth after 24 and 48 hrs incubation, -* no visible growth after 24 hrs incubation but visible growth after 48 hrs incubation

Table 3: Minimum Inhibitory Concentration (MIC) of most the active fractions in mg/ml

ESF				CSF			
S. aureus ATCC	*S. aureus* clinical	*E.coli* ATCC	*E.coli* Clinical	*S. aureus* Clinical	*S. aureus* Clinical	*E.coli* ATCC	*E.coli* Clinical
0.50000	0.50000	0.03125	0.03125	0.03125	0.03125	0.03125	0.03125

ESF = Ethyl acetate Soluble Fraction; CSF = Chloroform Soluble Fraction

RESULTS AND DISCUSSION

The result of the phytochemical screening of *Harungana madagascariensis* seed extract and fractions in table 1 showed that depending on their polarity index, tannins, flavonoids, anthraquinones, reducing compounds, terpenoids and sterols were detected in the seeds extract and fractions. Except for the crude methanol extract and aqueous fraction, all other fractions tested negative to Molisch reagents. However, all fractions and the crude methanol extract gave positive Fehling's test. This is an indication that whereas carbohydrates are present in the crude methanol extract and retained in its aqueous fraction, the non polar HSF, CSF, and ESF showing negative Molisch test but positive Fehling's test are unlikely to contain carbohydrates but compounds with reducing functional groups like aldehydes. Several terpenoids/steroids, phenolics and other phyto-constituents are known to exist as aldehydes. As expected, tannins were observed to be present only in the aqueous fraction ASF. Hydrolysable tannins are water soluble polyphenols. This explains their presence in the polar aqueous fractions. Similar reason could also be adduced for the presence of non-polar terpenoids/steroids in the non-polar n-hexane and chloroform fraction as against their absence in the polar and moderately polar aqueous and ethyl acetate fractions respectively as well as the absence of polar flavonoids in the n-hexane fraction. The observed positive $FeCl_3$ test result for the moderately polar ethyl acetate fraction could be attributed to other phenolic compounds like flavonoids. This is corroborated by its testing negative for the presence of phlobatannins but positive for flavonoids. Anthraquinones were detected only in the chloroform fraction. The antimicrobial susceptibility screening test result in table 2 showed that at the test concentration of 4.25 mg/ml, the crude methanol extract, CME inhibited the growth of the gram negative *E.coli* strains and not those of the gram positive *S. aureus.* However all the fraction at the test concentration of 1 mg/ml inhibited the growth of the test pathogenic organism after 24 hours of incubation. This could be attributed to increased concentration of the bioactive principle(s) in the fractions compared to the crude extract. This inhibition was observed to be sustained by only the ESF and CSF after 48 hrs of incubation. This indicate that at the test concentration of 1 mg/ml, whereas a somewhat bacteriocidal mechanism is being exhibited by the CSF and ESF, the HSF and ASF exerted their inhibition probably through a bacteriostatic mechanism. This is an indication that notwithstanding the observed overlap in phyto-constituents class among CSF/ HSF and CSF/ ESF/ ASF considering triterpenoid/ steroids and flavonoids class of phyto-constituents, the bacteriocidal principles are more likely to be high in the CSF and ESF. The observed overlap could be attributed to variation in concentration and/or structural conformation which could in turn influence bioactivity by way of structure activity relationship. On the basis of this, the Minimum inhibitory concentration (MIC) of the CSF and ESF were determined. The MIC determination (table 3) showed the MIC of the ESF against *S. aureus* to be at concentrations 0.5 mg/ml and that against *E. coli* to be < 0.03125 mg/ml. The CSF on the other hand exhibited a relatively higher degree of antibacterial effect compared to the ESF as seen in its observed MIC against the test *E. coli* (< 0.03125 mg/ml) and *S. aureus* (< 0.03125 mg/ml) strains. This observed spectrum is an indication that the most active antibacterial principle(s) are present in the CSF fraction. From the phytochemical screening result in table 1, although flavonoids, terpenoids and steroids were detected in the CSF like ESF/ASF and HSF respectively as seen in table 1, anthraquinones were detected only in the most bioactive CSF. This is an indication that the most active antibacterial principle in the seed could be an anthraquinone with some additive antibacterial activity effect from the flavonoids and terpenoid constituents depending on their structural conformation. The antibacterial activity of the seeds could be attributed to one or more of the detected secondary metabolites. The leaves of *Harungana madagascariensis* have been reported to contain the flavonoids[4] which have been reported to inhibit the growth of some bacterial species[11.] The anthraquinones derivatives: Harunmadagascariensis A , B, C and D, harunganol B and haningin anthrone, kengaguinine and kenganthranols A, B and C, kenganthranol D and the pentacyclic triterpenes: friedelin, lupeol and betulinic acid have been isolated from stem bark of the plant[12]. Bazouanthrone, a novel anthrone derivative, has been isolated also from the root bark of *Harungana madagascariensis*, together with feruginin A, harunganin, haninganol A, harunganol B, friedelane-3-one and betulinic acid[13].

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