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Urease Inhibitory activity of fractions from *Harungana madagascariensis* fruit Lam. Ex Poir.(Hyperiaceae)

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Abstract

Harungana madagascariensis is used in ethnomedicine to treat stomach diseases, bacterial infections some of which are due to ureolytic pathogens, and anemia among others. This study is a urease inhibition activity guided fractionation of the crude ethanol extract of the fruits of Harungana madagascariensis. The fruit of H. madagascariensis was extracted with 70% aqueous ethanol by cold maceration. The crude aqueous ethanol extract (CEE) was defatted with n-hexane and further partitioned with ethyl acetate to give n-hexane portion (HSF), ethyl acetate portion (ESF) and aqueous portion (ASF). Fractionation of most active ESF was done using chromatographic techniques and it yielded four fractions EA1 –EA4. The modified Berthelot's colorimetric method was used for the in vitro urease inhibition assay. Standard phytochemical methods were used for phytochemical screening. The trend of percentage (%) inhibition for the crude extract and its partitioned fractions was: CEE(35.03±0.05)>ESF(33.29±0.00)>ASF(17.10±0.06)>HSF(12.97±0.06) at 2.00 mg/ml while the trend of activity for the chromatography fractions from the most active ESF at 2.00 mg/ml was observed thus; EA3(60.74±0.00) > EA4 (26.69± 0.06)>EA2(16.79±0.06)>EA1(14.16±0.06). The most active chromatography fraction EA3 had an IC_{50} of 1.0 mg/ml. phytochemical investigation of EA3 revealed the presence of anthraquinones and phenolics. This supports the use in ethnomedicine practice, of H. madagascariensis to treat various stomach and urinary diseases and also a potential lead source of urease inhibitors for sustainable agronomy.

Keywords: *Harugana madagascariensis*, Hypericaceae, urease inhibitors, anthraquinones, phenolics.

Introduction

Approaches based on urease inhibition have been a great breakthrough in the treatment of diseases such as: gastritis, stomach cancer diseases, urolithiasis, urinary tract infection, pyelonephritis, arthritis, and peptic ulcer that are pathogenically associated with ureolytic organisms[1,2]. The urease enzyme causes the release of ammonia which leads to elevation of the physiological pH thus bringing about alkalinizing effect to promote the survival of these pathogenic bacteria[3,4]. It is hence, one of the contributing factors to antibiotic resistance. Aside clinical relevance, urease inhibitors are also utilised as additives in urea-based fertilisers to prevent nitrogen loss as ammonia to the atmosphere due to the activity of soil urease and the attendant food insufficiency and environment damage[5-7].

Nature is blessed with plant secondary metabolites with diverse bioactivities including urease inhibitory activities. Thus, research into natural products that have urease inhibitory activity has contributed immensely to the treatment of infections caused by urease producing organisms, and the development of eco-friendly urease inhibitors for sustainable agriculture. Some natural Products found to possess this activity include the stilbene reserveratrol[8], catechins[9], flavonoids from *Allium cepa* and *Psidium guajava*[10]. Previous reports have shown the prospect of Nigerian flora as source of urease inhibitors[11,12]. In a continued drive to bio-prospect for urease inhibition lead compounds from the Nigerian flora that, this present study investigates the fruits of *Harungana madagascariensis*, a tropical shrub found in Africa widely used in ethno-medicine practice in the treatment of human diseases like bacterial infection[13,14], gastrointestinal diseases[15]. This is with the view of establishing its potential; as source of urease inhibitors for the development of drugs and eco-friendly agrochemicals.

Materials and methods

Reagents and Instruments: Reagents and solvents used in this study were of analytical grade and are products of JHD, ethanol, DCM ethyl acetate, urease assay kit, thiourea, silica gel 200-400mesh, open glass column. UV-Visible spectrophotometer (N4 series).

Sample collection and extraction: The sample, fruit of *H. madagascariensis* was collected from the Medicinal plant Garden of the Department of Pharmacognosy and Phytotherapy

University of Port Harcourt. It was authenticated by a Taxonomist with voucher specimen deposited in the herbarium of the same department. It was air dried and pulverized. A 600g of the pulverized sample was extracted with 70% aqueous ethanol by cold maceration for 72hours with filtration and change of solvent done every 24hours. After the extraction, it was concentrated with rotary evaporator at 40^0C to at least one-tenth of its volume. The crude extract was further dried in a glass dessicator. The crude ethanol extract (CEE) was defatted with n-hexane and further partitioned with ethyl acetate to give n-hexane portion (HSF), ethyl acetate portion (ESF) and aqueous portion (ASF).

Fractionation of the most active ESF using column chromatography: Further fractionation of the ESF was done using chromatographic techniques. Briefly, 3g of ESF was pre-adsorbed by mixing in silica gel (4g) and loaded on a conventional column (internal diameter 4 cm), dry packed with silica gel (200–400 mesh, India) to a height of 15 cm. The mobile phase gradient (500 ml of each) used comprised of n-hexane (4:0 v/v); n-hexane: ethyl acetate (3:1, 2:2, 1:3, 0:4 v/v); ethyl acetate: ethanol (3:1, 2:2 v/v). The eluted fractions were collected at 10 ml intervals and pooled based on observed R_f of resolved spots and color reaction with chromogenic spray reagent from TLC. Four fractions were obtained for EA1(eluted with mobile phase gradient range of: n-hexane: ethyl acetate (3:1,-1:3,v/v)), EA2 (eluted with mobile phase gradient range of: n-hexane: ethyl acetate (1:3 v/v), EA3 (eluted with mobile phase gradient range of: n-hexane: ethyl acetate (1:3 - 0:4 v/v) and EA4 (eluted with mobile phase gradient range of: ethyl acetate: ethanol (4:0-2:2 v/v). All the fractions were screened for anti-urease activity.

Urease inhibition assay: The test was based on the Berthelot method with modification[12,16]. Briefly, 100mg of thiourea was dissolved in 10 ml of ethanol to afford a 10 mg/ml thiourea stock solution. A 1ml aliquot of this thiourea stock solution was further diluted to 10ml giving the 1mg/ml thiourea reference standard test solution. The Urease enzyme was reconstituted with 100ml of distilled water. Test tubes were labeled with the name of different fractions, standard (Thiourea) and negative control. To test tubes labeled for crude extract (CEE), n-Hexane soluble fraction (HSF), ethyl acetate soluble fraction(ESF) and aqueous soluble fraction (ASF), 0.5ml of enzyme and 0.1 ml of 2 mg/ml of each fraction were added followed by 0.2ml of urea and incubated for 10mins while to the test tube labeled standard, 0.5ml of enzyme and 0.1 ml of thiourea were added followed by 0.2 ml of urea, and incubated as above.. After 10 mins incubation of the samples, 0.5ml of the colour developer was added to all the test tubes and incubated for another 10 mins. The absorbance of the samples was taken in duplicate at a wavelength of 630 nm. The above procedure was also repeated on all the fractions from the most active ESF. The percentage urease inhibition was calculated as

$$\% \text{ Urease Inhibition} = \frac{[A_{(negative\ control)} - A_{(sample)}]}{A_{(negative\ control)}} \times 100$$

Where: $A_{(negative\ control)}$= Absorbance of the negative control solution (containing all the reagents except the test fractions), $A_{(sample)}$] = Absorbance of the test fraction.

Further assay was carried out on fractions with promising result at different concentrations and the IC_{50} was obtained by regression analysis from a plot of % inhibition against concentration.

Phytochemical analysis: This was done using standard reagents and methods used for phytochemical screening reagents as reported[17,18]

Statistical analysis: The student t-test and one way analysis of variance was used to test for significance (p=0.05).

Results and discussions

The phytochemical screening carried out on the crude extract showed that the phytoconstituents in the fruit of *H. madagascariensis* include flavonoid, tannins, anthraquinone, triterpenoids and carbohydrates. The trend in percentage (%) inhibition: CEE (35.03 ± 0.05) > ESF (33.29 ± 0.00) > ASF (17.10 ± 0.06) > HSF (12.97 ± 0.06) at 2.00 mg/ml was observed (Table-1). This is showing that of the three fractions from CEE, the ethyl acetate fraction ESF is the most active. Further chromatography fractionation of the ESF gave four fractions with a trend of activity at 2.00 mg/ml is thus; EA3(60.74 ± 0.00) > EA4(26.69± 0.06) > EA2 (16.79 ± 0.06) > EA1 (14.16±0.06) (Figure-1). Although these were significantly ($p < 0.05$) lower compared to the reference standard thiourea. The chromatography fraction EA3 which is the most active against the urease enzyme was further found to have an IC_{50} of 1.0 mg/ml (Figure-2). Phytochemical investigation of EA3 revealed the presence of anthraquinones and phenolics. This corroborates earlier qualitative and quantitative phytochemical analysis reports[19]. Some Phenolic rich plant extracts such as from: pomegranate peel[20] and several others[21] with potent anti-urease activity are documented. Anthraquinone natural products isolated from *Rumex hastatus* have been shown to possess anti-urease activity[22].

Organisms that secret urease include: *Helicobacter pylori, Yersinia enterocolitica,* species of: *Proteus, Klebsiella, Pseudomonas, Staphylococcus* and *Mycobacteria* among others[3,4]. Depending on the organism, diseases such as: gastritis, stomach cancer diseases, urolithiasis, urinary tract infection, pyelonephritis, arthritis, and peptic ulcer are associated clinical manifestations[1,2]. It has also been established that urease is a virulence factor in several pathogenic bacteria that contributes to antibiotic resistance.

Thus strategies based on urease inhibition could prove useful in the treatment of diseases that are caused by these ureolytic organisms[1,2].

Gastrointestinal disorders such as duodenal ulcer, peptic ulcer, gastritis and gastric cancer as well as urinary stone formation and pyelonephritis are mainly caused by *H. Pylori*. This organism inhabits the acidic medium of the stomach by using urease to generate an alkaline environment. The urease function in *Mycobacteria tuberculosis* nitrogen metabolism has been reported to be crucial for its pathogenesis and survival in nutrient-limited micro-environments[4]. This portends a direct relationship between urease presence and *Mycobacteria tuberculosis* survival. Recently, the antimycobacterial activity of an eluate from the n-hexane fraction of *H. madagascariensis* has been reported[23] and in this study it has been shown to possess a mild urease inhibitory activity as shown in Table-1.

Table-1: Anti-urease activity of the crude extract and fractions at 2.00 mg/ml.

Extracts/Fractions	% Inhibition (Mean ± S.D)
Crude 70% aqueous ethanol extract	35.03 ± 0.05
N-hexane soluble fraction	12.97 ± 0.06
Ethylacetate soluble fraction	33.29 ± 0.00
Aqueous soluble fraction	17.10 ± 0.06
Thiourea (1mg /ml) (reference)	88.77 ± 0.07

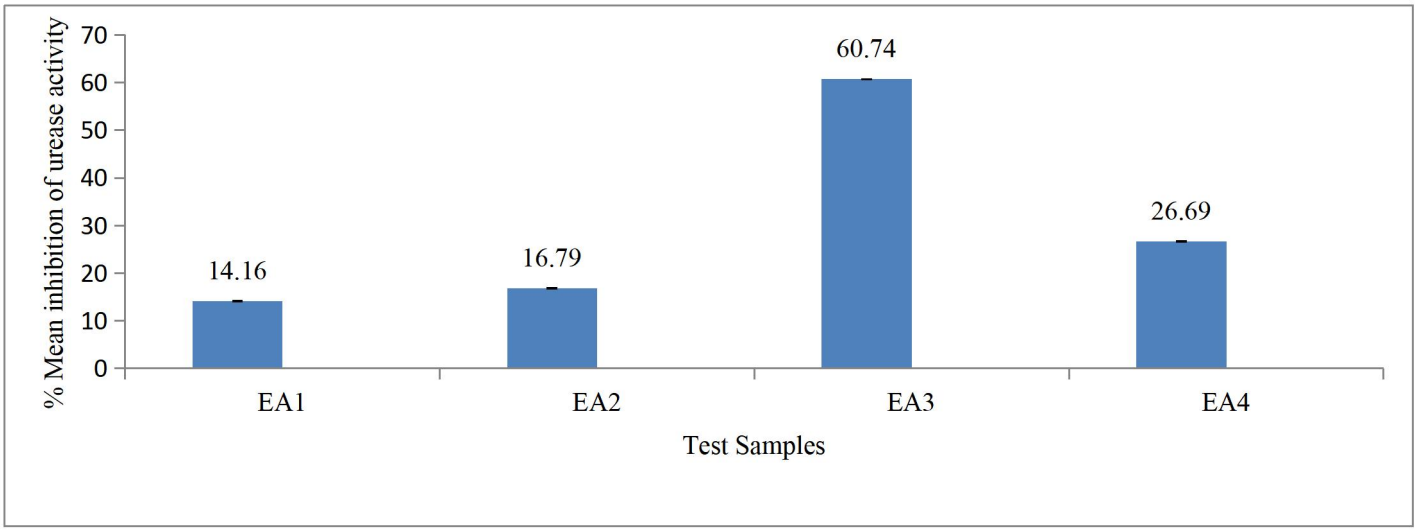

Figure-1: Percentage inhibition of urease by the Chromatography fractions at 2.00 mg/ml. Key to Figure 1: EA1, EA2, EA3 and EA4 are the chromatography fractions from the ethyl acetate portion (ESF) of *Harungana madagascariensis* fruits.

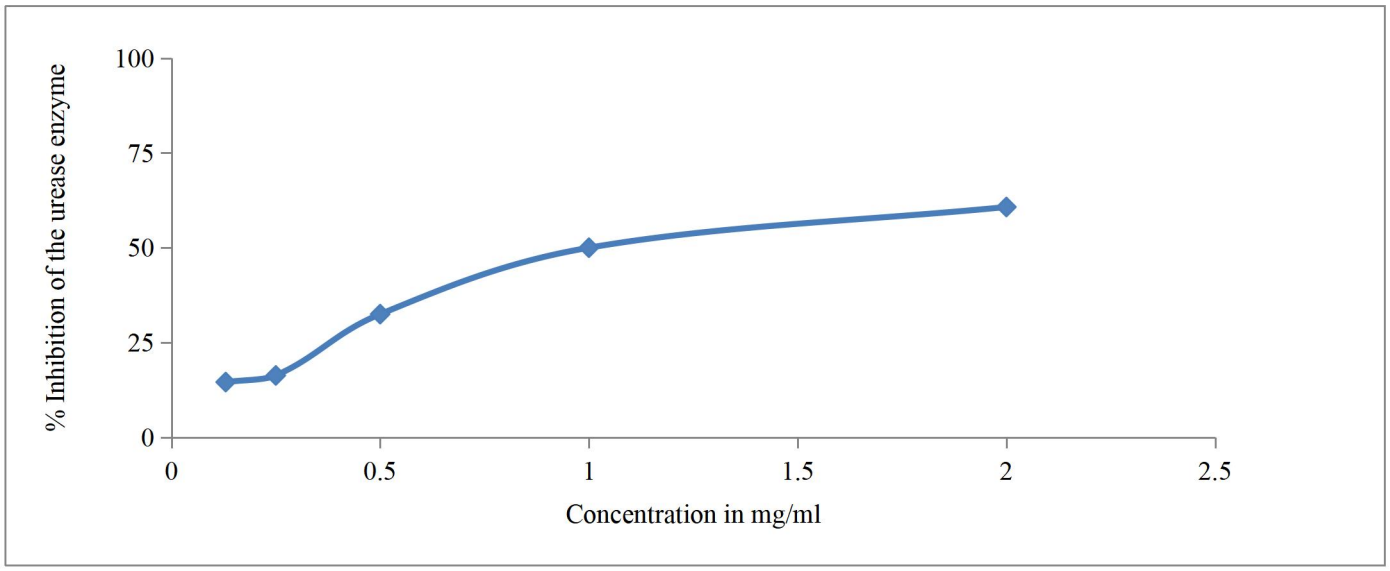

Figure-2: Concentration dependent urease inhibition activity of the most active chromatography fraction. EA3 from the ethyl acetate fraction ESF of *H. madagascariensis* fruits 70% aqueous ethanol extract**.**

Conclusion

H.madagascariensis has been used in treatment of various stomach and urinary diseases. The observed inhibition of urease activity in this study could also offer in part a rationale for the use of *H.madagascariensis* in the treatment of various stomach and urinary diseases. This study is the first time that anti-urease activity of the plant is being reported. Further work is on-going to isolate the urease inhibitor constituent(s) from the active chromatography fraction for drugs and eco-friendly agro-chemical development.

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