Cell Membranes and Free Radical Research

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AIM AND SCOPES

Cell Membranes and Free Radical Research is a print and online journal that publishes original research articles, reviews and short reviews on the molecular basis of biophysical, physiological and pharmacological processes that regulate cellular function, and the control or alteration of these processes by the action of receptors, neurotransmitters, second messengers, cation, anions, drugs or disease.

Areas of particular interest are four topics. They are;

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C- Interaction Between Oxidative Stress and Ion Channels
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Keywords
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In vitro antioxidant activity of extracts from the leaves of *Hura crepitans* (Euphorbiaceae) - a comparison of two assay methods

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List of abbreviations

A_{DPPH}; Absorbance of DPPH
BHA; Butylatedhydroxyanisole
DNA; Deoxyribonucleic acid
DPPH; 2, 2-diphenylpicrylhydrazyl radical
HCl; Hydrochloric acid
NaOH; Sodium hydroxide
ROS; Reactive oxygen species
RSA; Radical scavenging activity
TLC; Thin Layer Chromatography
UV; Ultraviolet Visible Spectrophotometer
PBS; Phosphate-buffered saline

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Abstract

Hura crepitans (Sandbox tree) of the family of Euphorbiaceae has enjoyed many ethnomedicinal applications but little is known about its chemistry and pharmacology. This research reports the *in vitro* antioxidant activity of this plant using two different assay methods. Scavenging effect on 2, 2-diphenylpicrylhydrazyl (DPPH) radical at 517 nm and on hydroxyl radical generated by hydrogen peroxide at 285 nm in a UV-Visible spectrophotometric assay. Butylatedhydroxyanisole (BHA), vitamin C and α-tocopherol were used as reference standards. There is generally decrease in absorption of DPPH caused by the extracts. The percent inhibition of the crude extract increases with a decrease in concentration in the DPPH photometric assay. The percent inhibition of hexane, ethylacetate and butanol fractions was low except for the butanol fraction (50.7 % at 1.0 mg/ml) when compared with standards vitamin C (90.8 % at 1.0 mg/ml) and BHA (95.4 % at 1.0 mg/ml). In the hydrogen peroxide assay however, the hexane, ethylacetate and butanol fractions scavenged hydroxyl radical more effectively than the standards. The crude extract possessed maximum % inhibition in both the DPPH and hydrogen peroxide free radicals. Therefore, *H. crepitans* has very weak activity as a hydrogen donor but its activity as hydroxyl radical scavenger is high when compared to standards used. This study suggests that the crude and fractions obtained from *H. crepitans* possess antioxidant activities which can counteract or prevent oxidative damage in biological systems caused by the presence of hydroxyl radical.

Keywords

UV-Visible, Antioxidant, 2,2-diphenylpicrylhydrazyl, Hydrogen peroxide , *Hura crepitans,* Euphorbiaceae

Introduction

The growing interest in alternative therapies especially in the use of natural products derived from plants was as a result of many pharmacologically active compounds of natural origin. Examples of important drugs obtained from plants are digoxin from *Digitalis spp.,* quinine and quinidine from *Cinchona spp.,* vincristrine and vinblastine from *Catharanthus roseus,* atropine from *Atropa belladana* and morphine and codeine from *Papaver somniferum.* It is estimated that 60% of anti-tumor and anti-infectious drugs already in the market or under clinical trial are of natural origin (De Pasquale, 1984; Vulto and Smet, 1988; Hamburger and Hostettmann, 1991; Simson and Ogorzaly, 1995; Williamson *et al.,* 1996; Yue-Zhong Shu, 1998). Medicinal plants find applications in pharmaceutical, cosmetic, agricultural and food industries. Recently, research has supported biological activities of some medicinal herbs. Cancer is such a segment where researchers are expecting new molecules from herbs that can provide tools for fighting the dreaded disease. The concept of antioxidant is also catching up and the latest research has shown that a number of herbal derivatives have excellent antioxidant action. Oxidation is essential to many living organisms for the production of energy to fuel many biological processes and generating reactive oxygen species (ROS) but excess has been shown to be harmful. Scavenging of free radical is one basic mechanism of inhibition of oxidative processes. Radical scavenging assays involve two fundamental aspects; the generation of test radical and the monitoring of radical reactions. Bacoside A derived from *Bacopa monnieri* has been shown to be a strong antioxidant which reduced several steps of free radical damage (Halliwell, 1989; Ayuveda, 2005; Bors and Saran, 1991; Potterat, 1997, Halliwell and Gutteridge, 1984).

Hura crepitans (Sandbox tree) of the family of Euphorbiaceae has enjoyed many ethnomedicinal applications as emetic, purgative, antimicrobial, anti-inflammatory and in the treatment of leprosy. The juice from the plant contains two lectins which have haemagglutinating activity that inhibits protein synthesis. Huratoxin, a piscicidal constituent (widely used to catch fish in different parts of the world) was isolated from the milky sap of *H. crepitans* along with hexahydrohuratoxin and keto-enal, crepitin is also a toxalbumin derived from *H. crepitans* and has been shown to be toxic (Kawazu, 1972; Burkill, 1985; Sofowora, 2008). The plant has not enjoyed much investigation in terms of the nature of secondary plant metabolites and pharmacological activity.

DPPH is a stable free radical and accept electron or hydrogen radical to become stable diamagnetic molecule (Frankel et al., 1996; Soares *et al.,* 1997; Mensor et al., 2001). It has absorption characteristics of 517 nm which confers on it a violet color. This color disappears quickly when the DPPH is reduced by a group of radicals and there is a decrease in absorption at 517nm when measured with a UV – Visible spectrophotometer. The bleaching of DDPH absorption occurs when the odd electron is paired. While hydroxyl radical is an extremely reactive free radical formed in biological systems (Hochstein and Atallah; 1988) and reacts rapidly with molecules of almost every type found in living cells, such as sugars, amino acids, phospholipids, DNA bases and organic acids, excess production of hydroxyl radical causes oxidative damage (Aitken and Clakson 1988, Halliwell and Gutteridge, 1984).

The aim of this research work therefore is to carry out *in vitro* antioxidant screening on *H. crepitans* by using two methods scavenging effect on 2, 2-Diphenyl-1-picrylhydrazyl radical (DPPH) and hydroxyl radical generated by hydrogen peroxide and compare the possible mode of action in each case. The activity is compared with the following reference standards: butylatedhydroxyanisole (BHA), Vitamin C and α-tocopherol. These assays are newly reported for *H. crepitans* but are widely used to evaluate antioxidant effect of plant extract as well as pure compounds (Koleva *et al.,* 2002; Gow-chin and Pin-Der, 1994; Nakayana et al., 1994).

MATERIALS AND METHODS

Chemicals and Reagents: Hexane, ethyl acetate, methanol, butanol, and chloroform, hydrochloric acid, ammonia solution, naphthol, bismuth nitrate, potassium iodide, sodium hydroxide, copper acetate, NaOH, sodium chloride, copper sulphate pentahydrate, ferric chloride, conc. tetraoxosulphate (VI) acid, conc. HCl, ammonia solution, sodium potassium tartarate, potassium chloride, glacial acetic acid, disodium hydrogen phosphate, and dihydrogen potassium phosphate were all BDH general purpose chemicals and distilled prior to use. Dimethylsulphoxide (M&B, England) and hydrogen peroxide (Merck, Germany) and 2, 2 - diphenyl-1-picrylhydrazyl radical (DPPH), ascorbic acid, butylatedhydroxylanisole or 2-tert-butyl-4-methoxyphenol (BHA) and α-tocopherol were obtained from Sigma Chemical Co (St Louis, MO).

Equipment and Apparatus: Soxhlet apparatus, Mettler analytical balance H80 (UK), Water Bath (Gallenkamp), condenser, Rotavapor RII0 (Buchi, England), silica gel

GF_{254} (precoated aluminium sheets - Merck Germany), pH meter (Jenway model), Astel Hearson Oven (Gallenkamp), UV-Visible spectrophotometer (Unico1200 & Perkin Elmer lambda 25 models, Germany).

Plant collection and identification

Fresh leaves of *Hura crepitans* were collected in September 2009 at the Botanical Gardens, University of Ibadan, Oyo state. The specimens were identified and authenticated at the Herbarium unit of the Department of Botany and Microbiology, University of Ibadan, Nigeria.

Sample preparation

The leaves of *Hura crepitans* were weighed and air-dried for 3 weeks until the weight was constant and then pulverized using mill machine at the Wood extraction laboratory, Department of Chemistry, University of Ibadan. The pulverized samples were weighed and kept for further analysis.

Extraction and fractionation procedure

Pulverized dried leaves (3.5 kg) of *H. crepitans* were extracted with 4.5 L of distilled methanol using soxhlet apparatus. The extracts were collected and concentrated with the aid of a Bucchi rotavapor at 37 ^{0}C and stored in a desiccator prior to further analysis. Thin Layer Chromatography (TLC) was employed using silica gel 60 F_{254} pre-coated plates and solvent system: Ethyl acetate/ methanol (8:2) to detect antioxidant activity by using 2, 2-diphenyl-1-picrylhydrazylradical (DPPH) as a spray reagent. Yellow coloration on the spots on the TLC plates indicates that the crude extracts of *H. crepitans* have antioxidant activity. The crude methanolic extracts obtained were partitioned into various fractions with distilled water, hexane, ethylacetate and butanol successfully. This was done to separate the plant constituents according to their polarity. Thereafter, quantitative free radical scavenging activity test were carried out on the fractions using the following spectrophotometric experiments; scavenging effect on 2, 2 - diphenyl-1-picrylhydrazyl radical (DPPH) and scavenging effect on hydroxyl radical generated by hydrogen peroxide.

Determination of Scavenging effect of *H. crepitans* leaves extracts on DPPH radical

Antioxidant activity or the capacity to scavenge the "stable" free radical DPPH was determined by the method described by Oloyede and Farombi (2010). A 3.94 mg of 2, 2-diphenyl-1-picryhydrazyl radical (DPPH), a stable radical was dissolved in methanol (100 ml) to give a 100 μM solution. To 3.0 ml of the methanolic solutions of DPPH was added 0.5 ml of each of the fractions with doses ranging from 1.0 to 0.0625 mg/ml (Gulcin *et al.,* 2002; Mutee *et al.,* 2010 and Oloyede *et al.,* 2010). The mixture was shaken well and left to stand for 10 minutes. The absorbance of the solution of DPPH only was measured spectrophotometrically at 517 nm. The reduction in absorption at 517 nm of DPPH was measured 10 minutes later. The actual decrease in absorption induced by the test extract was calculated by subtracting that of the control. The radical scavenging activity (RSA) was calculated as a function of the percentage inhibition of DPPH discoloration using the equation:

$$\% \text{ RSA or } \% \text{ inhibition} = \{(A_{DPPH} - A_S)/A_{DPPH}\} \times 100$$

where AS is the absorbance of the solution when the sample extract has been added at a particular concentration to the DPPH, and ADPPH is the absorbance of the DPPH solution. All tests and analyses were run in triplicates and the results obtained were averaged (Hatano et al., 1988). The analysis was carried out for the crude methanolic extract, hexane, ethylacetate and butanol fractions of *H. crepitans* leaves. BHA, vitamin C and α-tocopherol were used as antioxidant standards.

Determination of scavenging effect of plant extracts on hydroxyl radical generated by hydrogen peroxide

Spectrophotometric determination of extracts from the leaves of *H. crepitans* to scavenge hydroxyl radical generated by hydrogen peroxide was carried out at 285 nm in a UV-Visible spectrophotometer. A solution of 2 mM hydrogen peroxide was prepared in phosphate-buffered saline (PBS) at pH of 7.4. The fractions at the following concentrations; 0.1 - 0.00625 mg/ml was added to the hydrogen peroxide solution. Reduction in absorbance of hydrogen peroxide at 285 nm was determined spectrophotometrically 10 minutes against a blank solution containing the different extracts in PBS without hydrogen peroxide. All tests were run in triplicate and averaged. The same experiment was carried out on Butylatedhydroxyanisole (BHA), vitamin C and α-tocopherol (Soares *et al.,* 1997; Oloyede and Farombi, 2010).

Statistical analysis

Statistical analysis was carried out with Statistical Package for Social Sciences (SPSS). Data were expressed as the mean ± standard deviation and a probability of less than 0.05 ($p < 0.05$) was considered to be statistically significant. Graph was drawn using Microsoft Office excel, 2007 software.

RESULTS

Antioxidant Activity

Scavenging effects on DPPH

The reduction in absorbance of DPPH at 517nm caused by the samples was measured in triplicate after 10min. DPPH is known to be a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares *et al.,* 1997). There is generally decrease in absorption of DPPH caused by the extracts, absorption decreases as the concentration decreases for the crude extract while for the fractions and standards, the absorbance values increases as the concentration is decreased. The result is presented in Table 1. Percentage inhibition of DPPH by the methanolic extract, fractions of *H. crepitans* and standards; Vitamin C, BHA and α-Tocopherol are presented in Figure 1.

Scavenging effects on Hydrogen peroxide (H_2O_2)

The scavenging activities of the extracts and standard antioxidants such as Vitamin C, Butylated hydroxyanisole (BHA) and α-tocopherol on hydroxyl radical generated by hydrogen peroxide is shown in Table 2 and Figure 2. Scavenging effects on hydrogen peroxide was measured in triplicates after 10min of incubation at 285 nm.

DISCUSSION

The percentage inhibition of the crude extract increases with a decrease in concentration in the DPPH

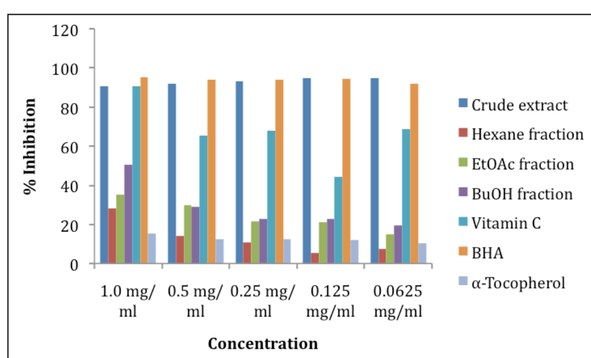

Figure 1. %Inhibition of DPPH by the methanolic extract, fractions of *H. crepitans* and standards; Vitamin C, BHA and α-Tocopherol.

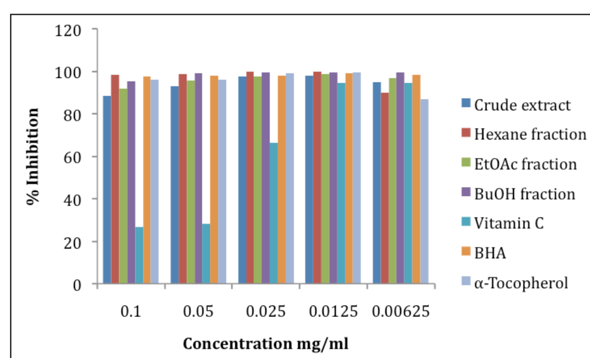

Figure 2. Inhibition of Hydroxyl radical by extracts obtained from *H. crepitans* and standards (Vitamin C, BHA and α-Tocopherol).

Table 1 . Absorbance values (nm) of plant extracts, vitamin C, BHA and α-Tocopherol at 517 nm

Conc (mg/ml)	Crude extract	Hexane fraction	EtOAc fraction	BuOH fraction	Vitamin C	BHA	α-Tocopherol
1.0	0.095±0.005	0.780±0.092	0.648±0.011	0.492±0.038	0.085±0.009	0.037±0.006	0.680±0.029
0.5	0.079±0.003	0.858±0.068	0.707±0.002	0.703±0.029	0.320±0.082	0.048±0.002	0.704±0.004
0.25	0.069±0.006	0.889±0.107	0.784±0.057	0.772±0.053	0.498±0.124	0.049±0.004	0.705±0.007
0.125	0.054±0.005	0.946±0.024	0.787±0.028	0.769±0.043	0.515±0.015	0.046±0.008	0.721±0.012
0.0625	0.051±0.002	0.924±0.061	0.848±0.043	0.802±0.048	0.289±0.128	0.065±0.003	0.707±0.007

*Absorbance measurement of hexane, ethyl acetate and Butanol (BuOH) fractions of leave, Ascorbic Acid, BHA and α- Tocopherol at 517nm. (DPPH absorbance = 0.999±0.011, n=5)

Table 2 . Scavenging effects on hydrogen peroxide by *H. crepitans* Leaves Extract (nm)*

Conc (mg/ml)	Crude extract	Hexane fraction	EtOAc fraction	BuOH fraction	Vitamin C	BHA	α-Tocopherol
0.1	0.430±0.010	0.023±0.004	0.302±0.002	0.174±0.001	2.759±0.049	0.095±0.003	0.155±0.061
0.05	0.267±0.001	0.049±0.003	0.168±0.005	0.033±0.001	2.924±0.211	0.074±0.015	0.181±0.015
0.025	0.097±0.029	0.012±0.001	0.094±0.006	0.020±0.002	1.265±0.119	0.113±0.014	0.032±0.045
0.0125	0.074±0.005	0.286±0.005	0.054±0.001	0.058±0.042	0.203±0.004	0.042±0.016	0.063±0.032
0.00625	0.197±0.004	0.355±0.004	0.122±0.001	0.025±0.002	0.195±0.001	0.062±0.019	0.494±0.017

* Absorbance values of plant extracts, vitamin C, BHA and α-Tocopherol (hydrogen peroxide absorbance = 3.7692 ± 0.021, n=5) at 285 nm

photometric assay. The highest value (94.9 % at 0.0625 mg/ml) was greater than those exhibited by the standards vitamin C (90.80 % at 1.0 mg/ml) and α-tocopherol (15.42 % at 1.0 mg/ml), but lower than the scavenging activity of the standard BHA (95.4 % at 1.0 mg/ml) (Figure 1). The percentage inhibition of hexane and ethylacetate fractions was low except for the butanol fraction (50.7 % at 1.0 mg/ml) when compared to those of standards vitamin C (90.8 % at 1.0 mg/ml) and BHA (95.4 % at 1.0 mg/ml) (Figure 1). In the hydroxyl radical scavenging assay however, the extracts from the leaves scavenged hydroxyl radical in a concentration dependent manner and the activity was comparable to BHA and α-tocopherol at concentration of 1.0 - 0.0065 mg/ml (Table 2). The scavenging effects of the crude extract increased with decrease in concentrations, at 0.0125 mg/ml, the percentage inhibition was 98.0 %, but very slightly lower than the scavenging effects of the standards BHA (98.9% at 0.0125 mg/ml) and α-tocopherol (98.7%). The percentage inhibition of hexane fraction increased with increase in concentration. It had the highest percentage inhibition of 99.67 % at 0.0125 mg/ml. The highly polar butanol fraction of the leaves extract has the highest % inhibition at 0.00625 mg/ml. All the extracts scavenged the hydrogen peroxide radicals more effectively than vitamin C (Figure 2). This result revealed that *H. crepitans* has strong ability as a hydroxyl radical scavenger. Hydrogen peroxide has only a weak activity to initiate lipid peroxidation, but its activity as an active - oxygen specie comes from its potential to produce the highly reactive hydroxyl radical through the Fenton reaction. Generally, flavonoids or phenolic compounds have been shown to be responsible for antioxidant activities of plants (Gow-chin and Pin-Der., 1994; Alan and Miller, 1996), the absence of this class of secondary metabolites in the leaves extract of *H. crepitans* may have been responsible for the weak activity observed in some fractions. The Butanol fraction however can be a source of natural anti-oxidant and useful in the therapy of ailments caused by oxygen reactive species.

Although oxidation reactions are crucial, their damaging effect cannot be overemphasized hence, plants and animals maintain complex systems of multiple types of antioxidants like vitamin C and E, glutathione, and enzymes such as superoxide dismutase, catalase and various peroxidase. Oxidation reaction produces free radicals which in turn start chain reactions that damage cells and biological macromolecules. Interaction between the various cell systems of a cell as well as the different types of protein biosynthesis, DNA and RNA and structural/ enzymatic function is tampered with. This invariably leads to oxidative stress, an important negative factor in many human diseases. Low levels of antioxidants or inhibition of the antioxidant enzymes are known to cause this oxidative stress that damage or kill cells. The usefulness of antioxidants in molecular biology is therefore intensively studied, particularly as treatments for stroke and neurodegenerative diseases.

CONCLUSION

Two different assay methods, scavenging effect on 2, 2-diphenylpicrylhydrazyl (DPPH) at 517 nm and on hydroxyl radical generated by hydrogen peroxide at 285 nm using UV-Visible spectrophotometer was used in this study to investigate and compare the activity of different extracts obtained from *H. crepitans.* There is no report yet in literature about the antioxidant activity of this plant. This study therefore revealed the mode of action of this plant on oxidants. *H. crepitans* extracts had weak percentage inhibition in the reaction involving DPPH indicating that *H. crepitans* has very weak activity as a hydrogen donor but its activity as hydroxyl radical scavenger is high when compared to standards used. In the hydrogen peroxide assay, the hexane, ethylacetate and butanol fractions scavenged hydrogen peroxide more effectively than the standards Vitamin C, BHA and α-Tocopherol. This study suggests that the crude and fractions possess antioxidant activities which can counteract or prevent the oxidative damage in biological systems caused by the presence of hydroxyl radical. Further work is going on to isolate the pure antioxidant compounds from *H. crepitans* and the chemical compounds isolated from this plant can be useful in the therapy of diseases involving oxidative damage thus, proving the medicinal importance of *H. crepitans.*

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