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Original article:

PHYTOCHEMICAL INVESTIGATION, CYTOTOXICITY AND FREE RADICAL SCAVENGING ACTIVITIES OF NON-POLAR FRACTIONS OF *ACALYPHA HISPIDA* (LEAVES AND TWIGS)

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ABSTRACT

Free radicals are involved in the process of lipid peroxidation and play a cardinal role in numerous chronic diseases like cancer, coronary heart disease and ageing. Thus the ability to scavenge free radicals in order to minimize oxidative damage to living cells is very important. The hexane extract of *Acalypha hispida* (Burn F.) was screened for phytochemical constituents and was found to contain flavonoids, carbohydrates, phenols and alkaloids. Fractions obtained from chromatographic separation were screened for free radical scavenging activities using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) and hydrogen peroxide. Out of the sixteen pooled fractions (S_1-S_{16}) screened, compounds S_{10} (91.8 %), S_{11} (93.8 %), S_{14} (92.5 %) and S_{15} (91.4 %) at a concentration of 0.1 mg/ml had significant antioxidant activities when compared to the known antioxidant ascorbic acid (90.9 %). However, in the analysis using hydrogen peroxide, S_1 (99.5 %), S_9 (99.2 %), S_{10} (95.4 %),S_{11} (95.8 %) and S_{15} (95.6 %) gave better activity than ascorbic acid (94.8 %), while only S_1 and S_9 were more effective than butylated hydroxylanisole (98.9 %) and α-Tocopherol (99.1 %) at the same concentration. The cytotoxicity analysis using the Brine Shrimp lethality test gave LC_{50} values greater than 1000 µg/ml for some of the fractions indicating very low level of toxicity. The better scavenging activity of *A. hispida* could be linked to the presence of secondary plant products like flavonoids and phenols, which have the ability to scavenge hydroxyl radicals, superoxide anions and lipid peroxy radicals.

Keywords: Phytochemical constituents, free-radical, cytotoxicity, flavonoids, Acalypha hispida, 2,2-diphenyl-1-picrylhydrazyl radical

INTRODUCTION

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many based on their uses in traditional medicine. Various medicinal plants have been used for years in daily life to treat diseases all over the world. The widespread use of herbal remedies and healthcare preparations, such as those described in ancient texts like the Vedas and the Bible has been traced to the occurrence of natural products with medicinal properties. In fact, plants produce a diverse array of bioactive molecules, making them a rich source of diverse type of medicines (Durga et al., 2009). Thus, natural products with pharmacological or biological activities still play a very important role in medicine (Cragg et al., 1997). In particular, they are important in

the treatment of life-threatening conditions (Newman et al., 2000).

Oxidation reactions initiated by excess free radicals have been shown to lead to the formation of tumours, damage of DNA, RNA, proteins, enzymes, cause cancer, cardiovascular diseases, nervous disorders, premature ageing, Parkinson's and Alzheimer's diseases, rheumatic and pulmonary disorders (Cooke et al., 2002). The need therefore for systematic screening of medicinal plants for antioxidant activity cannot be overemphasized.

The plant *Acalypha hispida* of the family Euphorbiaceae, is found naturally in the tropics of Africa, America and Asia. It is claimed to have many folklore uses, as a laxative, diuretic, expectorant in asthma, in the treatment of leprosy and kidney ailments.

Previous phytochemical screening of the aqueous and leaf methanolic extracts of *A. hispida* revealed the presence of phenolics, flavonoids, glycosides, steroids, saponins, phlobatannins, and hydroxyanthraquinones (Iniaghe et al., 2009; Okorondu et al., 2009). The antifungal properties of extracts of leaves of *A. hispida* have also been established (Ejechi and Soucey, 1999). Gallic acid, corilagin, cycloartane-type triterpenoids, flavonoids like quercetin and kaempferol derivatives have been isolated from the plant (Adesina et al., 2000; Bergitte et al., 2003; Gutierrez-Lugo et al., 2002). In addition, alcoholic extracts of *A. hispida* have been reported to be biologically active against *P. aeruginosa, E. coli* as well as *S. aureus* and *S. typhii* (Okorondu et al., 2009).

In continuation of our studies in our laboratory of bioactive components and derivatives from Nigerian medicinal Euphorbiaceae plants and search for source of new antioxidants and therapeutic drugs from plant source (Onocha et al., 2004; Oloyede et al., 2010a; Onocha and Ali, 2010; Onocha and Olusanya, 2010), we now report the cytotoxicity and free-radical scavenging activities of the non-polar fractions of *A. Hispida* (leaves and twigs). Cytotoxicity of fractions obtained from chromatographic separation of *A. hispida* was evaluated using Brine shrimp lethality assay. The free-radical scavenging activity of the hexane extract was achieved by subjecting the extract to *in vitro* antioxidant assays, which include the determination of the effect on 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) at 517 nm in visible spectroscopy and the scavenging effect on hydrogen peroxide at 285 nm.

MATERIALS AND METHODS

Reagents and chemicals

Hexane, ethyl acetate, methanol, butanol and chloroform, hydrochloric acid, ammonia solution, naphthol, bismuth nitrate, potassium iodide, sodium hydroxide, sodium chloride, copper sulphate pentahydrate, 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), hydrogen peroxide and silica gel 60-120 microns (Merck, Germany) and 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, butylated hydroxylanisole (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), Rotavapor RII0 (Buchi, England), silica gel GF_{254} (precoated aluminium sheets - Merck Germany), pH meter (Jenway model), UV-Visible spectrophotometer (Unico1200 & Perkin Elmer lambda 25 models), Glass Column chromatographic materials and fraction collectors.

Plant material

Fresh leaves of *A. hispida* were collected in September (2009) in Ibadan, North Local Government Area of Oyo State, Nigeria and specimens were identified and authenticated at the Forestry Research Insti-

tute of Nigeria, Ibadan where a voucher specimen (FHI107319) was deposited. The leaves were air-dried for 2 weeks, ground into fine powder with a Hammer Mill (Ashai 7500) and subjected to solvent extraction.

Extraction and fractionation procedure

The powdered leaves (1 kg) of *A. hispida* were extracted with methanol by cold extraction. The mixture was filtered, evaporated to dryness in a rotary evaporator at 37 °C and stored in desiccators prior to use. Thin Layer Chromatography (TLC) was employed using silica gel 60 F_{254} precoated plates and solvent system: Ethyl acetate/methanol (8:2) to detect antioxidant activity with DPPH as spray reagent. Yellow coloration on the spots on the TLC plates indicated that the methanolic extract of *A. hispida* had antioxidant activity. The crude methanolic extract (53.8 g) of *A. hispida* was partitioned into hexane (non-polar) and ethylacetate (moderately polar). The yields of the residue left after evaporation were noted.

The hexane fraction gave a better antioxidant colour reaction and was then phytochemically screened for the following chemical constituents: alkaloids, saponins, flavonoids, tannins, phenols, anthraquinones, cardiac glycosides, sterols, proteins and carbohydrates (Harborne, 1998). 12 g of the hexane fraction *A. hispida* was preadsorbed with 5 g of silica gel prior to column chromatographic separation. 80 g of silica gel was packed into the column and the adsorbed hexane mixture was added on top in the dry state. The mobile phase was gradiently introduced to elute the material. The proportion of the more polar solvent was increased gradually in the non-polar one. Solvents used were hexane, ethylacetate and methanol. This produced the stock solutions used with the gradient mixer. 10 ml each of the effluent were collected in the fraction collectors and analysed by TLC. Silica gel 60-120 microns was used as adsorbent. The following solvent systems were employed for TLC: EtOAc: MeOH (9:1), EtOAc: MeOH (7:3), EtOAc: MeOH (5:5), Hexane: EtOAc (6:4), Hexane: EtOAc (2:8) and adsorbent for TLC was silica gel $60F_{254}$ precoated aluminium plates. The retention factor R_f calculated from TLC analysis of the 247 fractions obtained was used as the basis for bulking the fractions accordingly, thus: 1-13 (S_1), 14-28 (S_2), 29-42 (S_3), 43-83 (S_4), 84-88 (S_5), 89-95 (S_6), 96-102 (S_7), 103-123 (S_8), 124-137(S_9), 138-149 (S_{10}), 150-169 (S_{11}), 170-190 (S_{12}), 191-218 (S_{13}), 219-222 (S_{14}), 223-228 (S_{15}), 229-247 (S_{16}).

Subsequently, cytotoxicity test was carried out on the bulked fractions using Brine shrimp lethality assay while free radical scavenging activity tests were carried out on the bulked fractions using the following spectrophotometric experiments: scavenging effect on DPPH and scavenging effect on hydroxyl radical generated by hydrogen peroxide. DPPH radical gives strong absorption at 517 nm (deep violet colour) in visible spectroscopy. The absorption vanishes or is decolourized as the electron becomes paired off in the presence of a free radical scavenger. Scavenging effect on hydrogen peroxide on the other hand was carried out at 285 nm.

Scavenging effect on DPPH

2,2-diphenyl-1-picryhydrazyl radical (3.94 mg), 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 bulked fractions with doses ranging from 1.0 mg/ml to 62.5 µg/ml (Gulcin et al., 2002; Mutee et al., 2010; Oloyede et al., 2010b). The decrease in absorption at 517 nm of DPPH was measured 10 minutes later. The actual decrease in absorption was measured against that of the control and the percentage inhibition was also calculated. The same experiment was carried out on butylated hydroxylanisole (BHA), α-tocopherol and ascorbic acid which are known as antioxidants. All tests and analyses were run in triplicates and the results obtained were averaged.

Scavenging effect on hydrogen peroxide

Spectrophotometric determination of the hexane fractions of *A. hispida* was carried out at 285 nm. A solution of 2 mM hydrogen peroxide was prepared in phosphate buffered-saline (PBS) pH 7.4. Varying concentrations of the fractions ranging from 0.01 mg/ml to 6.25 µg/ml, was added to the H_2O_2 solution. Decrease in absorbance of H_2O_2 at 285 nm was determined spectrophotometrically 10 minutes later against a blank solution containing the test extract in PBS without H_2O_2. All tests were run in triplicates and averaged (Soares et al., 1997; Oloyede and Farombi, 2010).

CYTOTOXICITY ANALYSIS

Brine shrimp lethality test

Cytotoxicity of fractions obtained from chromatographic separation of A. hispida was evaluated using Brine shrimp lethality assay (Meyer et al., 1982). The shrimp's eggs were hatched in sea water for 48 h at room temperature. The nauplii (harvested shrimps) were attracted to one side of the vials with a light source. Solutions of the extracts were made in DMSO, at varying concentrations (1000, 100, and 10 µg/ml) and incubated in triplicate vials with the brine shrimp larvae. Ten brine shrimp larvae were placed in each of the triplicate vials. Control brine shrimp larvae were placed in a mixture of sea water and DMSO only. After 24 h the vials were examined against a lighted background and the average number of larvae that survived in each vial was determined. The concentration at fifty percent mortality of the larvae (LC50) was determined using the Finney computer programme.

RESULTS AND DISCUSSION

A 54.83 % w/w hexane extract was obtained from the partitioned methanol extract. Phytochemical screening of hexane extract of *A. hispida* (non-polar fraction) revealed the presence of flavonoids, carbohydrates, phenols, anthraquinones, cardiac glycosides, proteins, alkaloids and absence of tannins, sterols and saponins. This result agrees favorably with the result previously obtained by Iniaghe et al. (2009). Phenolic compounds are synthesized in plants as secondary metabolites. They exhibit several biological activities such as: antioxidation, anti-inflammation, anti-ageing, as well as inhibition of angiogenesis and cell proliferation. Most of these biological activities have been associated with their intrinsic reducing capability towards pro-oxidants (Han et al., 2007).

The biological functions of flavonoids on the other hand, include protection against allergies, inflammation, free radicals scavenging, platelets aggregation, microbes, ulcers, hepatoxins, viruses and tumors (Okwu and Omodamiro 2005; Okwu and Emenike, 2006). The free radical scavenging capability of *A. hispida* has been explored by this research due to the presence of phenolic compounds in the plant.

Free radical scavenging activities

Scavenging activities of all the bulked samples (semi-pure compounds) of the hexane fractions of *A. hispida* on 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) and hydrogen peroxide(H_2O_2) are as shown in Tables 1and 2. Their free radical scavenging activities were compared with the activities of known antioxidants namely: ascorbic acid (AA), butylated hydroxylanisole (BHA) and α-tocopherol. The activities were determined as a function of their %Inhibition (%I) which was calculated using the formula:

$$\%I = \frac{A_{Control} - A_{sample}}{A_{control}} \times 100$$

The stable radical 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) and hydrogen peroxide (H_2O_2), which are known to generate free radicals were used as the radical sources. From analysis, a larger percentage of the samples exhibited the ability to scavenge the free radical used in a concentration dependent manner, as their %I decreased with decrease in concentration.

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Scavenging effects on DPPH

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). The reduction in absorbance of DPPH at 517 nm caused by the samples was measured in triplicate after 10 min. The percentage inhibition showed a decreasing trend with decrease in concentration of the samples. The results revealed (Table 1) that the compounds S_{10} (91.8 %), S_{11} (93.8 %), S_{14} (92.5 %), S_{15} and S_{16} (91.4 %) at a concentration of 0.1 mg/ml had significant antioxidant activities on comparison with the known antioxidants, ascorbic acid (AA) (90.9 %) and α-Tocopherol (15.4 %). At 500µg/ml, S_1 (85.0 %), S_2 (87.2 %), S_9 (70.4 %), S_{10} (89.8 %), S_{11} (88.8 %), S_{12} (83.7 %), S_{13} (81.9 %), S_{14} (88.3 %), and S_{15} (79.4 %) had better activities than AA (68.7 %). At 250 µg/ml, S_1 (71.0 %), S_2 (86.5 %), S_{11} (78.2 %) and S_{12} (82.9 %) were better inhibitors than AA (67.8 %). Only S_2 (77.9 %) showed better scavenging activity than AA (65.4 %) at 125 µg/ml while at the least concentration of 62.5 µg/ml, S_2 (50.0 %) and S_9 (49.0 %) were better inhibitors than AA (44.3 %). It was also observed that at all concentrations tested, BHA had a better scavenging activity than α–tocopherol. S_5 on the other hand did not exhibit significant activity at all the concentration tested. The better scavenging activity of most of the fractions of *A. hispida* could be linked to the presence of secondary metabolites such as flavonoids and phenolics (Alan and Miller, 1996).

Scavenging effects on hydrogen peroxide (H_2O_2)

The scavenging activities of fractions collected from chromatographic separation of *A. hispida* and known antioxidants ascorbic acid (AA), butylated hydroxylanisole (BHA) and α-tocopherol on H_2O_2 is as shown in Table 2.

Table 1: %Inhibition of non-polar fractions of *A. hispida* and known antioxidants at absorbance$_{517nm}$ on 2,2-diphenyl-1-picrylhydrazyl radical (DPPH)

	Concentrations				
Samples	0.1 mg/ml	500 µg/ml	250 µg/ml	125 µg/ml	62.5 µg/ml
S_1	85.3	85.0	71.0	46.0	34.0
S_2	89.0	87.2	86.5	77.9	50.0
S_3	79.8	53.1	31.8	21.9	15.3
S_4	64.1	43.3	27.7	13.6	12.5
S_5	05.0	02.5	02.2	01.6	01.1
S_6	27.3	19.0	18.8	18.1	16.9
S_7	42.9	23.0	21.8	20.5	19.5
S_8	62.1	54.8	43.2	42.2	41.2
S_9	80.4	70.4	58.7	55.2	49.0
S_{10}	91.8	89.8	67.3	40.0	30.6
S_{11}	93.8	88.8	78.2	53.5	33.9
S_{12}	86.7	83.7	82.9	51.8	31.0
S_{13}	86.7	81.9	63.1	32.0	24.2
S_{14}	92.5	88.3	52.5	31.4	19.3
S_{15}	91.4	79.4	54.4	31.7	19.6
S_{16}	91.4	54.2	35.5	26.6	19.0
AA	90.9	68.7	67.8	65.4	44.3
BHA	95.4	94.3	94.0	93.9	91.9
α-T	15.4	12.4	12.4	12.1	10.4

AA = Ascorbic acid, BHA = Butylated hydroxylanisole, α-T = α-tocopherol

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Scavenging effects on H_2O_2 was measured in triplicates after 10min of incubation at 285 nm. Namiki (1990) showed that H_2O_2 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 (Cohen and Heikilla, 1974). Interestingly, one of the known antioxidants (α-T) showed an exceptionally high antioxidant activity in scavenging OH radical when compared to its activity in DPPH.

The result of the analysis as shown in Table 2 revealed that at a concentration of 0.01 mg/ml, S_1 (99.5 %), S_9 (99.2 %), S_{10} (95.4 %),S_{11} (95.8 %) and S_{15} (95.6 %) gave better activity than AA (94.8 %) while only S_1 and S_9 were significantly greater than BHA (98.9 %) and α-tocopherol (99.1 %) at that concentration. At 25 µg/ml, none of the samples scavenged better than BHA (98.0 %) and α-tocopherol (95.9 %), although, S_1 (71.6 %), S_9 (68.4 %), S_{10} (71.1 %), S_{11} (70.5 %), S_{12} (67.9 %), S_{13} (67.9 %) and S_{14} (75.3 %) were better in activity than AA (66.5 %). At 12.5 µg/ml, all the samples exhibited better activity than AA (26.8 %) except S_5 (2.6 %) and S_6 (19.8 %) while none of the samples was higher in activity than either BHA (97.5 %) or α-tocopherol (95.2 %). However, at 6.25 µg/ml no sample had higher activity than BHA (97.0 %) and α-tocopherol (86.9 %) but S_1 (41.5 %), S_2 (39.5 %), S_3 (28.6 %), S_4 (39.6 %), S_7 (33.0 %), S_8 (37.5 %), S_9 (54.5 %), S_{10} (46.4 %), S_{11} (43.5 %) and S_{12} (39.1 %) had higher activity than AA (22.4 %). Only S_5 in H_2O_2 revealed extremely low %inhibition at all the concentration tested. Consequently, the potential antioxidant activities of the fractions obtained from the chromatographic separation of the non polar extract of *A. hispida* and their ability to scavenge hydroxyl radicals contributes to its medicinal value, thereby making the plant a potential source of useful drugs.

Table 2: %Inhibition of non-polar fractions of *A. hispida* and known antioxidants at absorbance$_{285nm}$ on H_2O_2

	Concentrations				
Samples	0.01 mg/ml	50 µg/ml	25 µg/ml	12.5 µg/ml	6.25 µg/ml
S_1	99.5	72.3	71.6	70.4	41.5
S_2	93.8	67.1	39.9	39.8	39.5
S_3	66.6	63.8	41.8	39.8	28.6
S_4	75.0	68.7	62.7	42.5	39.6
S_5	19.1	19.0	05.2	02.6	01.1
S_6	40.1	33.5	29.5	19.8	13.4
S_7	41.2	39.7	39.4	37.4	33.0
S_8	66.0	58.1	54.2	38.8	37.5
S_9	99.2	71.0	68.4	65.4	54.5
S_{10}	95.4	88.7	71.1	70.2	46.4
S_{11}	95.8	95.5	70.5	46.3	43.5
S_{12}	86.7	69.5	67.9	57.6	39.1
S_{13}	85.0	80.0	67.9	40.1	11.5
S_{14}	93.8	85.1	75.3	52.0	18.5
S_{15}	95.6	57.7	46.4	38.4	18.2
S_{16}	81.2	66.4	40.7	33.2	18.1
AA	94.8	94.5	66.5	26.8	22.4
BHA	98.9	98.4	98.0	97.5	97.0
α-T	99.1	98.3	95.9	95.2	86.9

AA = Ascorbic acid, BHA = Butylated hydroxyanisole, α-T = α-tocopherol

CYTOTOXICITY ANALYSIS

Cytotoxicity analysis as determined by Brine shrimp lethality test revealed that fractions S_1, S_3, S_8, S_9, S_{13}, S_{14} and S_{16} were not toxic as their LC_{50} values were all greater than 1.000 µg/ml. However, S_2 (LC_{50} = 153.8551 µg/ml), S4 (LC_{50} = 214.3895 µg/ml), S5 (LC_{50} = 89.7831 µg/ml), S6 (LC_{50} = 134.3995 µg/ml), S7 (LC_{50} = 0.0220 µg/ml), S10 (LC_{50} = 732.2560 µg/ml), S11 (LC_{50} = 87.6732 µg/ml), S12 (LC_50 = 0.0000 µg/ml) were all toxic. The cytotoxic ability of this plant makes it a valuable entity in the therapy of diseases involving cell or tumour growth.

CONCLUSION AND RECOMMENDATION

The results obtained from investigating the antioxidant and cytotoxicity activities of *A .hispida* has proven that the semi-pure compounds present in the fractions are useful potential source of antioxidants and can be used in the therapy of diseases like cancer, coronary heart disease, ageing and any other disease related to oxidative stress. Further work therefore needs to be carried out on the fractions in order to isolate, purify and characterize the active chemical compounds which could be subjected to further toxicological analysis.

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