



Antioxidant Potential and Quantitative Estimation of Content of Phenolic Compound in Crude and Fractionated Extract of *Caesalpinia bonduc*

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ABSTRACT

Currently there is an increase of interest in phytochemicals as a new source of natural antioxidants to be used in Pharmaceutical preparations to replace synthetic antioxidants. The present study investigated the phytochemical screening, antioxidant capacity and total content of phenolic compound in crude and fractionated extract of *Caesalpinia bonduc*. The antioxidant activity of samples was evaluated by DPPH (2, 2, Diphenyl -1-picryl hydrazyl) scavenging method. The folin-ciocalteu reagent and aluminum chloride colorimetric technique were used to estimate the total the phenolic and flavonoid contents of the extract respectively. The IC₅₀ of the DPPH scavenging activity ranged from 15 µg/ml to 130 µg/ml while that total phenolic and flavonoid contents ranged from 64.74 ± 0.002 to 388.24 ± 0.002 mg gallic acid equivalents (GAE)/g extract and from 33.38 to 42.91mg quecetin equivalent (QE)/g extract respectively. The ethyl acetate fraction was found to have the highest total phenolic, total flavonoid and highest antioxidant property (lowest IC₅₀). The coefficient of determination in a regression analysis between DPPH radical scavenging activity (IC₅₀) and the total phenolic and flavonoid contents (R² = 0.9456 and R² = 0.8292, respectively) suggested that phenolics and flavonoids in the extracts may be partly responsible for the radical-scavenging capacity. In conclusion, the leaves of extract of *C. bonduc* especially the ethyl acetate fraction possesses good antioxidant activity and research is in progress to isolate and characterize the antioxidant compound in ethyl acetate fraction.

Key Words: antioxidant, extract, phenolic content, flavonoid.

INTRODUCTION

Reactive oxygen Species (ROS) such as superoxide anion (O₂⁻), Hydrogen peroxide (H₂O₂), and hydroxyl radical (HO·) are produced as by-products of oxidative process of our body's metabolism. Oxidative stress occurs when there is excess production of these free radicals [1]. There are great deal of evidence indicating that oxidative stress, oxidation cellular thiols of proteins, peroxidation of membranes lipids, damage of DNA or RNA and alteration of the balance between the formation of reactive oxygen species and amount of antioxidants are actively involved in the pathogenesis of many diseases,[2]. These diseases including atherosclerosis, coronary heart disease, cancer, neurodegenerative disorder, malaria, diabetes, acquire immunodeficiency syndrome (AIDs), arthritis, rheumatoid, and degenerative

diseases, [10-14]. This situation has prompted the continuous search for various plant sources with these medical values. Antioxidants are vital substance which posses the ability to protect the body from damage caused by free radicals[15]. Number of synthetic antioxidants such as butylated hydroxyanisole, BHA) butylated hydroxytoluene, BHT) tertiary butylated hydroxy quinine and gallic acid esters have been suspected to be carcinogenic,[16,17,9]. Hence strong limitations have been placed on their use and there is a trend to replace them with naturally occurring antioxidants,[18]. Plant derived antioxidants such as ascorbic acid, α - tocopherol, polyphenols such as phenolic acid, flavonoids, proanthocyanidins are becoming increasingly suggested as important



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dietary factors[19,20]. Polyphenols, especially flavonoids which are generally known as the antioxidants agent in plant extracts[21]) are plant secondary metabolites, which play an important role in diseases – resistance protection against pests. In the literature, many crude extracts and pure natural compounds have been reported to have antioxidant potential[22-26] This has captured our interest to explore local medicinal plants for valuable antioxidant properties. *Caesalpinia bonduc* a plant of the family of Caesalpiniaceae are used as remedies in treating many diseases. The plant was found to have anti-inflammatory[27], anthelmintic, antimalaria [28]. The main aim of the study is to evaluate antioxidant potential and quantitative estimation total phenolic and flavonoid content in the leaves extract of *C. bonduc*.

MATERIALS AND METHODS

Plant Material

Fresh leaves of *Caesalpinia bonduc* was collected in June 2011 from Igueben in Igueben Local Government Edo State. It was identified and authenticated by Ugbogu O.A and Shasanya O.S. of Forestry Research Institute of Nigeria (FRIN). A Specimen (109493) was deposited at the FRIN, Herbarium. The fresh leaves were air dried and the dried material was grounded into powder using an electric blender (pye Unicam, Cambridge, England) and stored in an air-tight container for further use. The powder (1800 g) was macerated in 15 litres of methanol for 72 hours in the dark. The solution was evaporated to dryness using a rotary evaporator at a pressure below 40°C to 9.6 % w/w. The dried methanol extract was suspended in methanol and successively partitioned with petroleum ether, chloroform and ethyl acetate. The percentage yield of the three fractions were 11.04 %, 8.33 % 3.96 % for petroleum ethyl, chloroform and ethyl acetate respectively.

Total Phenol Assay

The total phenol content of crude and its fractionated extract were determined using the folin-ciocalteu reagent in a method described by [29]. 0.5 ml of the each extract solution at concentration of 1 mg/ml were mixed with 4.5 ml deionised water and 0.5 ml of Folin ciocalteu,s reagent (1:10 v/v diluted with distilled water) was then added to the solution. After mixing the tubes, they were maintained at room temperature for 5 min followed by the addition of 5 ml of 7 % sodium carbonate and 2 ml of deionized water was added. The mixture was allowed to stand for another 90 minutes kept in the dark with intermittent shaking and the absorbance

or the blue colour that development was measured at 750 nm using spectrophotometer. The experiment was carried out in triplicates.

Gallic acid was used for constructing the standard curve (50 to 1000 µg/ml). The amount of total phenolic content was expressed as mg/g gallic acid equivalent using the expression obtained from the calibration curve in figure 1. $Y = 0.00051x + 0.016$ $R^2 = 0.994$. Where X is the gallic acid equivalent in mg/g extract and Y is the absorbance.

Total Flavonoid Assay

The total flavonoids were determined using the method of [30]. 0.5 ml of each extract (5 mg/ml) was mixed with 1.5 of methanol and then, add 0.1ml of 10 % aluminum chloride followed by 0.1ml of potassium acetate and 2.8 ml of deionized water. The mixture was incubated at room temperature for 30 min. The absorbance was measured by a spectrophotometer at 415 nm. Total flavonoids content was calculated as quercetin equivalent mg/g using the equation obtained from the standard curve in figure 2. $Y = 0.009x + 0.069$, $R^2 = 0.995$. Where x is the quercetin equivalent and Y is the absorbance. The standard curve was prepared by quercetin in different concentration (10, 25, 50, 75, 100, 150 µg/ml).

Preparation of Quercetin Calibration Plot

A calibration plot, using quercetin with concentrations ranging 10 to 150 µg/ml was prepared from the quercetin stock solutions (5 mg/ml) by pipetting out volumes ranging from 0.002 to 0.030 ml into test tubes. The final volumes were made to 1ml with methanol in each test tube. 0.5 ml of different concentrations of the resultant quercetin solution were used for experiment as describe above.

Radical Scavenging Activity

The method of [31] was used for the determination of scavenging activity of DPPH free radical in the extract solution. 3 ml of each extract with seven different concentrations 20, 50, 150, 200, 250, 300 µg/ml were mixed with 1ml of a 0.1 Mm methanolic solutions of 2, 2, - diphenyl -1-picryl-hydrazyl (DPPH). The absorbance was measured by a spectrophometers at 517 nm at 30 intervals against a blank and ascorbic acid and gallic acid were used as standards. The scavenging ability of each of these extracts was calculated using this equation.

DPPH scavenging activity (%) =

$$\frac{[\text{Abs control} - \text{Abs sample}]}{(\text{Abs control})} \times 100$$
 were Abs control is the absorbance of DPPH plus

methanol, Abs sample is the absorbance of DPPH radical plus sample (extract or standard).

Statistical Analysis

The results were expressed as mean \pm standard deviation (SD) of three replicates. The data were subjected to one way analysis of variance (ANOVA) and differences between samples were determined by Duncan's multiple range test. P value < 0.05 were considered as significant and correlation between DPPH scavenging activity and total phenolic and flavonoid contents were established by regression analysis at a 95 % significance level $p \leq 0.05$ was considered as a significant difference.

RESULTS

Phytochemical test of the crude extract revealed the present of phenolic, flavonoids, triterpens, alkaloids, saponins, steroid, cardiac glycosides and tannins (Table 2). Several studies have reported a significant correlation between antioxidant activities and phytochemicals, the terpenoids have been found to possess antioxidant properties in various studies [32-35]. The cardiac glycosides have been exhibited antioxidant property in various studies [36,-37]. In many studies conducted by [38, 39] indicated that in various plant extract that showed the presence of steroids and these have been found to possess antioxidant potentials. Tannins are known to possess potent antioxidant activity in many studies[40-45]

Table 3 shows the contents of total phenols that were measured by folin ciocalteus reagent and was expressed in terms of mg gallic acid equivalent/g of the extract. The standard curve equation gave $Y = 0.00051x + 0.016$ $R^2 = 0.994$ (fig 1). The total flavonoid contents were measured by aluminum chloride colorimetric technique and were expressed in terms of mg quercetin equivalent per gram of the extract. The standard curve equation gave $Y = 0.009x + 0.069$, $R^2 = 0.995$ (fig 2). Each value in the table was obtained by calculating the average of three experiment \pm SD. The crude and fractionated extract of the leaves of *C. bonduc* were tested for antioxidant activity using the DPPH Assay and Table 4 shows their percentage inhibition. As shown in table 4, there is a wide range for free radical scavenging activity of crude and fractionated extract. The values ranged from 60.54 – 93.38 %, 32.84 – 85.78 %, 29.41 – 55.15 % and 38.72 – 67.89 % for ethyl acetate, chloroform, petroleum ether and crude extract respectively (tab.4). The variation of the free radical scavenging activity may

be due to the differences in their secondary constituents [46] as result of difference in polarities of the solvent.

DISCUSSION

The present study revealed high level of total phenols and flavonoids in crude and fractionated extract. Total phenolics content of the extracts were expressed as mg gallic acid, equivalent (GAE) per gram of the extract. As shown in table 3, the total phenolics content ranged from 64.71 ± 0.02 to 388.24 ± 0.007 mg GAE/g extract. Ethyl acetate fraction showed the highest total phenolic content of 388.24 ± 0.007 mg GAE/g extract, while petroleum ether fraction was the lowest one in total phenolic content (64.71 ± 0.002 mg GAE/g extract). Folin cocalteus assay gives a crude estimate of the amount of phenolic compounds present in an extract. It is not specific to polyphenolic but many interfering compounds may react with the reagent giving elevated apparent phenolic concentration [47]. Various phenolics compounds responded differently in this assay, depending on the number of phenolic groups they have and total phenolics content does not incorporate necessarily all the antioxidant that may be present in an extract. The total flavonoid content of the extracts of plant species was expressed as milligram quercetin per gram extract. The total flavonoid content ranged from 33.38 ± 0.07 – 42.91 ± 0.003 mg QE/g extract (table3.). Antioxidant activities of crude and fractionated extract were evaluated to find a new natural source of antioxidant. DPPH radical scavenging method is the most popular and widely used method for screening the free radical scavenging ability of compounds. DPPH is a stable radicals, with a strong absorption maximum at 517 (purple colour) in the UV spectrum. In the presence of an antioxidant which acts as a hydrogen donor, DPPH radical is reduced by accepting an electron from the antioxidant and accompanied by change of purple colour to yellow.

Table 4 shows the free radical scavenging activity of the crude and fractionated extract. Ascorbic and gallic acids were used as standards. IC_{50} valued, which is defined as the concentration of substrate at 50 % inhibition [48] was determined by plotting graph of percentage inhibition against concentration. The IC_{50} of the positive control (ascorbic and gallic acid) were the same ($IC_{50} = 8.5$ μ g/ml). The ethyl acetate fraction demonstrate the significantly highest activity with IC_{50} of 15 μ g/ml as compared with 70 μ g/ml, 90 μ g/ml and 130 μ g/ml for chloroform fraction, crude extract and petroleum

ether fraction respectively (tab.3). The IC_{50} values of the analyzed extracts were higher than that of the standards. The lower IC_{50} for Ascorbic and gallic acids were as result of their highly purified state as against complex plant species analyzed. The findings suggested that further purification is needed to achieve better antioxidant capacities. The result from DPPH Assay is the consonant with the results of many antioxidants studies using DPPH assay in recent years conducted by[49-53] In these Assays the highest antioxidant activity has been reported for *Rupus ulmifolious*, IC_{50} value $5.1\mu\text{g/ml}$ [53], whereas the lowest antioxidant was reported for *Thelesperma megapotamicam*, IC_{50} values $2000\mu\text{g/ml}$ [51]. Coefficient of determination between the DPPH scavenging ability (IC_{50}) and both the total phenolic and the total flavonoid contents of the crude and fractionated extract was determined (figures 4 and 5). Significant ($P < 0.05$) and negative correlations were observed between the IC_{50} values for the DPPH Assay and total phenolic content. ($Y = -0.334x + 139.67$, $R^2 = 0.9456$ (fig.4). A correlation though not significant was also found between the IC_{50} and flavonoid compounds in crude and fractionated extract($Y = -9.7984X + 431.84$, $R^2 = 0.8292$) (fig.5). The coefficient of determination between the IC_{50} values for the DPPH Assay and total phenolic content was 0.9456 whereas the total flavonoid content was 0.8292. These results suggested that the total phenolic and flavonoid compounds contributed 95 % and 83 % to free DPPH radical scavenging of the crude and fractionated extract. Scavenging effects may not be limited to flavonoid compounds. The activity also comes from the presence of other antioxidant secondary metabolized in the extract.

Table 1: Preparation of different concentration of quercetin for calibration plot

	Quercetin mg/ml	Quercetin stock solution (ml)	methanol (ml)
1	10	0.002	0.998
2	25	0.005	0.995
3	50	0.010	0.990
4	75	0.015	0.985
5	100	0.020	0.980
6	150	0.030	0.970

Table 2: Phytochemical analysis of the plant extracts

Phytochemical constituent	Results
Alkaloids	+
Cardiac glycosides	+
Flavonoids	+
Phenolic	+
Steroids	+
Tannins	+
Saponins	+
Triterpenes	+

+ = Present

Table 3: The total Phenolic, Flavonoid content and DPPH IC_{50} of crude and fractionated extract

Extract	total phenolic mg GAE/g extract	total flavonoid mg QE/g extract	IC_{50} $\mu\text{g/ml}$
Crude	139.22 ± 0.01	34.16 ± 0.02	90
PET. Ether fraction	64.71 ± 0.02	33.38 ± 0.07	130
Chloroform fraction	166.67 ± 0.01	34.71 ± 0.02	70
Ethyl acetate fraction	388.24 ± 0.007	42.91 ± 0.003	15.00

\pm ; standard deviation of triplicate

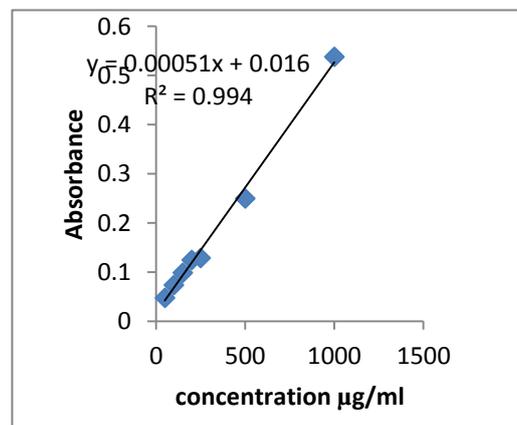


Figure 1: Calibration Plot of Gallic Acid.

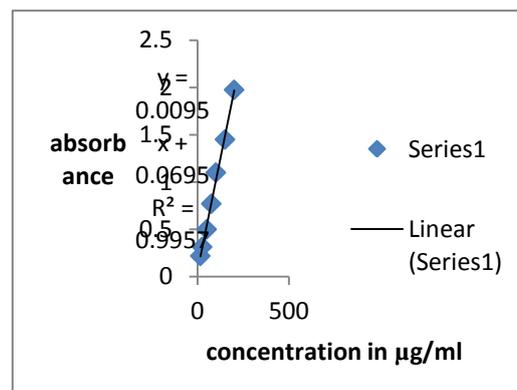


Figure 2: Calibration Plot of Quercetin.

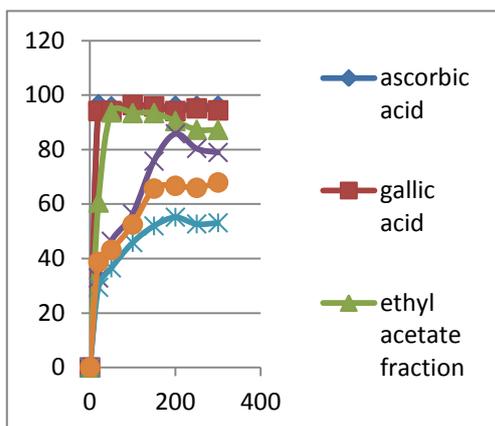


Figure 3: DPPH free radical scavenging activity of crude, fractionated extract and standards (ascorbic and gallic acids)

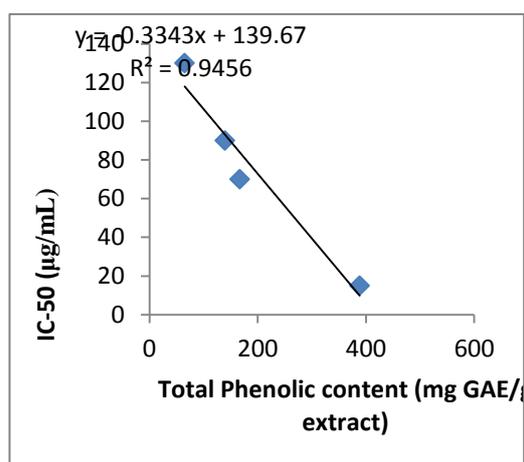


Figure 4: Correlation between DPPH Radical Scavenging Activity (IC₅₀) and Total Phenolic Contents.

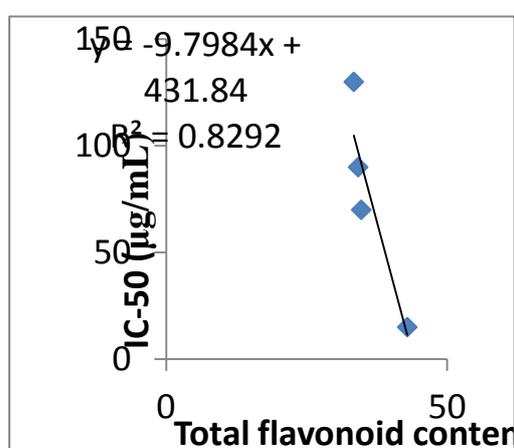


Figure 5: Correlation Between DPPH Radical Scavenging Activity (IC₅₀) and Total Flavono Contents.

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