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In-vitro Antioxidant Analysis and Phytochemical Evaluations of The Aqueous Extracts of *Hymenocardia acida* (orupa) Leaf, Stem, Root & Bark.

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Abstract - Hymenocardia acida (H.acida) is a plant that is widely used in African complementary medicine. Natural products of this plant have been used in folklore for the treatment of many illnesses and diseases. They have been the most productive source of leads for the development of many effective drugs now available in orthodox medicine. The various phytochemical tests revealed the presence of tannins, alkaloids, flavonoids, saponins, terpenoids and cardiac glycosides whereas steroid was not present in the whole plant, it was only present in the leaf and bark stem. The result also showed that phenol was not present in the stem and bark stem and also the whole plant did not contain chalcone. This research provided detailed information on the Phytochemical and antioxidant properties of the Hymenocardia acida (leaf, stem, root and bark stem).The antioxidant analysis revealed that Hymenocardia acida root extract had the highest total phenolic content (1.55 ± 0.02mgGAE/100g), Hymenocardia acida was higher in total phenolic content (1.35 ± 0.25mgGAE/100g) than hymenocardia acida stem (1.00 ± 0.00mg GAE/100g) and Hymenocardia acida leaf being the lowest (0.90 ± 0.01 mgGAE/100g). The total flavonoid assay showed the stem extracts with highest value (3.50 ± 0.00mg QE/100g), the bark stem extract was higher (2.15 ± 0.07mg QE/100g) than the leaf extract (1.47 ± 0.04mg QE/100g) and the root extract being the lowest in flavonoid content (1.30 ± 0.42mg QE/100g). The ABTS assay also revealed that Hyemnocardia acida leaf, stem, root and bark stem had (75 ± 35.36mmol TEAC/100g, 25.64 ± 1.32mmol TEAC/100g, 90.5 ± 0.71mmol TEAC/100g and 21.70 ± 16.50) respectively. The ferric reducing antioxidant property (FRAP) results showed that the Hymenocardia acida root had the highest FRAP ability (13.75 ± 12.37mmolAAE/g), while hymenocardia acida leaf had higher ability (4.95 ± 0.11mmolAAE/g) than hymenocardia acida bark stem (2.46 ± 0.06 mmolAAE/g) and Hymenocardia acida stem being the lowest (0.21 ± 0.12mmolAAE/g). The result also revealed that the hymenocardia acida stem extract had the best scavenging ability (35.83 ± 1.70mgMAE/ml), and the leaf extract showed better scavenging ability (25.91 ± 16.70mgMAE/ml) than hymenocardia bark stem extract (14.38 ± 6.53mgMAE/ml) and the result shows that the hymenocardia acida root extract have good scavenging ability (1.71 ± 1.46mgMAE/ml).

keywords: phytochemical, antioxidant, hymenocardia acida, leaf, stem.

1. Introduction

Hymenocardia acida (H. acida) belongs to the family of Phyllanthaceae, a native to tropical Africa, and a famous medicinal plant that has been reported to be used in folk medicine for many years in Nigeria and some other parts of Tropical Africa (Igoli *et al*., 2008). Hymenocardia acida (Euphorbiaceae) is very popular in African Trado-medicine. It is called "Heart-fruit" in English (Schmelzer *et al*., 2008), "ii-kwarto" in Tiv, "emela" in Etulo, "Uchuo" in Igede (Agishi *et al*., 2004), "enanche" in Idoma (Abu *et al*., 2011). It is commonly known as "jan yaro" in Hausa, "yawa satoje" in Fulani, "ikalaga" in Igbo, and "Orunpa" in Yoruba (Ibrahim *et al*., 2007). Majorly, all parts of the plant can used as remedies for many ailments (Abu *et al*., 2011). Boiling of the leaves and other parts of this plant alone or mixed with other plant species are used for chest complications, abdominal disorder, and menstrual pains (Sofidiya *et al*., 2010). When the leaves are infused together with the roots, they are used in treatment of small pox (Olotu *et al*; 2010). Ngo et al; 2011 reported that the

leaves, bark and roots of H. acida can be used either in infusion or powdered form to treat hypotension, diabetes, sickle cell, epilepsy, schizophrenia. It is one of the most common plants used in the management of sickle cell anaemia in Nigeria (Ameh 2012 & Ibrahim 2007). Decoction of the leaves and bark is used to treat skin disease and as spice (Ige 2011). Igoli et al; 2008 also reported that the plant's stem bark is also widely applied in traditional medicine. It is reportedly used for bone setting or as an anti-inflammatory agent by traditional bone healers and in the treatment of chest pains. As reported by Olotu et al; 2010, that the Hausa tribe in Northern Nigeria has over the years been using the decoction of leaves and stem bark or root bark for the management of pain of different categories such as migraine, sickle cell crisis and menstrual pain, while Igoli et al; 2005 reported that among the Idoma and Igede people of North Central Nigeria, decoction of roots and stem bark is being used in the treatment of diabetes. The *Hymenocardia acida* possessed a panel of antioxidant compounds and antioxidant enzymes (Awad *et al.*,2011). Still, some related studies have been reported on Hymenocardia acida from, Tunisia (Amira *et al.*,2012), Oman (Al-Farsi *et al.*,2007), Algeria (Mansouri *et al.*,2005) and Iran. The *Hymenocardia acida* is considered very rich source of total phenolic contents among other consumable plants (Biglari *et al.*,2008). It has been reported that Hymenocardia acida leaf extract exerts pharmacological activity in rats, by interaction with antioxidant enzymes, reactive oxygen species and extra cellular calcium (Henrietta *et al.,* 2010). Furthermore, it has been demonstrated that aqueous ethanolic extract of Hymenocardia acida stem bark in female rats has anti-fertility activity. The results suggest that, it could induce inhibitory effects on reproductive functions in female albino rats (Abu and Uchendu, 2011).

2.0 Materials and methods

2.1 Samples collection

Hymenocardia acida (orupa) leaf, stem, root & bark stem were collected in the bush at the south campus of, The Federal Polytechnic Ede, Ede, Nigeria. The identification and authentication of the plant was done in the Department of Biology, Federal University of Technology, Akure and a voucher specimen was deposited at the Departmental Herbarium The fresh plants were washed under running tap-water and dried to a constant weight and pulverized to powder using laboratory electric blender.

2.2 Sample treatment

5g each of the powdery substance of H. acida (leaf, stem, root & bark) was weighed on analytical weighing balance and dissolved in 50ml of distilled water. It was poured in a reagent bottle and placed on HY-2 speed adjusting multi-purpose vibrator/mechanical shaker for 24hrs for thorough shaking and mixing. Then, each dissolved sample was filtered, using a whatmann No1 filter paper and funnel. The filtrate was centrifuged in medical low speed centrifuge at the speed of 3500 for 15mins. The supernatant was poured into a clean reagent bottle and kept in the refrigerator for further analysis while the sediments were discarded.

2.3 Qualitative phytochemical analysis

Qualitative phytochemical analysis of aqueous extracts of H. acida (leaf, stem, root & bark) was carried out on the extracts using standard procedure to detect the bioactive constituents as described by Sofowora (1993), Trease and Evans (1989a) and Harbone (1973).

2.3.1 Test for Taninns: 1ml of extract was boiled in 20ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed green or a blue – black coloration which confirmed the presence of tannin.

2.3.2 Test for Saponin: About 5ml of the extract was boiled in 20ml of distilled water in a water bath and filtered. 10ml of the filterate was mixed with 5ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion which confirmed a positive presence of Saponin.

2.3.4 Test of Flavonoids: 3ml of 1% Aluminium chloride solution was added to 5ml of each extract. A yellow coloration was observed indicating the presence of flavonoids. 5ml of dilute ammonia solution was added to the above mixture followed by addition of concentrated H_2SO_4. A yellow coloration disappeared on standing. The yellow coloration which disappeared on standing indicated a positive test for flavonoids.

2.3.5 Test for Steroids: 2ml of acetic anhydride was added to 2ml extract of each H.acida sample, followed by careful addition of 2ml H_2SO_4. The change in color from violet to blue or green indicated the presence of steroids.

2.3.6 Test for Terpenoids (Salkowski test): 5ml of each the extract was mixed with 2ml of chloroform, and 3ml concentrated H_2SO_4 was carefully added to form a layer. A reddish brown coloration on the interface was formed to show positive results for the presence of terpenoids.

2.3.7 Test for Cardiac Glycosides and Cardenolides: 5ml of each H.acida extract was treated with 2ml of glacial acetic acid containing one drop of ferric chloride solution. This was underplayed with 1ml of concentrated sulphuric acid. A brown ring at the interface indicated a deoxysugar characteristics of cardenolides which confirms a positive presence of cardenolides. A violet-green ring appearing below the brown ring, in the acetic acid layer, indicates the positive presence of glycoside.

2.3.8 Test for Alkaloids: 1ml of each extract was stirred with 5ml of 1% aqueous HCl on a steam bath and filtered while hot. Distilled water was added to the residue and 1ml of the filtrate was treated with a few drops of Wagner's reagent (solution of iodine in Potassium iodide). The formation of a cream colour gave a positive test for alkaloids.

2.3.9 Test for Chalcones: 2ml of ammonia solution was added to 5ml of H.acida extracts. Formation of a reddish color confirmed presence of chalcones.

2.3.10 Test for Phenol: 5ml of each H.acida extract was pipetted into a 30ml test tube, then 10ml of distilled water was added. 2ml of ammonium hydroxide solution and 5ml of concentrated amyl alcohol were also added and left to react for 30min. Development of bluish -green color was taken as a positive presence of phenol.

2.4 Quantitative phytochemical analysis

2.4.1 Determination of total alkaloids: It was carried out according to the method of Harborne, (1973). 5g each of H. acida (leaf, stem, root & bark) was weighed into a 250ml beaker and 200ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4hours. This was filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue was the alkaloid, which was dried and weighed.

2.4.2 Determination of total saponins: It was carried out according to the method of Obdoni et al; (2001). 20g of each H. acida (leaf, stem, root & bark) was put into a conical flask and 100cm3 of 20% aqueous ethanol was added. The samples were heated over a hot water bath for 4 hours with continuous stirring at about 55°C. The mixture was filtered and the residue was re-extracted with another 200ml of 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90°C. The concentrate was transferred into a 250 ml separator funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water

bath. After evaporation the samples were dried in the oven to a constant weight and the saponin content was calculated.

2.5 *in-vitro* antioxidant assays

2.5.1 Determination of total phenol content

The total phenol content was determined according to the method of Singleton et al. (1999). Briefly, appropriate dilutions of the watermelon extracts were oxidized with 2.5 ml 10% Folin-Ciocalteau's reagent (v/v) and neutralized by 2.0 ml of 7.5% sodium carbonate. The reaction mixture was incubated for 40 min at 45°C and the absorbance was measured at 765 nm using UV-VIS spectrophotometer (Gold spectrumlab 53). The total phenol content was subsequently calculated as gallic acid equivalent.

2.5.2 Determination of total flavonoid content

The total flavonoid content was determined using a slightly modified method reported by Meda et al. (2005). Briefly 0.5 mL of appropriately diluted H.acida extracts were mixed with 0.5 mL methanol, 50 µL 10% $AlCl_3$, 50 µL 1 M Potassium acetate and 1.4 mL water, and allowed to incubate at room temperature for 30 min. The absorbance of the reaction mixture was subsequently measured at 415 nm using UV-VIS spectrophotometer (Gold spectrumlab 53). The total flavonoid content was subsequently calculated using quercetin as standard.

2.5.3 2, 2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical scavenging ability

The $ABTS^{\cdot}+$ (2, 2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) scavenging ability of the H. acida (leaf, stem, root & bark) extracts were determined according to the method described by Re et al. (1999). The $ABTS^{\cdot}$ was generated by reacting an (7 mmol/L) ABTS aqueous solution with $K_2S_2O_8$ (2.45 mmol/L, final concentration) in the dark for 16 h and adjusting the Absorbance at 734 nm to 0.700 with ethanol. 0.2 mL of appropriate dilution of the watermelon fruit extract was added to 2.0 mL $ABTS^{\cdot}+$ solution and the absorbance were measured at 734 nm after 15 min using UV-VIS spectrophotometer (Gold spectrumlab 53). Trolox was used as standard and trolox equivalent antioxidant capacity (TEAC) was subsequently calculated.

2.5.4 Determination of reducing property

The reducing property of the aqueous extracts from the H. acida (leaf, stem, root & bark) was determined by assessing the ability of the extracts to reduce FeCl3 solution as described by Oyaizu (1986). 2.5ml aliquot was mixed with 2.5 ml 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml 1% potassium ferricyanide. The mixture was incubated at 50^0C for 20 min. and then 2.5 ml 10 % trichloroacetic acid was added. This mixture was centrifuged at 650 rpm for 10 min. 5 ml of the supernatant was mixed with an equal volume of water and 1 ml 0.1% ferric chloride. The absorbance was measured at 700 nm. The ferric reducing antioxidant property was subsequently calculated.

2.5.5 1, 1-diphenyl–2 picrylhydrazyl radical scavenging ability (DPPH)

The free radical scavenging ability of the H.acida extracts against 1,1-diphenyl–2 picrylhydrazyl (DPPH) free radical was evaluated as described by Gyamfi et al. (1999). Briefly, appropriate dilution of the extracts (1 ml) was mixed with 1 ml, 0.4 mM methanolic solution containing DPPH radicals, the mixture was left in the dark for 30 min and the absorbance was taken at 516 nm. The DPPH free radical scavenging ability was subsequently calculated as percentage of the control.

2.6 Statistical analysis

All the analysis was done in triplicates and the results were analyzed statistically. It was expressed as mean (n=4) ± standard deviation.

3.0 Results

Table 1. Qualitative phytochemical screening of the aqueous extracts of hymenocardia acida (leaf, stem, root & bark).

Phytochemical Assay	H. acida leaf	H. acida stem	H. acida root	H. acida bark
Saponin	+ve	+ve	+ve	+ve
Tannin	+ve	+ve	+ve	+ve
Alkaloid	+ve	-ve	+ve	+ve
Glycoside & Cardinocides	+ve	+ve	+ve	+ve
Steriods	+ve	-ve	-ve	+ve
Terpenoids	+ve	+ve	+ve	+ve
Flavonoid	+ve	+ve	+ve	+ve
Phenol	+ve	-ve	+ve	-ve
Chalcone	-ve	-ve	-ve	-ve

+ve = Present
-ve = Not present

Table 2. Quantitative phytochemical screening of the aqueous extracts of hymenocardia acida (leaf, stem, root & bark).

Phytochemical Assay	H. acida leaf	H. acida stem	H. acida root	H. acida bark
Total saponin (%)	1.05	1.50	0.69	1.80
Total Alkaloid (%)	2.68	2.30	1.00	0.90

Table 3. Total phenol, total flavonoid, ABTS, and FRAP of aqueous extracts of hymenocardia acida (leaf, stem, root & bark).

Sample	Total Phenol (mgGAE/100g)	Total Flavonoid (mgQE/100g)	ABTS (mmol/TEAC/100g)	FRAP (mmolAAE/g)
H. acida leaf	0.90 ± 0.01	1.47 ± 0.04	75 ± 35.36	4.95 ± 0.11
H. acida stem	1.00 ± 0.00	3.50 ± 0.00	25.64 ± 1.32	0.21 ± 0.12
H. acida root	1.55 ± 0.02	1.30 ± 0.42	21.70 ± 16.50	13.75 ± 12.37
H. acida bark	1.35 ± 0.25	2.15 ± 0.07	90.50 ± 0.71	2.46 ± 0.06

Values represent means ± standard deviation of triplicate readings.
GAE = Gallic Acid Equivalent
QE = Quercetin Equivalent
AAE = Ascorbic Acid Equivalent
TEAC = Trolox Equivalent Antioxidant Capacity

Table 4. 2,2-diphenyl-picrylhydrazyl (DPPH) radicals scavenging abilities of the aqueous extracts of hymenocardia acida (leaf, stem, root & bark).

Assay	H. acida leaf	H. acida stem	H. acida root	H. acida bark
DPPH (mgMAE/ml)	25.91 ± 16.70	35.83 ± 1.70	1.71 ± 1.46	14.38 ± 6.53

Values represent means ± standard deviation of triplicate readings.

4.0 Discussion

Phytochemicals are a powerful group of chemicals obtained from natural sources, especially from plants origin. In plants, phytochemicals play significant roles in their growth and development. The phytochemical analysis showed that saponin, tannin, terpenoids, flavonoids, and alkaloids (except H.acida stem) were present in H. acida leaf, stem, root & bark. This is an indication that every part of H.acida is medicinally valuable. As reported by Osagie et al; (1998) that saponins possessed cholesterol-lowering ability in the blood by preventing its reabsorption which makes it viable in the management of cardiovascular disease. It has also been reported that saponins have anti-tumor and anti-mutagenic potentials thereby lowering the the risk of human cancers, thus, prevent the growth of cancer cells (Roa *et al.*, 1995). Flavonoids possess antioxidant potentials and health-promoting abilities such as anti-thrombotic, anti-inflammatory, vasoprotective, anti-allergic, tumor inhibitory and anti-viral potentials (Trease and Evans, 2002b). Tannin rich medicinal plants are used as healing agents in a number of diseases. In Ayurveda, formulations based on tannin-rich plants have been used for the treatment of diseases like leucorrhoea, rhinnorhoea and diarrhea (Dhir *et al*., 2002). Okwa and Josiah (2006) reported tannin to promote wound-healing. Firn (2010) had documented terpenoids to be anti-cancer agent. Duke (1992) also documented terpenoids to be anti-diabetic agent due to its ability to inhibit glucose uptake. Clinical studies had also documented alkaloid to possess analgesic activities due to its ability to reduce pains (Sarker & Nahar, 2007). The presence of these bioactive constituents in H. acida leaf, stem, root & bark is in conformation with the study carried out by Tor-Anyii Terrumn Amom et al, (2013).

Antioxidants protect cells against the damaging effects of reactive oxygen species otherwise called, free radicals such as singlet oxygen, super oxide, peroxyl radicals, hydroxyl radicals and peroxynite which results in oxidative stress leading to cellular damage (Mattson & Cheng, 2006). Natural antioxidants play a key role in health maintenance and prevention of the chronic and degenerative diseases, such as atherosclerosis, cardiac and cerebral ischema, carcinogenesis, neurodegenerative disorders, diabetic pregnancy, rheumatic disorder, DNA damage and ageing (Uddin *et al*., 2008; Jayasri *et al*., 2009).

The antioxidants results of this study revealed that the neglected parts (root and bark) had the most increased total phenol content 1.55 ± 0.02 mgGAE/100g and 1.35 ± 0.25 mgGAE/100g respectively, while stem and leaf had 1.00 ± 0.00 mgGAE/100g and 0.90 ± 0.01 mgGAE/100g respectively. On the other hand, the stem and bark possessed highest total flavonoid content, 3.50 ± 0.00 mgQE/100g and 2.15 ± 0.07 mgQE/100g respectively. This agreed in line with the studies carried out by Hour et al, (1980) and Singleton et al, (1999), where they both submitted that plants rich in phenols and flavonoids, reduce the risk of coronary heart disease, protect human body from free radicals, prevent oxidative cell damage, and have high potent anti-cancer activity. The reducing power (FRAP) of H.acida was most significant in the root with 13.75 ± 12.37 mmolAAE/g while the stem recorded the least value 0.21 ± 0.12 mmolAAE/g. FRAP measures the tendency of an antioxidant which acts as a reducing agent and contribute a single electron to the Fe^{3+} in a redox-linked colorimetric reaction based on the breaking of the free radical chain in order to stabilize and terminate the radical chain reactions (Prior *et al*., 2005). Apart from the H.acida root that recorded 1.71 ± 1.46 mgMAE/ml as DPPH value, other parts (leaf, stem, and bark) of H.acida exhibited great free radicals scavenging abilities with the stem having the highest scavenging ability of 35.83 ± 1.70 mgMAE/ml. This is in line with the submission of Ahmadi et al; 2007 which stated that plants with significant DPPH values have high tendencies to mop up free radicals in human body, thereby preventing the damage of DNA cells.

5.0 Conclusion

The study revealed that H. acida (leaf, stem, root & bark) possessed bioactive components which can be utilized in the management of various degenerative diseases such as diabetes, renal dysfunction, hypertension, etc. Also, its antioxidant potentials exhibited that it could effectively scavenge free radicals in human body, which may prevent the damage of DNA cells.

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