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Studies of *In vitro* Antioxidant and Cytotoxic Activities of Extracts and Isolated Compounds from *Parinari curatellifolia* (Chrysobalanaceae)

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Abstract
Parinari curatellifolia (family: chrysobalanaceae) is a plant used in Nigerian folk medicine for treatment of cancer and other diseases. In our search for pharmacologically active compounds, we have isolated and characterized compounds through column chromatography and spectroscopic techniques (MS, NMR and IR). We have evaluated the *in vitro* antioxidant activity of the different extracts and the pure compounds using the 2,2-diphenyl-1-picrylhydrazyl (**DPPH)** free radical model. The *in vitro* cytotoxic activity of the extracts and the pure compounds using the sodium 3'-[1-(phenylamino-carbonyl) -3,4-tetrazolium]-bis-[4-methoxy-6-nitro] benzene sulfonic acid hydrate (**XTT)** colorimetric method on cervical cancer **(HeLa)** cell line have also been assessed. The extracts and pure compounds have displayed moderate to excellent antioxidant and cytotoxic activities. The biological results suggest that these compounds may be suitable candidates for further drug development and investigation. The results obtained have provided a promising scientific basis for the use of *P. curatellifolia* in traditional medicine for treatment of cancer.
Keywords: *Parinari curatellifolia,* Cytotoxicity, HeLa Cell line, Antioxidant, DPPH Free Radical

1.0 Introduction

Plants have been used since antiquity to treat numerous diseases. The ancient people used plants selectively to treat specific diseases. *P. curatellifolia* is one of the numerous plants used by Traditional Medicine Practitioners in Northern Nigeria. It is used for treatment of numerous diseases among which is cancer. Scientific researches have indicated that the bioactive components of plants can reduce the risk of cancer through their antimicrobial, antioxidant, and antitumorogenic activity and their ability to directly suppress carcinogen bioactivation (Kaefer and Milner, 2008).

An antioxidant is a compound that could act as hydrogen donor, electron donor, peroxide decomposer, singlet oxygen quencher, enzyme inhibitor, or metal chelating agent. Antioxidants are capable of offsetting the harmful effects of the reactive oxygen species (ROS) through endogenous enzymatic defence systems such as the superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) present in the human system. These antioxidants are considered as possible protection agents against the oxidative damage of human body by ROS and retard the process of many chronic diseases as well as lipid peroxidation (Peryor, 1991; Kinsella *et al*., 1993; Lai *et al*., 2001). Antioxidants which scavenge active oxygen species (free radicals) are found in variety of food stuffs and are commonly referred to as scavengers (Rajendra and Shakti, 2009). Plants are endowed with free radical scavenging molecules (antioxidants), such as vitamins, terpenoids, phenolic acids, lignins, stilbenes, tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains and other metabolites which are rich in antioxidant activity (Zheng and Wang, 2001; Cai *et al*., 2003). Studies have shown that many of these antioxidant compounds possess anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial, and antiviral properties (Sala *et al*., 2002; Rice-Evans, 1995). The ingestion of natural antioxidants have been associated with reduced risks of cancer, cardiovascular disease, diabetes and other diseases associated with ageing (Ashokkumar *et al*., 2008; Veerapur *et al*., 2009). In recent years, there has been a worldwide trend towards the use of the natural phytochemicals present in berry crops, teas, herbs, oil seeds, beans, fruits and vegetables (Kitts *et al*., 2000; Muselík *et al*., 2007; Wang and Jiao, 2000). Antioxidants can terminate or retard the oxidation processes (chain reactions) by scavenging free radicals. Recently, the polyphenols are found to be beneficial as strong antioxidants (Wang *et al*., 1997). Plant-derived antioxidants are regarded as effective in controlling the effects of the oxidative damage cause to living cells by ROS, and hence

have influenced people's dietary behaviours (Viana *et al*., 1996; Pinder and Sandler, 2004). The antioxidative effect of these plant-derived antioxidants is mainly due to the presence of phenolic components, such as flavonoids (Pietta, 1998), phenolic acids, and phenolic diterpenes (Shahidi *et al*., 1992). The antioxidant ability of phenolic compounds are mainly ascribed to their redox properties, which play an important role in the absorbing and deactivating of free radicals, quenching of singlet and triplet oxygens, or decomposing of peroxides (Osawa, 1994). In most cases, people prefer natural antioxidants due their safety and economical related issues (Mandal, 2009). The extraction method, the isolation and characterization of β-sitosterol, stigmast-4-en-3-one, stigmasterol, betulin and betulinic acid from petroleum ether and ethyl acetate extracts of the stem bark of *Parinari curatellifolia* have been reported in the first and second parts of this research (Halilu *et al*., 2013a; Halilu *et al*., 2013b). This present paper, reports the *in vitro* antioxidant and cytotoxic activities of the extracts and the isolated compounds.

2.0 Materials and Methods

2.1 Preparation of Plant Extracts and Pure Compounds for In vitro Determination of Antioxidant Activity

The crude plant samples (2 mg) were respectively dissolved in 200 µL of ethanol to afford a 500 µg/mL concentration of the stock solutions. The pure compounds (2 mg) were respectively dissolved in 1 mL of ethanol to give a 100 µg/mL concentration of the stock solutions. Vitamin C was also prepared to a concentration of 100 µg/ml as the positive control. DPPH solution was prepared by dissolving 20 mg of the powder in 500 ml of ethanol to give a 0.04 µg/mL concentration of the stock solution (Du-Toit *et al*., 2001; Danielle and Lall, 2012,).

2.2 In Vitro Antioxidant Activity (DPPH Assay)

The method of Du-Toit *et al* (2001); Danielle and Lall (2012) were followed to determine the radical scavenging capacity (RSC) of the crude extracts and the pure compounds. In this method 96 well plates were employed. To each well in the top row, 200 µL of distilled water was added. This was followed by the addition of 110 µL of distilled water to the remaining wells. The extracts/pure compounds (20 µL) were added separately to the top wells of the 96 well plates in triplicate. Which was then followed by serial dilutions with the following concentrations: 3.90, 7.81, 15.62, 31.25, 62.5, 125.0, 250.0 and 500.0 µg/mL for the extracts and 0.781, 1.562, 3.125, 6.25, 12.5, 25.0, 50.0 to 100 µg/mL for the isolated compounds. Vitamin C was diluted serially in the same manner with concentrations ranging between 0.781 to 100 µg/mL. Finally, 90 µL prepared DPPH stock solution was added to each well, with the exception of the negative control where distilled water was added. The plates were allowed to develop in a dark room for 30 minutes.

2.3 Spectrophotometric assay

The radical scavenging capacity of the extracts/pure compounds were determined using ELISA multi well plate reader to measure the disappearance of purple colour of DPPH at 515 nm. The results were analyzed using the KC junior computer program.

2.4 Statistical Analysis

The statistical analysis of the results was carried out using Graph Pad Prism 4 software. The means, the standard deviations and the IC_{50} values of each extracts/ pure were determined by linear regression at 95 % confidence level. The vitamin C equivalent for each extract/ pure compound was calculated as follows:

$$\text{Vitamin C equivalent} = \frac{(IC_{50} \text{ of extract } \mu g/ml \times 200 \text{ mg vitamin C})}{IC_{50} \text{ of vitamin C.}}$$

2.5 In vitro Determination of Cytotoxic Activity

Preparation of HeLa Cell Culture

The HeLa cells were maintained in culture flask containing Eagle's Minimum Essentail medium supplemented with 10 % heat-inactivated fetal bovine serum (FBS) and 1% antibiotics (100 µg/mL penicillin,100 µg/mL streptomycin and 250 µg/mL fungizone). The cells were grown at 37 ^{o}C in a humidified incubator set at 5% CO_2. The HeLa Cells were sub-cultured after they have formed a mono-layer on the flask. The cells were detached from the flask by treating them with trypsin-EDTA (0.25% trypsin containing 0.01% EDTA) for 10 minutes and then by adding a complete medium to inhibit the reaction.

Preparation of Plant Extracts and Pure Compounds for In vitro cytotoxicity Studies

The crude extracts/pure compounds (2 mg) were dissolved in 0.1mL of DMSO to give a concentration of 20 mg/mL. The extracts were further dissolved in a complete medium to a concentration of 400 µg/mL. The pure compounds were further dissolved in a complete medium to a concentration of 200 µg/mL.

2.6 In vitro Cytotoxicity Studies on Cervical Epithelial Carcinoma (HeLa) Cell Line

Cytotoxicity was measured by the XTT (sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium] -bis-[4-methoxy-6-nitro]benzene sulfonic acid hydrate) colorimetric method using the cell proliferation Kit II. The method described by Zheng *et al* (2001), was used to evaluate the cytotoxic activity. The HeLa cells were seeded

(100 µL) in a 96-well micro titre plate (concentration 1 × 10^5 cells / mL). The plates were incubated for 24 h at 37 ^oC and 5% CO_2 to allow the cells to attach to the bottom of the wells. The extracts/ pure compounds were each prepared to a stock solution of 20 mg/mL and added to the micro titre plate. Serial dilutions were made to concentrations of 3.125, 6.25, 12.5 25.0, 50.0, 100.0, 200.0 and 400.0 µg/mL for the crude extracts and 1.563, 3.125, 6.25, 12.5, 25.0, 100.0 and 200 µg/mL for the pure compounds. The micro titre plates were incubated for a further 72 h. The negative control wells included vehicle-treated cells exposed to 2% DMSO and the positive control (Actinomycin D) with concentrations ranging between 0.5 µg/mL and 0.002 µg/mL. After the 72 h incubation period, the XTT reagent (50 µL) was added to a final concentration of 0.3 µg/mL and the plates were then further incubated for another 1 h. After the incubation, the absorbance of the colour complexes were analysed at 490 nm with a reference wavelength set at 690 nm using the ELISA multi well plate reader. The assay was conducted in triplicate in order to calculate an IC_{50} of the cell population for each of the extract/ pure compound. The results were analysed using the Graph Pad Prism 4 program.

3.0 Results

3.1 Qualitative Determination of Antioxidant Activity P. curatellifolia on DPPH

The qualitative screening of free radical scavenging activity was determined by thin layer chromatography. A yellow spot against a purple background on the TLC plate indicates free radical scavenging activity. The methanol extract, the ethyl acetate extract, compound C_7 and C_8 (unknown compounds) gave a positive result. The petroleum ether extract, betulin, β-Sitosterol, betulinic acid and Compound C_6 (unknown compound) did not give positive results. The results are shown in Table 1.

Table 1: Qualitative Antioxidant Screening with DPPH Using Pre-coated TLC Plates

S/ No.	Extracts/ Isolated Compounds	Results
1.	Ethyl acetate extract	+
2.	Methanol extract	+
3.	Pet. Ether extract	-
4.	Betulin	-
5.	β-Sitosterol	-
6.	Betulinic acid	-
7.	C_6	-
8.	C_7	+
9.	C_8	+

Note: +, indicates yellow spot against purple background; – , indicates no activity;C6 = Compound six; C7 = Compound Seven and C8 = Compound Eight

3.2 In Vitro Determination of Antioxidant Activity of P. curatellifolia on DPPH

The radical scavenging activity was expressed in terms of the amount antioxidant required to decrease the initial absorbance of DPPH by 50 % (IC_{50}). The ethyl acetate fraction showed significant dose dependent responses (95 % confidence level) with inhibitory concentration (IC_{50}) 13.47±0.928 µg/mL ethyl acetate fraction and 5.667±0.137 µg/mL for the methanol fraction. The petroleum ether fraction and the pure compounds showed relatively low antioxidant activity. The vitamin C equivalent is also presented. The results are shown in Table 2.

Table 2: Summary of the IC_{50} Values and Vitamin C Equivalents of Crude Extracts Isolated Compounds

S/No.	Extract/ Compounds	IC_{50} Values (µg/mL) ± S.D	Vitamin C Equivalent for 200 mg capsule
1.	Ethyl acetate extract	13.47 ± 0.928	1.3601
2.	Methanol extract	5.667 ± 0.137	0.5724
3.	Pet. Ether extract	>500	-
4.	Betulin	>100	-
5.	β-sitosterol	>50	-
6.	Betulinic acid	>etuli	
7.	C_6	>100	-
8.	C_7	10.92 ± 0.119	1.1030
9.	C_8	2.208 ± 0.0205	0.0223
10.	Vitamin C (+ve control)	1.98 ± 0.006	-

Note: The lower the IC_{50} the better the antioxidant properties of the compound. Any IC_{50} value from 50 µg/mL shows that the compounds/ extracts have low antioxidant activities. IC_{50}: Is defined as the concentration sufficient to obtain 50% of a maximum effect estimate in 100%. SD = Standard deviation (Mensor *et al*., 2001).

3.3 In Vitro Determination of Cytotoxic Activity of P. curatellifolia

The assay is based on the ability of living cells to reduce the yellow water soluble XTT into an insoluble

formazan product (Berridge *et al*., 1996). From the results as shown in Table 3, the ethyl acetate extract, the methanol extract, the petroleum ether extract and the pure compounds all showed cytotoxic activity on the HeLa cells.

Table 3: Summary of IC_{50} Values of the Plant Extracts / Isolated Compounds on HeLa Cells

S/No.	Extracts/ Pure Compounds	IC_{50} µg/mL
1.	Ethyl acetate extract	Proliferation occurred at 50 µg/ml
2.	Methanol extract	48.5 ± 3.21
3.	Pet. Ether extract	Very toxic
4.	Betulin	Very toxic
5.	β-Sitosterol	Very toxic
6.	Betulinic acid	Very toxic
7.	C_6	10.41 ± 1.46
8.	C_7	26.39 ± 0.235
9.	C_8	Proliferation occurred at 200 µg/ml
10.	Actinomycin D(Positive Control)	0.002109 ± 0.0001

4.0 Discussion

The biological activity exhibited by plant extracts are related to type of phytomolecules the plant is composed of. In this study, DPPH assay was used for the evaluation of the antioxidant activity of the extracts/ pure compounds. The stable DPPH free radical assay is a rapid method of evaluating the antioxidant activity of the substances. Moreover, the method is reliable and the results obtained are reproducible under a given condition (Du-Toit *et al*., 2001). In order to investigate the antioxidant activity, preliminary qualitative screening of antioxidant activity was conducted using thin layer chromatographic plate which was sprayed with DPPH solution prepared in ethanol. Yellow spot against a purple background was taken as a positive test for the presence of free radical scavenging compound(s). The test showed that the methanol extract, and the ethylacetate extract had free radical scavenging activities. On the other hand, the petroleum ether extract did not give a positive result (Table 1). Therefore, it can be inferred that the free radical scavenging activity demonstrated by the methanol extract, and the ethyl acetate extract may be due to the presence of the polar poly-phenolic compounds (flavonoids and tannins) which are good scavengers of free radicals (Zheng and Wang, 2001; Cai *et al*., 2003). The free radical scavenging activity demonstrated by the extracts is directly related to their antioxidant activity. Previous studies on the preliminary phytochemical constituent of *P. curatellifolia* have shown that the plant contains these flavonoids and tannins (Halilu *et al*., 2010). The lack of activity demonstrated by the petroleum ether extract may be due to the absence of these phytochemicals in the extract. This observation may be accredited to the inability of the petroleum ether to extract polar phyto-constituents. From Table 1, compounds C_7 and C_8 showed free radical scavenging activities may be for the reason that they were isolated from the ethyl acetate extract. Ethylacetate is a moderately polar solvent and therefore, it has the ability to extract both polar and non-polar phyto-constituents. The rest of the compounds did not give positive results (Table 1) which may be attributed to their non-polar nature. The *in vitro* determination of antioxidant activity (DPPH assay) was carried out in order to determine the IC_{50} of the extracts and the pure compounds. Four extracts and six pure compounds were screened for the antioxidant activity. From the results (Table 2), the ethyl acetate extract had IC_{50} value of 13.47±0.928 µg/mL and methanol extract gave a IC_{50} value of5.667±0.137 µg/mL. The free radical scavenging activity demonstrated by these extracts may be attributed to their phenolic contents (Zheng and Wang, 2001). The petroleum ether extract did not scavenge for 50% of the free radical and hence the IC_{50} could not be determined (Table 2). The low activity exhibited by the petroleum ether extract may be due to its non polar constituents (steroids and triterpenes). Compound C_7 exhibited excellent free radical scavenging activity with IC_{50} of 2.208 ± 0.0205 µg/mL. This result can be compared favourably with the IC_{50} of vitamin C (IC_{50} 1.98±0.006 µg/mL; Table 2). Compound C_8 exhibited good free radical scavenging activity with IC_{50} of 10.92±0.119 µg/mL (Table 2). Betulin, β-sitosterol, betulinic acid and Compound C_6 did not scavenge for 50 % of the free radical and hence their IC_{50} could not be determined (Table 2). The results obtained from the *in vitro* studies are in agreement with the observation made from the preliminary qualitative screening for free radical scavenging activity. From Table 2, it can be deduced that, compound C_7 showed the highest free radical scavenging activity, having a notably lower IC_{50} value, thus showing a relatively good free radical scavenging ability.(Danielle and Lall, 2012; Mensor *et al*., 2001). Compound C_7 thus show promise as an antioxidant agent. The vitamin C equivalent of the extracts and compound C_7 and C_8 were also calculated (Table 2). This was done because there are no standard units for measuring antioxidants and it is convenient to express the free radical scavenging capacity in relation to vitamin C which is a standard antioxidant (Du-Toit *et al* ., 2001). The vitamin C equivalent is the measurement of the amount of extract/ compound in gram that would be required to have a radical scavenging capacity that is equivalent to that of 200 mg vitamin C.

The cytotoxicity studies were conducted in order to validate the ethnomedical claim of P. *curatellifolia* in cancer treatment. Antioxidants have been shown to reduce the risk of cancer through their ability to scavenge for free radicals which have been identified as one of the major causes of cancer. The methanol extract, the ethylacetate extract, petroleum ether extract, β-sitosterol, betulin, betulinic acid, and compounds C_6, C_7 and C_8 were tested against the cervical epithelial carcinoma (HeLa) cell line using the XTT colorimetric assay. From the results of the assay (Table 3), the methanol extract, the ethyl acetate extract and the pure compounds, all showed cytotoxic activity except for the ethyl acetate extract and compound C_8 which cell proliferation occurred up to 50 µg/mL and 200 µg/mL respectively. On the other hand, the methanol extract showed a good cytotoxicity with IC_{50} of 48.5 ± 3.21 (Table 3). The cytotoxicity exhibited by the crude methanol extract can be attributed to its high polyphenolic contents (Danielle and Lall, 2012; Ewelina *et al*., 2008; Suhailah *et al*., 2011). Furthermore, the petroleum ether extract was found to be very toxic even at the lowest concentration of 1.503 µg/mL. This high cytotoxicity exhibited may be due to the presence of triterpenoids/steroids which are found to be present in the plant extract (Halilu *et al* ., 2010). The pure compounds: betulin, β-sitosterol and betulinic acid were also found to be very toxic even at the lowest concentration of 1.503 µg/mL. Compounds C_6 and C_7 also showed a good cytotoxicity with IC_{50} of 10.41± 1.46 and 26.39 ± 0.235 (Table 3) respectively. Generally, the lower the IC_{50} the better the cytotoxicity. The IC_{50} of actinomycin D (a well known anticancer drug) which was used as positive control was found to be 0.002109 ± 0.0001. The activity showed by the pure compound can be compared with actinomycin D (Table 3).

5.0 Conclusion

P. curatellifolia is used in Nigerian folk medicine for treatment of cancers. On the basis chromatographic and spectroscopic techniques, it is found to contain, betulin, β–sitosterol, stigmast-4-en-3-one, stigmasterol and betulinic acid as bioactive compounds. The extracts and the bioactive compounds demonstrated moderate to excellent free radical scavenging activity. Furthermore, the extracts and the pure compounds exhibited cytotoxic activity on the cervical cancer cell line. The compounds isolated and characterized are reported for the first time as constituents of *P. curatellifolia.* The free radical scavenging activity and the cytotoxic activity are also reported for the first time. Even though some of the compounds tested against the cervical cancer (HeLa) cells demonstrated undesirable cytotoxicity, modification to the scaffold structure can be investigated in order to decrease the high cytotoxicity and hence may be a drug with promising inhibitory activity and less side-effect can be synthesised.

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