ORIGINAL RESEARCH ARTICLE

Phytochemical and antioxidant evaluation of *cassia sieberiana* D.C. stem bark extracts

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ABSTRACT

Cassia sieberiana D.C. belongs to the Fabaceae family and it is used ethnomedically in the management of cancer, diabetes and other diseases. There is a growing concern about the scourge of diseases caused by excessive free radicals in the body and lack of standardization of medicinal herbal products. Therefore, this research is aimed at evaluating the phytochemical and free radical scavenging activities of the plant extracts with the view to identify the chemical constituents and antioxidant properties of the extracts. The powdered stem bark was evaluated for its pharmacognostic profiles and then extracted successively with the aid of Soxhlet extractor using n-hexane, ethyl acetate and methanol. The extracts obtained were concentrated using rotary evaporator and the percentage yields of the extracts were determined. The extracts were screened for their phytochemical constituents using standard protocols. The thin layer chromatography (TLC) profiles of the resulting extracts were determined using suitable solvent systems and the retention factor (R_f) values of separated spots of compounds were calculated. The total phenolic content of the ethyl acetate and methanol extracts were measured using Folin-Ciocalteu reagent and gallic acid was used as standard. The antioxidant (free radical scavenging) activity of the extracts were measured using DPPH (2, 2-diphenyl-1- picryl hydrazyl) and ascorbic acid was used as standard. The results were expressed in terms of percentage inhibition. The percentage yields of the extracts were: 0.52 %, 6.58 % and 10.52 % respectively for n-hexane, ethyl acetate and methanol. The preliminary phytochemical screening of the extracts revealed the presence of tannins, cardiac glycosides, saponins, flavonoids and triterpenoid/steroids. Also, the total phenolic contents were found to 7.64 mg and 2.97 mg gallic acid equivalents for ethyl acetate and methanolic extract respectively. The free radical scavenging activity of the extracts revealed higher activity in the ethyl acetate extract. The results indicated that the extracts contain phenolic compounds which may be responsible for the antioxidant activity.

***Key words**: DPPH, Chromatography and Pharmacognostic*

INTRODUCTION

Natural products have played a very important role in health care and prevention of diseases for thousands of years (Phillipson, 2001). This trend has continued to this very generation were 80 % of the human population in the developing world rely mainly on traditional medicines derived from plants for their health care needs (WHO, 2011; Gopal, 2013; Mohamed *et al*., 2015; Haidan *et al*., 2016).

There is a growing concern about the burden of diseases caused by excessive free radicals in the body. These free radicals cause oxidative stress in the biological systems which is believed to be a major contributor to the pathogenesis of a number of chronic and degenerative diseases such as cancer, atherosclerosis, Parkinson's, Alzheimer's, diabetes mellitus, renal, respiratory, eye and coronary heart disease (Pong, 2003; Afolayan *et al*., 2008; Sandhya *et al*., 2010; Andrés *et al*., 2020). Therefore, there is an urgent need to screen, and standardize medicinal plants with antioxidant potentials. Antioxidants in biological systems have multiple functions, including defense against oxidative damage and in the major signaling pathways of cells. The major action of antioxidants in cells is to prevent damage caused by the action of free radicals such as the reactive oxygen species [e.g. superoxide radical (O_2·), hydroxyl radical (OH·), peroxide radical (ROO·) and nitric acid radical (NO^-)] (Halilu *et al*., 2013; Andrés *et al*., 2020; Sergio and Paola, 2020). Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are synthetic antioxidants which are commercially available and are currently in use but due to their carcinogenicity (Emad *et al*., 2017), there is need to develop effective antioxidants of plant origin which are assumed to be safer (Emad *et al*., 2017). The natural antioxidants (Phenolic compounds) play a key role in antioxidative defense mechanisms in biological systems and they act as free radical scavengers (Frankel *et al*., 1996; Climpoiu, 2006).

Plants are a huge reservoir of chemical substances that can be used to combat these diseases. One of such reputable plant is *Cassia sieberiana* which belongs to the family Fabaceae. The plant is known to have a wide variety of medicinal application in African traditional medicine. The entire plant is used as purgative and diuretic. Extracts from various parts of the plant are used to treat fever, malaria, diarrhoea, leprosy, bilharzias, stomach pain and as a diuretic, jaundice, improvement of lactation after child birth, treatment of rheumatic condition, vermifuge, laxative, wound dressing, pain, inflammation, toothache, stomach-ache, ulcers, diarrhoea, gonorrhoea, HIV/AIDS, headache, eczema, haemorrhoids, dropsy, dysentery, control insect pests, sleeping sickness, venereal disease, sterility, dysmenorrhea, aphrodisiac and peptic ulcer (Dalziel, 1956; Akah and Nwabie, 1993; Akah *et al*.,1998; Modusolumuo *et al*., 1999; Ra'ed, 1999, Nwafor and Okwuasaba, 2001; Belmain *et al*., 2001, Mary-Ann *et al*., 2019)

Extracts from various parts of *Cassia species* have been reported to demonstrate antibacterial and antifungal activities. The medicinal value of these plants lies on some chemical substances that produces a definite physiological effect. These substances include alkaloids, anthraquinones, flavonoids, glycosides, tannins, terpenoids, saponins, phenols and oils (Abo *et al.*, 1999; Modusolomuo *et al.*, 1999; Abo *et al.*, 2000; Ayo and Amupitan, 2007; Mary-Ann *et al*., 2019).

Previous studies on the ethanol root extract of *C. sieberiana* had demonstrated anti-parasitic and myorelaxant effects, anti-spasmodic, anti-microbial, analgesic and anti-inflammatory activities (Silva *et al*., 1997; Fall *et al.*, 2005; Guato *et al.*, 2009). Fractions from the root extract have

been reported to demonstrate free radical scavenging activity (Halilu *et al*., 2017). The leaf extracts of *C. sieberiana* were found to be active against *Staphylococcus lutea, Mycobacterium phlei, Bacillus subtilis* and *Proteus sp*. but not against *Staphylococcus albus, Pseudomonas aeruginosa* or *Escherichia coli* (Silva *et al*., 1997). The flavones from the leaf extract have been reported to induce diuresis, and have demonstrated antibacterial and anti-inflammatory activity (Silva *et al*., 1997).

C. sieberiana stem bark have been used extensively in traditional medicine for treatment of diseases associated to free radicals without scientific validation. Therefore, this research is designed to systematically evaluate *C. sieberiana* stem bark extracts with regards to its pharmacognostic, phytochemical and antioxidant property.

MATERIALS AND METHODS

Collection, Identification, and Preparation of Plant Material

The leaves, the flowers and the stem bark of *Cassia sieberiana* were collected in August 2016 in Tureta Local Government, Sokoto State-Nigeria. The plant was identified by a pharmacognocist (Dr. H. E. Mshelia) at the Department of Pharmacognosy and Ethnopharmacy, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria. Voucher specimen was prepared and assigned voucher number (PCG/UDUS/CAES/0002) was deposited at the herbarium of the department for reference purposes. The stem bark of the plant was chopped and air dried for 14 days. It was ground to coarse powder using wooden pestle and mortar. The powder was stored in a plastic container for future use.

Macroscopic Examination of Stem bark of *C. sieberiana*

The macroscopic identification of plant was based on the shape, size, colour, surface characteristics, texture, fracture characteristics, odour and taste of the dried powder bark. The macroscopic characters of the samples were examined according to the method described by Wallis, (1985), Evans (2008), (Halilu *et al*., 2008) & WHO (2011).

Microscopic Examinations of the Stem bark of *Cassia sieberiana*

The microscopic examinations of the powdered bark were conducted according to the method described in Halilu *et al*. (2008); Sam *et al*. (2011) & WHO (2011).

Chemomicroscopical detection of cell contents and cell wall materials

The powder bark was treated separately with appropriate chemical reagents on microscopic slide and observed under the microscope for the presence of cellulose, hemicelluloses, lignin, calcium oxalate crystals, calcium carbonate, starch, mucilage, tannins, fixed oils and fats (Evans, 2008; Halilu *et al*., 2008 & WHO, 2011).

Physicochemical Evaluation of Powdered Stem bark of *C. sieberiana*

The moisture content, total ash, acid insoluble ash, alcohol soluble and water soluble extractive values were determined as described by Halilu *et al*. (2008) & WHO, (2011). The experiments were conducted in triplicates.

Successive Extraction of Plant

The powdered plant material (90g) was first extracted using n-hexane (500 mL) with the aid of Soxhlet extractor and was allowed to run for 16 hours. The extract was concentrated using rotary

evaporator at 60 ^{o}C. The soxhlet extraction was repeated using ethyl acetate (500 mL) and methanol (500 mL) respectively. The percentage yield of extracts were calculated (Halilu *et al*., 2017).

Preliminary Phytochemical Screening of Extract of *C. sieberiana*

Phytochemical screening of the extracts was conducted to detect the presence of carbohydrates, alkaloids, tannins, anthraquinones, saponins, flavonoids, steroids/triterpenes and cardiac glycosides. The tests were carried out according to the methods described by Sofowara (2008) and Evans (2008).

Thin layer chromatographic studies of extracts of *C. sieberiana*

The extracts were studied in order to ascertain their separation profiles in various solvent systems. The n-hexane extract was best resolved in n-hexane: ethyl acetate (7:3 and 8:2). The ethyl acetate extract was resolved in Hexane: Ethyl acetate (2:8 and 4:6). The methanol extract was resolved in water, methanol and ethyl acetate (1:1:8) and Chloroform: Methanol (8:2). The results were expressed in terms of R_f values (WHO, 2011).

Total Phenolic Content

The assay was carried out according to the methods described by Macdonald *et al*. (2001) and Wolfe *et al*. (2003) using the folin- ciocalteu reagent.

Antioxidant studies (DPPH antioxidant assay)

The qualitative and quantitative assays were carried out according to the methods described by The method of Du-Toit *et al*. (2001) as reported by Halilu *et al*. (2013) were followed to determine the radical scavenging capacity (RSC) of the crude extracts. In this method 96 well plates were utilized. 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 (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. 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. The radical scavenging capacity of the extracts were determined using ELISA multi well plate reader to measure the disappearance of purple colour of DPPH at 515 nm.

Data Analysis

The data obtained were analyzed using SPSS version 20 statistical software and Microsoft Excel 2016.

RESULTS

***Macroscopy* of *Cassia sieberiana* Stem Bark**

The results showed the various macroscopic characters of the stem bark. It was observed that the stem bark had characteristic odour, bitter taste and quill curvature (Table I).

Table I: Macroscopic Characters of Cas*sia sieberiana* Stem Bark

Parameter	Character/feature
Whole drug	
Shape	Cylindrical
Surface	Smooth
Texture	Rough
Curvature	Quill
Fracture	Fibrous
Powdered drug	
Taste	Bitter
Odour	Characteristic
Colour	Brown

Chemomicroscopical studies of Cassia sieberiana powdered Stem bark

The chemomicroscopic studies of the stem bark revealed the presence of starch, calcium oxalate, tannins, cellulose, hemicellulose, lignin and mucilage (Magnification ×100). This is presented in Table II and plates A-F.

Table II: Chemomicroscopical characters

Test reagents	Observation	Inference
Starch	Blue-black colour	Present
Calcium oxalate	No effervescence	Present
Tannin	Greenish black colour	Present
Fixed oil and fat	Brownish pink stain	Present
Lignin	Cherry red colour	Present
Mucilage	Pink colour	Present

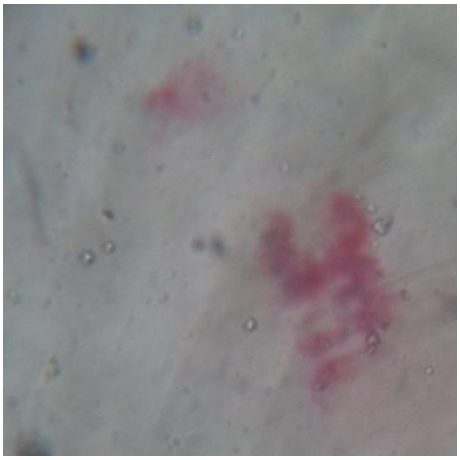

Plate A: Lignified cell walls

Plate B: Clusters of prismatic calcium oxalate crystals

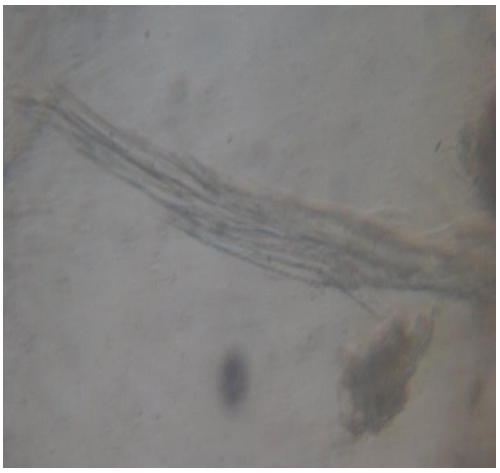

Plate C: Pericyclic fibers

Plate D: Cork cells

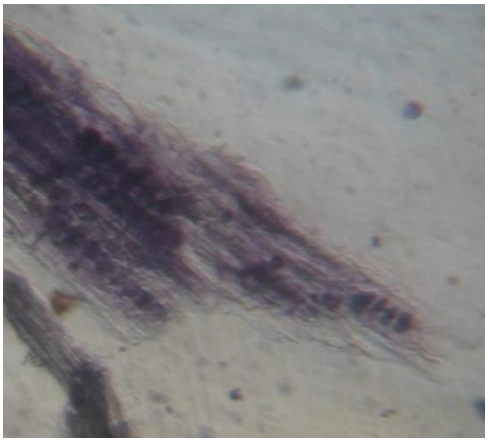

Plate E: Starch grain parenchyma

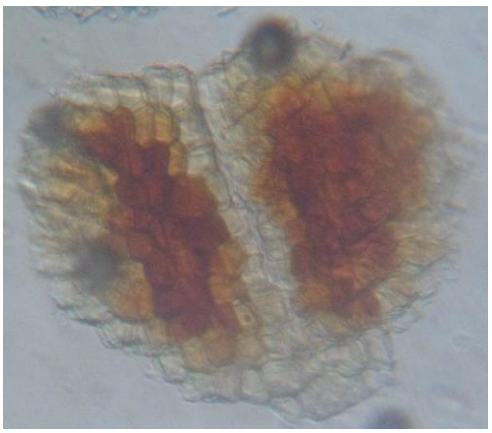

Plate F: Cork cells with cortical

Physicochemical Evaluation of Powdered Stem Bark

The result showed moisture content of 6.5 % and water soluble extractive value of 16.0 %. The alcohol soluble extractive value was 22.0 % which is higher than water soluble extractive value. The results are presented in Table III.

Table III: Physicochemical Parameters of *C. sieberiana* Stem Bark

Parameter	% w/w (mean ± S.D)
Moisture content	6.5 ± 0.1
Total ash	4.0 ± 0.2
Acid insoluble ash	1.5 ± 0.1
Alcohol soluble extractive	22.0 ± 0.2
water soluble extractive	16.0 ± 0.2

Extraction of Plant Material

The percentage yields of the extracts increases from the non-polar, to moderately polar and to the polar solvent. The n-hexane has the least and methanol has the highest percentage yield. The results are presented in Table IV.

Table IV: Mass and percentage of Extracts

Extract	Color	Mass of extract (g)	% Yield
n-Hexane	Greenish Brown	0.470	0.52
Ethyl acetate	Brown	5.926	6.58
Methanol	Dark Brown	9.472	10.52

Phytochemical Evaluation

The presence of some secondary metabolites have been detected in the extracts and are presented in Table V.

Table V: Phytochemical Screening of Various Extracts of *C. sieberiana* Stem Bark

Constituent	n-Hexane	Ethyl acetate	Methanol
Saponins	-	+	+
Flavonoids	-	+	+
Tannins	-	+	+
Cardiac glycosides	-	+	+
Alkaloids	-	+	+
Triterpenoids	+	+	+

Key: + = Present; - = Absent

Thin Layer Chromatography (TLC)

The results showed the presence of some compounds with varying R_f values in the extracts. The n-hexane extract was best resolved in solvent system [n-hexane: ethyl acetate (7:3)]. The methanol extract in both solvent systems [Chloroform: methanol (8: 2); Ethyl acetate Methanol: Water (8:1:1). The ethyl acetate extract was best resolved in n-hexane: Ethyl acetate (8:2). The results are shown in Table VI.

Table VI: TLC Profiles of Various Extracts of *C. sieberiana*

Extract /Solvent System	Spot/R_f value
n-Hexane/ Hexane: Ethylacetate (8:2)	Seven Spots/ R_f = 0.19; 0.30; 0.41;0.522; 0.61; 0.72;0.83
n-Hexane/ Hexane: Ethylacetate (7:3)	Nine Spots/R_f = 0.10; 0.16; 0.26; 0.43; 0.55; 0.65; 0.71; 0.84; 0.94
Methanol/ Chloroform: Methanol	Four Spots/ R_f = 0.13;0.63;0.75; 0.95)
Methanol/ Ethylacetate: Methanol: Water (8:1:1)	Four Spots/ R_f = 0.04;0.14;0.92; 0.98)
Ethylacetate/ Hexane: Ethyl acetate (2:8)	Eight Spots/ R_f = 0.191; 0.26; 0.38; 0.55; 0.64; 0.72; 0.85; 0.95
Ethylacetate/ Hexane: Ethyl acetate (4:6)	Seven Spots/ R_f = 0.11; 0.17; 0.29; 0.45; 0.57; 0.87; 0.94)

Total Phenolic Content

The total phenolic content of the methanol and ethyl acetate extracts as determined from the calibration curve (R^2 =0.989) at 200µg/ml were 2.97 mg and 7.64 mg gallic acid equivalents/g respectively. The calibration curve is presented in Figure 1.

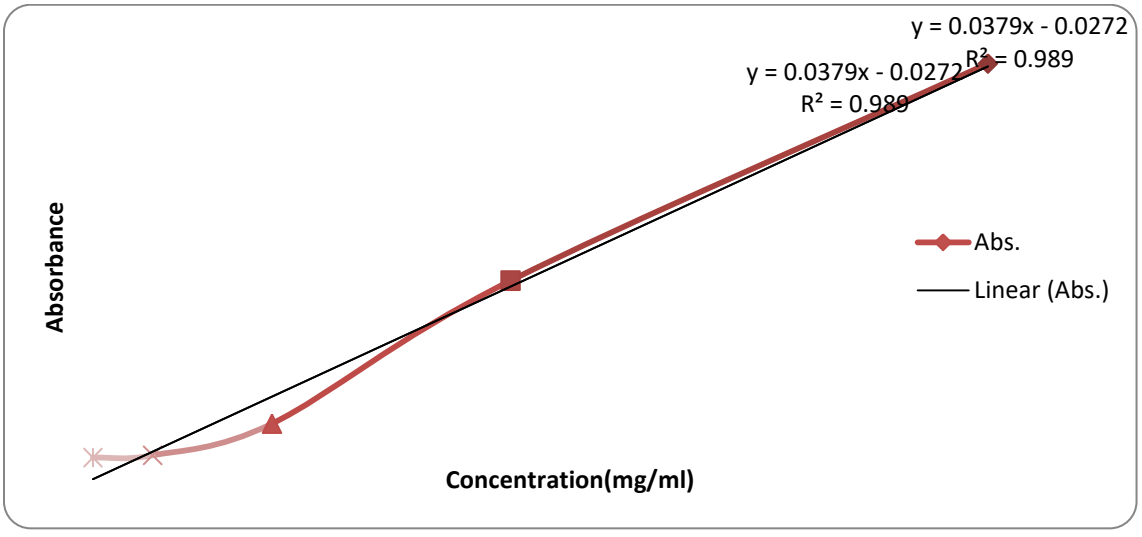

Fig. 1: Calibration curve of Gallic acid (Standard)

Qualitative Antioxidant Assay

The qualitative TLC screening for antioxidant provides the rapid detection of antioxidant compounds in plant extracts. The intensity of the yellow spots produced on the TLC Plate against a purple background after spraying with DPPH showed which compound is most active. It can be inferred that the ethyl acetate extract is the most active.

Quantitative Antioxidant Activity

The results of the quantitative antioxidant assay were expressed in terms of percentage inhibition and IC_{50}. The result showed that the ethyl acetate showed higher activity than the methanol extract. The activity of the ethyl acetate was high at the lowest concentration. The activity of the ascorbic acid was found to be concentration dependent. The results are presented in Figure 2. The IC_{50} of ascorbic acid was 123.02 µg/ml and that of the ethyl acetate was 7.45 µg/ml. That of the methanol could not be determined since it could not scavenge 50% of the free radical.

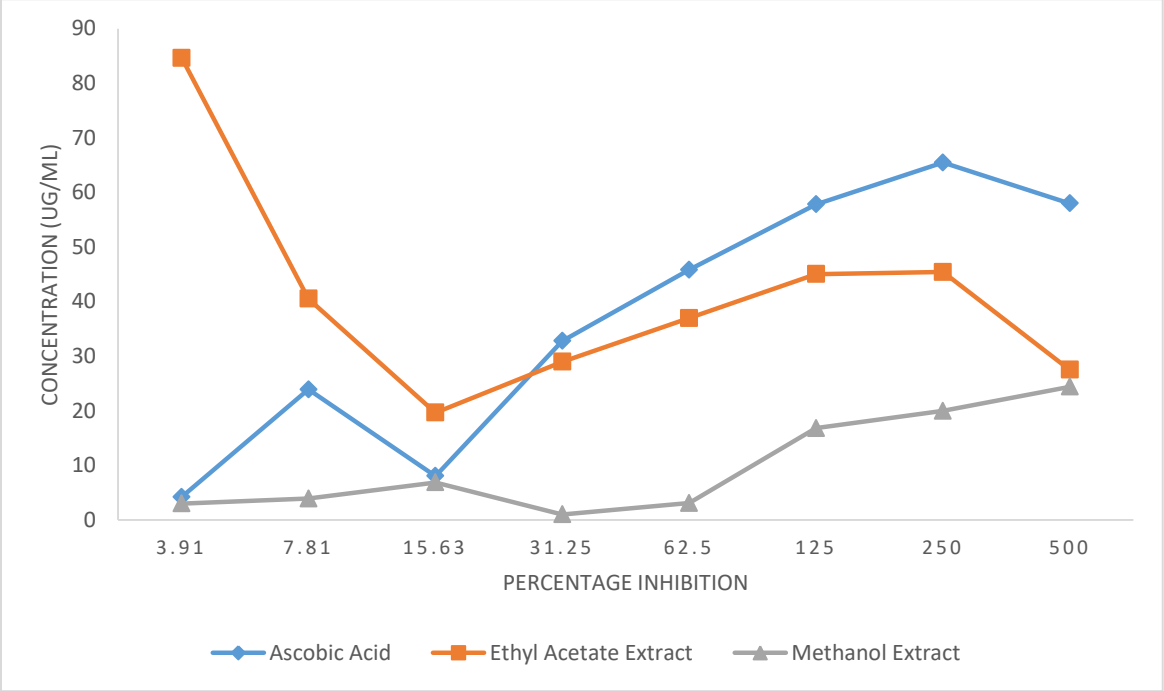

Fig. 2: Percentage Inhibition of Ethyl acetate and methanol Extracts

DISCUSSION

The primary steps required for establishing standards for any plant drug are the macroscopic and microscopic evaluations. According to WHO (2011), botanical standards should be proposed as a protocol for the diagnosis of herbal drugs. Macroscopic identity of medicinal plant materials is based on the characters they produce when they are fresh or dried or in powder form. The observed characters include: the shape, size, colour, texture and fracture. These are notable features used as diagnostic characters to distinguish the plant from other species. Findings from this research (Table I) showed the macroscopy of *C. sieberiana* and the results are in agreement

with the standards established by the WHO (2011). However, since these characteristics are judged subjectively and substitutes or adulterants may closely resemble the genuine material, these findings are usually substantiated by microscopy. According to the WHO 2011, microscopic characters are necessary to establish the botanical identity of commercial samples of medicinal plants and play an important role in checking adulteration and substitution. The results of the chemomicroscopy (Table II and Plates A-F) of the powdered stem bark gives a preliminary idea about the type of compounds present and their accumulation in the plant tissues. The result agrees with the report of Sam *et al*. (2011) which indicated the presence of prismatic calcium oxalate crystals and oval starch grains from the root bark of the plant. Chemomicroscopical studies of *C. sieberiana* are of great interest for quality control in basic research and drug production, especially for imported items and for raw material sold by traditional herbalists.

Physicochemical evaluation of powdered stem bark of *C. sieberiana* is useful in establishing standards on identification, purity and quality of the plant. The moisture content obtained from the study (Table III) represents the amount of water of crystallization present in the plant sample. The presence of excessive moisture encourages bacterial, fungal or yeast growths and deterioration of the active constituents following hydrolysis (WHO, 2011). The percentage moisture (Table III) was higher than that reported by Fatokun *et al*. (2017) who indicated moisture content of 4.5 % (w/w) from root bark of *C. sieberiana*. The difference may be due to storage environment. The ash value indicates the presence of inorganic salts occurring naturally in the crude drug or adhering to it or those deliberately added to it as adulterant (WHO, 2011). The total ash (Table III) obtained from this study was found to lower than that reported by Sam *et al*. (2011); Fatokun *et al*. (2017) who recorded 7.90 % (w/w) and 7.4 % (w/w) respectively from the root bark of *C. sieberiana*. This deviation may be due to the difference in the parts of the plant used for the study. The acid insoluble ash (Table III) was low when compared with the value reported by Sam *et al.* (2011) which indicated acid insoluble ash of 5.15% (w/w). This shows that the amount of silica present, especially as sand and siliceous earth are in very minute quantity. This variation may be attributed to the difference in the parts of the plant used for the investigation. Although the result abtained from this study agrees with result of Fatokun *et al*. (2017) who reported acid insoluble ash of 1.5 % (w/w) from the root bark of the plant.

Extractive values help in determination of the amount of active constituents that can be extracted using a given solvent. The alcohol soluble and water soluble extractive values (Table III) showed this result is in contrast to the findings of Sam *et al*. (2011) who showed that ethanol (70 %) had extractive value of 14.28 % (w/w) and water extractive value of 15.99 % (w/w) for the root of *C. sieberiana*. The result of this investigation showed higher percentage of alcohol soluble extractives which 70 % alcohol is a better solvent for extracting the constituents of the stem bark of *C. sieberiana*. This variation can also be due to duration of the extraction and part of the plant used. The trend observed from the result of this study is in agreement with Fatokun *et al*. (2017).

The results of the extraction (Table IV) showed an increase in percentage yields of the extracts from non-polar, semi-polar to polar solvent. This may be attributed to the extracting strength of this solvents. Polar solvents generally extract more phytochemicals than non-polar solvents (Halilu *et al*., 2013).

The preliminary phytochemical screening of *C. sieberiana* stem bark extracts (Table V) revealed the presence of some secondary metabolites. The ethyl acetate and methanol extracts contains the same classes of compounds. The ability of these extracts to contain these compounds may be due to the nature of extracting solvent where ethyl acetate is moderately polar and methanol is polar (Halilu *et al*., 2013). The n-hexane extract contains triterpenoids/steroids. This may be due to its non-polar nature. This result is in agreement with Modusolomuo *et al.* (1999); Sam *et al.* (2011) and Halilu *et al*. (2017) who reported the presence flavonoids, tannins, saponins and alkaloids in the root bark extract.

TLC studies performed on the n-hexane, ethyl acetate and methanol extracts of *C. sieberiana* stem bark showed the presence of several compounds as seen from the number of spots produced by each extract (Table V) which is in line with Halilu *et al*. (2017).

The total phenolic content (Figure 1) of the methanol and ethyl acetate extracts as calculated from the calibration curve (R^2 =0.989) at a concentration of 200µg/ml were 2.97 and 7.64mg gallic acid equivalents/g respectively as gallic acid equivalent of phenols. Phenolic compounds have been reported to demonstrate antioxidant and anticancer activities (Halilu *et al*., 2013; Halilu *et al*., 2017).

The qualitative determination of the antioxidant activity of the extracts using TLC revealed preliminary evidence for activity. The result showed that ethyl acetate had the highest activity as evidenced by the intensity of yellow spot produced against purple background (Halilu *et al*., 2017). The quantitative assay of the antioxidant activity (Figure 2) of the ethyl acetate and methanol extracts revealed antioxidant activity which compared favorably with ascorbic acid (standard) at different concentrations (Figure 2). The extracts produced concentration dependent effect which is seen on the increase in the percentage inhibition. The ethyl acetate and methanolic extracts at a concentration of 500 µg/mL showed a percentage inhibition of 27.53 and 24.42 respectively and for 3.91 µg/mL it was 84.62 and 3.02 respectively. The ascorbic acid at a concentration of 500 µg/mL exhibited a percentage inhibition of 58.01 and for 3.91 µg/mL 4.22 (Figure 3). An increase in percentage inhibition was observed for the ascorbic acid except for 7.81 and 500 µg /mL (Figure 2). The EC_{50} value of ascorbic acid was found to be 123.02 µg/ml. The EC_{50} value of ethyl acetate extract was 7.45 µg /mL indicating that it has better antioxidant potential than the ascorbic acid. The EC_{50} of methanol extract could not be determined because it could not scavenge for 50 % of the DPPH free radical. The antioxidant activity demonstrated by the extracts may be attributed to the phenolic compounds (flavonoids and tannins) present in the extracts. This is because of the phenolic hydroxyl groups confers free radical scavenging ability on the compounds (Zhu *et al*., 1997; Nayan *et al*., 2011; Halilu *et al*., 2013).

CONCLUSION

The preliminary phytochemical screening of the extracts revealed the presence of phenolic compounds and some other secondary plant metabolites which may be responsible for the antioxidant activity demonstrated by the extract of *C. sieberiana*. The ethyl acetate extract exhibited higher antioxidant activity with EC_{50} value of 7.45 µg /mL.

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