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Antioxidant Potentials of the Methanol Seed and Leaves Extracts Of *Chytranthus Macrobotrys* (Gilg) Exell & Mendonca

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Abstract: The antioxidant potentials of crude methanol seed extract (CMS/ME) as well as methanol and ethyl acetate leaves soluble fractions of *Chytranthus macrobotrys* Gilg, were studied. The antioxidant potentials of extract/different fractions were evaluated using different *in vitro* antioxidant models. In addition, total amount of polyphenolics compounds, Ferric-ion reducing antioxidant power (FRAP), Trolox equivalent absorbance capacity (TEAC) and Inhibition of Fe (II)-induced microsomal lipid peroxidation of crude extract and its different fractions were determined. From the two fractions of the ethyl acetate extract of *C. macrobotrys* leaves (CML/EE); fractions D_2 and D_3, fraction D_2 possessed total antioxidant capacity with the highest TEAC value, 15356.7 ± 1.75 µM TE/g and FRAP value, 185.76 ± 1.59 µM AAE/g. Polyphenol and flavonol were most prominent in CML/ME with values (4393.21 ± 8.04mg/g, 12966.07 ± 10.19mg/g) respectively.

Key Words: Antioxidant activity, *Chytranthus macrobotrys*, different fractions, free radical scavenging activity, polyphenol

INTRODUCTION

It has been established that oxidative stress is among the major causative factors in induction of many chronic and degenerative diseases including atherosclerosis, ischemic heart disease, ageing, diabetes mellitus, cancer, immunosuppression, neurodegenerative diseases and others.[1] Oxidative process is one of the most important routes for producing free radicals in foods, drugs and even in living systems.[2] Antioxidants are substances that may protect the body cells against the effects of free radicals. Antioxidants could include free radical scavengers, singlet oxygen quenchers, inactivators of peroxides and other reactive oxygen species (ROS), metal ion chelators, quenchers of secondary oxidation products and inhibitors of pro-oxidative enzymes.[3] Primary antioxidants such as tocopherols and some phenolic compounds inhibit the chain reaction of oxidation by acting as hydrogen donors or free radical acceptors and generation of more stable radicals.

Current research is now directed towards finding naturally occurring antioxidants particularly of plant origin. Currently available synthetic antioxidants like butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), tertiary butylated hydroquinone and gallic acid esters have been suspected to cause negative health effects. Hence, strong restrictions have been placed on their application and there is a trend to substitute them with naturally occurring antioxidants. Moreover, these synthetic antioxidants also show low solubility and moderate antioxidant activity.[4] BHA and BHT are suspected of being responsible for liver toxicity and carcinogenesis.[5,6] The use of traditional medicine is widespread and plants still present a large source of natural antioxidants that might serve as leads for the development of novel drugs.

Chytranthus macrobotrys (Gilg), (Family: Sapindaceae), native to Africa, is a forest shrub up to 35ft high, sparingly branched, pendulous with erect brownish flowers.[7] The fruits are edible, unripe fruits are green and become brightly yellow when ripe.[8]

Therefore, the objectives of present study were to determine the amount of total polyphenolic compounds and to evaluate the *in vitro* antioxidant activities of the crude methanol seed and the ethyl acetate leaves extracts of *C. macrobotrys* and its different fractions through different free radical scavenging assay.

MATERIALS AND METHODS

Collection and authentication of plant material: The plant was collected from a farm in Igbara-odo, Ekiti State (Nigeria) in May, 2016. It was identified and authenticated at the herbarium of the Department of Plant Science and Biotechnology, Ekiti State University, Ado-Ekiti. Voucher specimen was deposited, voucher number, UHAE.EPH 2:002.

Preparation of extract: The plant was air dried for a period of two weeks until constant weight. The sample was pulverized using mortar and pestle and blended with a blender (Marlex). The powdered form was weighed and extracted with ethyl acetate and methanol in a stoppered container for 72 hours. The resultant mixture was vacuum filtered with Whatman No.1 filter paper. The filtrate was concentrated to dryness using rotary evaporator at 40^0C. The fractions from the ethylacetate extract were obtained using varied proportions of *n*-hexane and ethylacetate.

Ferric-ion reducing antioxidant power (FRAP) assay: FRAP was done according to the method described previously.[9] In a 96-well clear microplate (visible range), 10 µL of the fractions (D_2 & D_3), methanol and ethylacetate extracts of the leaves of *C. macrobotrys* and methanol extract of the seeds were mixed with 300 µL FRAP reagent (a mixture of acetate buffer, 300 mM, pH 3.6; tripyridyl triazine (TPTZ), 10 mM in 40 mM HCl) and $FeCl_3.6H_2O$ (20 mM). After incubation at room temperature for 30 minutes, the plate was read at a wavelength of 593 nm in a Multiskan Spectrum plate reader (Thermo Fisher Scientific). L-Ascorbic acid was used as a standard with concentrations varying between 0 and 1000 µM. Further dilutions were done to the samples that were highly concentrated and such dilution factors were recorded and used for calculations of the affected samples. The results were expressed as µM ascorbic acid equivalent per milligram dry weight (µM AAE/g) of the test samples.

Trolox equivalent absorbance capacity (TEAC) assay: TEAC was done according to the methods previously described.[10,11] The stock solutions which contain 7 mM ABTS (2, 2'-Azino-bis (3-ethylbenzo thiazoline-6-sulphonic acid) diammonium salt) and 140 mM potassium-peroxodisulphate ($K_2S_2O_8$) was prepared and kept at -2 ^oC. The working solution was then prepared by adding 88µL $K_2S_2O_8$ solution to 5mL ABTS solution. The two solutions were well mixed and allowed to react for 24 hours at room temperature in the dark. Trolox (6-Hydroxyl-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) was used as the standard with concentrations ranging between 0 and 500 µM. After 24 hours, the ABTS mixture was diluted with ethanol to read a start-up absorbance (control) of approximately 2.0±0.1. The stock solution of the fractions (D_2 & D_3), methanol and ethylacetate extracts of *C. macrobotrys* leaves and methanol extract of *C. macrobotrys* seeds was allowed to react with 300 µL ABTS in the dark at room temperature for 30 min. The absorbance was read at 734 nm at 25 ^oC in the plate reader. The results were expressed as µM Trolox equivalents per milligram dry weight (µM TE/g) of the test samples.

Inhibition of Fe (II)-induced microsomal lipid peroxidation assay: Sepharose column with 0.01M potassium phosphate buffer; pH 7.4, supplemented with 1.15 % KCl was used to first isolate rat liver microsomes from an S9 liver fraction. The isolation of microsomes was done at -5 ^oC. A glass column (24/29) was parked with sepharose (30 cm) and eluted with phosphate buffer as described above. Microsomes obtained were homogenized and distributed into 2 mL eppendorf tube and kept at -80 ^oC prior to experiment. The protein content of the homogenized microsome was determined *in-situ*. A modified method was adopted.[12] The reaction mixture contained microsomes (1 mg of protein/mL in 0.01M potassium phosphate buffer; pH 7.4, supplemented with 1.15% KCl).

The positive control included microsomes, buffer and ferrous sulphate, in the absence of the samples to be tested. The sample stock solutions of (fractions (D_2 & D_3), methanol & ethylacetate extracts of the leaves of *C. macrobotrys* and methanol extract of the seeds of *C. macrobotrys* were prepared in methanol (1mg/mL, w/v). The working sample solutions were prepared in 0.01M potassium phosphate buffer; pH 7.4, supplemented with 1.15 % KCl diluted to 100, 50 and 25 µg/ml concentrations. 100 µL of each sample (working solutions) were dissolved in potassium phosphate buffer and pre-incubated with 500 µL microsomes at 37 ^oC for 30 minutes in a shaking water bath. 200 µL of KCl-buffer were added to the mixture, followed by 200 µL of a 2.5 mM ferrous sulphate solution and incubated at 37 ^oC for 1 hour in a shaking water bath. The reaction was terminated with 10% trichloroacetic acid (TCA) solution (1 mL) containing 125 µL butylated hydroxytoluene (BHT, 0.01 %) and 1 mM ethylenediamminetetraacetic acid (EDTA). Samples were centrifuged at 2000 rpm for 15 minutes, 1 mL of supernatant was mixed with 1 mL of 0.67 % thiobarbituric acid (TBA) solution. The reaction mixture was then incubated in a water bath at

90 ^{o}C for 20 minutes and the absorbance were measured at 532 nm using plate reader. The percentage inhibition of TBARS formation relative to the positive control was calculated by:

% inhibition of TBARS = $[(A_{control} - A_{sample}/ A_{control}] X 100$

Where A_{blank} is the absorbance of the blank, A_{sample} is the absorbance of the sample.

Total polyphenol content: The total polyphenol content of the extracts and fractions against tyrosinase was determined using the established Folin Ciocalteu method.[13] A volume of 25 ml of the extract and fractions was incubated in a clear 96-well flat bottom plate for 5 min with 125 ml freshly prepared 0.2 N. Folin Ciocalteu's phenol reagent was thereafter 100 ml 7.5 % sodium carbonate was added and incubated for 2 hours. The absorbance at 765 nm was measured on a plate spectrometer (Multiskan, Thermo Electron Corporation) and total polyphenols calculated using the standard Gallic acid in 10 % ethanol. Results were expressed as mg Gallic acid equivalents per gram.

Total flavanol content: The flavanol content of the extracts and fractions was determined colourimetrically at 640 nm and spectrophotometrically at 360 nm, respectively in accordance to the previous methods.[14,15] A freshly prepared 0.05 % DMACA solution was made by dissolving DMACA in 8 % HCl prepared in methanol. The samples (50 ml) were incubated in a clear 96-well flat bottom plate with 250 ml DMACA solution for 30 min.

Total flavonol content: A solution of 0.1 % HCl prepared in 95 % ethanol was made. The samples (12.5 ml) were incubated in a clear 96-well flat bottom plate with 12.5 ml of 0.1 % HCl-ethanol solution and 225 ml of 2 % HCl for 30 min. The absorbance was measured on a plate spectrometer (Multiskan, Thermo Electron Corporation). The total flavanol and flavonol was calculated using the standards catechin in methanol and quercetin in 95% ethanol, respectively.[14,15] Results were expressed as mg standard equivalents per gram.

RESULTS AND DISCUSSION

Antioxidants are tremendously important substances which possess the ability to protect the body from damage caused by free radical induced oxidative stress. The antioxidant potential of *C. macrobotrys* methanol extract of the seed, leaves and ethyl acetate extracts of leaves were investigated in the search for new bioactive compounds from natural resources. Results obtained in the present study revealed that the level of phenolic compounds in the methanol and ethyl acetate extracts of the seeds and leaves of *C. macrobotrys* were considerable (Table 1). Polyphenolic compounds are known to have antioxidant activity and it is likely that the activity of the extracts is due to these compounds.[16,17] The evaluation of the total polyphenol content was highest in the methanol extract of the leaves (CML/ME) (Table 1). Flavonol was only detected in the methanol extract of the leaves (CML/ME) and was not detected in the other extracts and fractions. Flavanol was not detected in any of the fractions and extracts.

The FRAP assay measures a sample's ability to reduce the intense blue ferric TPTZ (2, 4, 6- tri [2-pyridyl]-s-triazine, iron (III) chloride hexahydrate) complex to its ferrous form in an acidic medium thereby changing its absorbance.[9] The methanol extract of the seed, ethyl acetate and methanol extracts of the leaves and fractions D_2 and D_3 obtained from the ethyl acetate extract of the leaves were investigated for the ability to transfer electron to free radicals by measuring their antioxidant capacities on FRAP and TEAC. The

results in Table 2 show that fraction D_2 possessed similar antioxidant activity (TEAC value) when compared to the commercial antioxidant derived from green tea, EGCG. It is therefore evident that fraction D_2 possessed interesting antioxidant activities expressed by its trolox (TEAC) and ascorbic acid (FRAP) equivalents.

Table 1: Evaluation of the total polyphenol, flavonol and flavanol content of the extracts and fractions of *C. macrobotrys* seed and leaves

Sample	Polyphenol	Flavonol	Flavanol
CML/ME	4393.21±8.04	12966.07±10.19	nd
Fraction D_2	1457.87±4.79	nd	nd
CML/EE	46.08±1.84	nd	nd
CMS/ME	119.73±7.35	nd	nd
Fraction D_3	3675.50±5.27	nd	nd

nd: not detected

Table 2: Ferric ion reducing and trolox equivalent antioxidant capacities of the extracts and fractions of *Chytranthus macrobotrys* seed and leaves

Sample	FRAP (µM AAE/g)	TEAC (µM TE/g)
CML/ME	nd	3648.3±1.20
Fraction D_2	185.76±1.59	15356.7±1.75
CML/EE	101.12±2.37	66.7±0.39
CMS/ME	134.88±1.59	146.9±0.38
Fraction D_3	20.32±2.36	2150.3±0.08
EGCG	3326.45±5.76	11545.4±17.28

nd: not detected**, EGCG:** Epigallocatechingallate, **CML/ME:** methanol extract of *C. macrobotrys* leave**, CML/EE:** ethyl acetate extract of *C. macrobotrys* leaves, **CMS/ME:** methanol extract of *C. macrobotrys* seed

The over production of reacting oxygen species results in an attack of not only DNA but also other cellular components which are highly sensitive to oxidation.[18] Therefore, unsaturated fatty acids in cell membranes are susceptible to free mediated oxidation. Oxidative degradation of lipids is a common consequence of oxidative stress, a process whereby polyunsaturated lipids of the membranes are susceptible to oxidative damage via the reaction of free radicals which can lead to lipid peroxidation.[19] Products of lipid peroxidation such as malondialdehyde (MDA), 4-hydroxyl 2-nonenal and some other alkanals react with cell to form adducts with significant irreversible effects on cellular functions and could also promote ageing process. The ethyl acetate extract of the leave (CML/EE) demonstrated potent inhibitory activity against Fe^{2+} - induced lipid peroxidation in a competitive manner to that of EGCG at concentrations of 100, 50 and 25 µg/mL (Table 3) with IC_{50} values of 75.50 ± 0.02 µg/mL and 36.25 ± 0.01 µg/mL respectively. No significant IC_{50} value was observed by CML/ME, CMS/ME, fraction D_2 and D_3.

Table 3: Anti-lipid peroxidation activities of the extracts and fractions of *Chytranthus macrobotrys* seed and leaves

Sample	100 µg/mL	50 µg/mL	25 µg/mL
CML/ME	7.99	0	0
Fraction D_2	11.50	3.08	1.18
CML/EE	53.14	48.68	48.23
CMS/ME	18.94	11.22	8.92
Fraction D_3	36.06	29.98	29.16
EGCG	92.93	58.05	49.30

CONCLUSION

These findings show that the *C. macrobotrys* extracts possess antioxidant activity. The ethyl acetate leaves fraction D_2 possessed total antioxidant capacity with the highest TEAC value, 15356.7 ± 1.75 µM TE/g and FRAP value, 185.76 ± 1.59 µM AAE/g. Polyphenol and flavonol were most prominent in CML/ME with values (4393.21 ± 8.04mg/g, 12966.07 ± 10.19mg/g) respectively, which attributed to the antioxidant activity of *C. macrobotrys* extracts in this study. The seed and leaves extracts are promising candidates for use as natural products based antioxidant for the health of human being. To the best of our knowledge, this is the first report on the antioxidant potentials of seed and leaves extracts of *C. macrobotrys.*

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