Free radical scavenging and cytotoxic effects of methanol extract of *Theobroma cacao* L. (Sterculiaceae) seed

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ABSTRACT

Background: *Theobroma cacao* L. (Sterculiaceae) is a plant with great economic and health benefits. The plant is believed to possess diverse medicinal value among different populations.

Objective: The present study determined the radical scavenging and cytotoxic activities of the methanol seed extract of *Theobroma cacao* and its fractions.

Methods: Total phenolic, flavonoid content and the 2,2-diphenyl-2-picryl-hydrazyl (DPPH) radical spectrophotometric assays were used to determine the antioxidant potential of the *T. cacao* seed extracts. Cytotoxicity activity of the extracts was determined using the MTS assay against MCF-7 breast cancer cell lines.

Results: The seed extracts showed remarkably high scavenging effect on DPPH radical. The ethyl acetate fraction exhibited the highest scavenging effect with IC_{50} value of 5.00 µg/mL. The phenolic and flavonoid contents were significantly higher in the ethyl acetate fraction compared to other fractions ($p < 0.05$). The MTS assay revealed about 17.90 % and 22.00 % reduction in MCF-7 viability after 48 h exposure to 50 µg/ml of the crude methanol extract and the petroleum ether fraction respectively.

Conclusion: The above results illustrate the antioxidant ability of cocoa seed and show its potential cytotoxic effect on breast cancer cell line. This finding however needs substantiation by further experiments.

Key words: *Theobroma cacao,* radical scavenging, DPPH, cytotoxicity

Libre balayage radical et effects cytotoxiques de l'extrait de méthanol de la graine de *Theobroma cacao* L. (Sterculiaceae)

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RESUME

Contexte: *Theobroma cacao* L. (Sterculiaceae) est une plante qui présente d'énormes avantages économiques et médicaux. On croit que la plante possède une valeur médicale diverse chez plusieurs populations.

Objectif: La présente étude a déterminé le balayage radical et les activités cytotoxiques de l'extrait de la graine de méthanol du *Theobroma cacao* et ses fractions.

Méthodes: La teneur total phénolique, flavonoïde et les essais radicaux de spectrophotométries 2,2-diphenyl-2-picryl-hydrazyl (DPPH) furent utilisés pour déterminer le potentiel antioxydant des extraits de la graine du *T. cacao*. L'activité de cytotoxique des extraits fut déterminée à l'aide de l'essai MTS contre les lignes cellulaires du cancer du sein MCF-7.

Résultats: L'extrait a indiqué un effet de balayage remarquablement élevé sur le radical DPPH. La fraction de l'acétate d'éthyle a montré l'effet de balayage le plus élevé avec une valeur IC_{50} de 5,00 µg/mL. Les teneurs phénoliques et flavonoïdes étaient nettement plus élevées dans la fraction de l'acétate d'éthyle par rapport aux autres fractions ($p < 0.05$). L'essai MTS a révélé environ 17,90 % et 22,00 % de réduction dans la viabilité MCF-7 après 48 h d'exposition à 50 µg/ml d'extrait de méthanol brut et la fraction d'éther de pétrole respectivement.

Conclusion: Les résultats ci-dessus montrent la capacité antioxydant de la graine de cacao et indique son effet cytotoxique potentiel sur la ligne cellulaire du cancer du sein. Ce résultat a néanmoins besoin de preuves avec des essais supplémentaires.

Mots-clés: *Theobroma cacao,* balayage radical, DPPH, cyto-toxicité

INTRODUCTION

Medicinal plants have been identified and used throughout human history as source of food and for medication. The medicinal values of plants lies in their ability to produce a wide range of biologically active agents. [1,2]

A number of drugs currently used in modern medicine have their origin from plants. [3,4] Over the past few decades, there has been renewed interest in the search for new molecular entities for the treatment of the numerous diseases that man currently grapples with. The plant kingdom has remained a major area of interest in this search.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced as part of normal metabolic processes and by exposure to ionizing radiations and other environmental pollutants.[5,6] Oxidative stress, which represents a consequence of an imbalance between the production of free radicals and the body's ability to defend against them has been implicated in the development of many diseases such as cancer[7], neurodegenerative diseases [8], cardiovascular diseases [9], Sickle Cell Disease [10], fragile X syndrome [11] and autism. [12] *Theobroma cacao* L. (Sterculiaceae) commonly called cacao or cocoa is an important tropical rain forest specie mainly grown for its oil rich seed to produce cocoa and cocoa butter. Cocoa has several ethno-medicinal uses as remedy for alopecia, burns, cough, fever, listlessness, malaria, rheumatism, snake bite and wound infections.[13] Phytochemical studies have shown that cocoa is a rich source of fat, carbohydrate, protein, polyphenols, vitamins and minerals. Important compounds isolated from cocoa seed include theobromine; phenylethylamine and caffeine.[14] *T. cacao* has been reported to have antioxidant, anti-carcinogenic, cardioprotective, antimicrobial and neuro-protective effects. [15-19]

In the present study, we have investigated the free radical scavenging effect *viz a viz* the antioxidant activities of the crude methanol extract and fractions of the seed as well as the cytotoxic effect of the crude methanol extract of the seed on MCF-7 breast cancer cell line.

METHODS

Plant material

Fresh *Theobroma cacao* seeds were collected from a forest near Benin City, Nigeria. The plant material was identified and authenticated by the Department of Plant Biology and Biotechnology University of Benin. The seeds were dried under shade and ground into powder using an attrition mill. The crude powdered sample was stored in an air-tight container until ready for use.

Preparation of extract and fractions

The powdered sample (150 g) was macerated with methanol (750 mL) for 72 hours. The extract was concentrated using a rotary evaporator (RE 300, Bibby Scientific, UK) at reduced pressure. The crude methanol extract was subjected to prefractionation/partitioning using different solvents. The crude extract was first defatted with 1.5 L of petroleum ether (40 – 60°C), the ether insoluble portion was extracted with 1.2 L of chloroform followed by 750 mL of ethyl acetate. The various fractions were concentrated *in vacuo* and then stored in a refrigerator until required.

Determination of antioxidant activity

Determination of total phenol

Sample solution (0.5 ml) with a concentration of 1 mg/mL was added to 4.5 mL of deionized distilled water and 0.5 mL of Folin Ciocalteu's reagent (previously diluted with water 1:10, v/v).

The tubes were mixed and maintained at room temperature for 5 min followed by the addition of 5 mL of 7 % sodium carbonate and 2 mL of deionized distilled water. The tubes were mixed and then incubated for 90 min at room temperature. The absorbance was measured at 750 nm. All determinations were performed in triplicates with gallic acid utilized as the positive control. The total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per gram of extract (mg GAE/g extract). [20]

Determination of total flavonoid

Total flavonoid contents were estimated using the method described by Ebrahimzadeh *et al*.[21] Sample solution (0.5 ml) of concentration 1 mg/mL was mixed with 1.5 mL of methanol and then, 0.1 mL of 10 % aluminium chloride was added, followed by 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. The mixture was incubated at room temperature for 30 min and thereafter absorbance taken at 415 nm. Similar concentrations of quercetin, the positive control were used. The results were expressed as milligrams quercetin equivalents (QE) per gram of extract (mg QE/g extract).

DPPH radical scavenging assay

The DPPH radical scavenging effect was estimated using the protocol described by Jain *et al*.[22] A solution of 0.1

mM DPPH in methanol was prepared, and 1.0 mL of this solution was mixed with 3.0 mL of extract in methanol containing 0.01 – 0.2 mg/mL of the extract. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. Ascorbic acid was used as reference standard. The ability to scavenge DPPH radical was calculated using the following equation:

DPPH radical scavenging activity (%) = $[(A_0 - A_1)/(A_0)] \times 100$, Where A_0 was the absorbance of DPPH radical + methanol; A_1 was the absorbance of DPPH radical + sample extract/standard.

The 50% inhibitory concentration value (IC_{50}) is indicated as the effective concentration of the sample that is required to scavenge 50% of the DPPH free radicals.

Cytotoxic activity

Cell culture

Human breast cancer cell line MCF-7 (ATCC: HTB-22) was obtained from the American Type Culture Collection (Manassas VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Germany) with 10% fetal bovine serum (PAA Laboratories, GmbH, Germany) and antibiotic-antimycotic (1% penicillin G/streptomycin and 0.5% amphotericin B) solution (Ratiopharm, Germany). Cells were maintained at 37°C in a 5% CO_2 atmosphere. The medium was changed every two days. Confluent Cells were sub-cultured after treatment with 0.05% trypsin/0.02% EDTA to detach the cells.

MTS Assay

Cells were seeded in 96-well-plates in 100 µL medium and left to attach for 48 hours at 37°C. Subsequently, cells were treated with extracts at concentration of 50 µg/mL in culture medium. After 48-hour incubation at 37°C in a 5% CO_2 atmosphere, cells were assayed with 10 µL MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] solution (Promega Corp., Madison, WI) for 1 hour at 37°C. The vehicle DMSO (0.1%) was used in the same manner to serve as control. Assessment of metabolic activity was recorded as relative colorimetric changes measured at 492 nm. Correction for background absorbance was done by measuring the absorbance of the extracts and MTS solution without the cells. Raw data were transferred to Microsoft Excel and analyzed. The percentage cell viability was calculated by the formula;

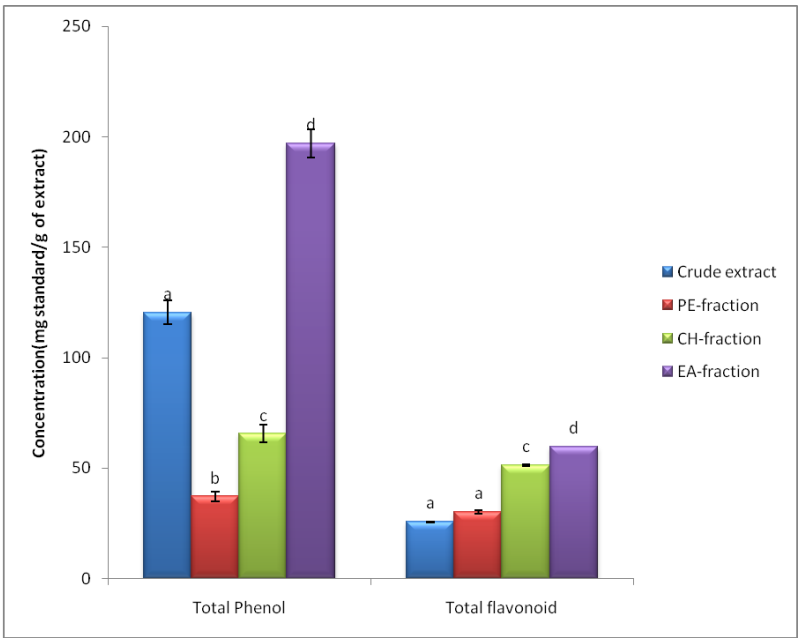

Figure 1: Total phenolic and flavonoid contents of extract and fractions of *T. cacao* seed. Different lowercase letters represent significant difference between means at P < 0.05.

Legend

PE-petroleum ether

EA-Ethyl acetate

CH- Chloroform

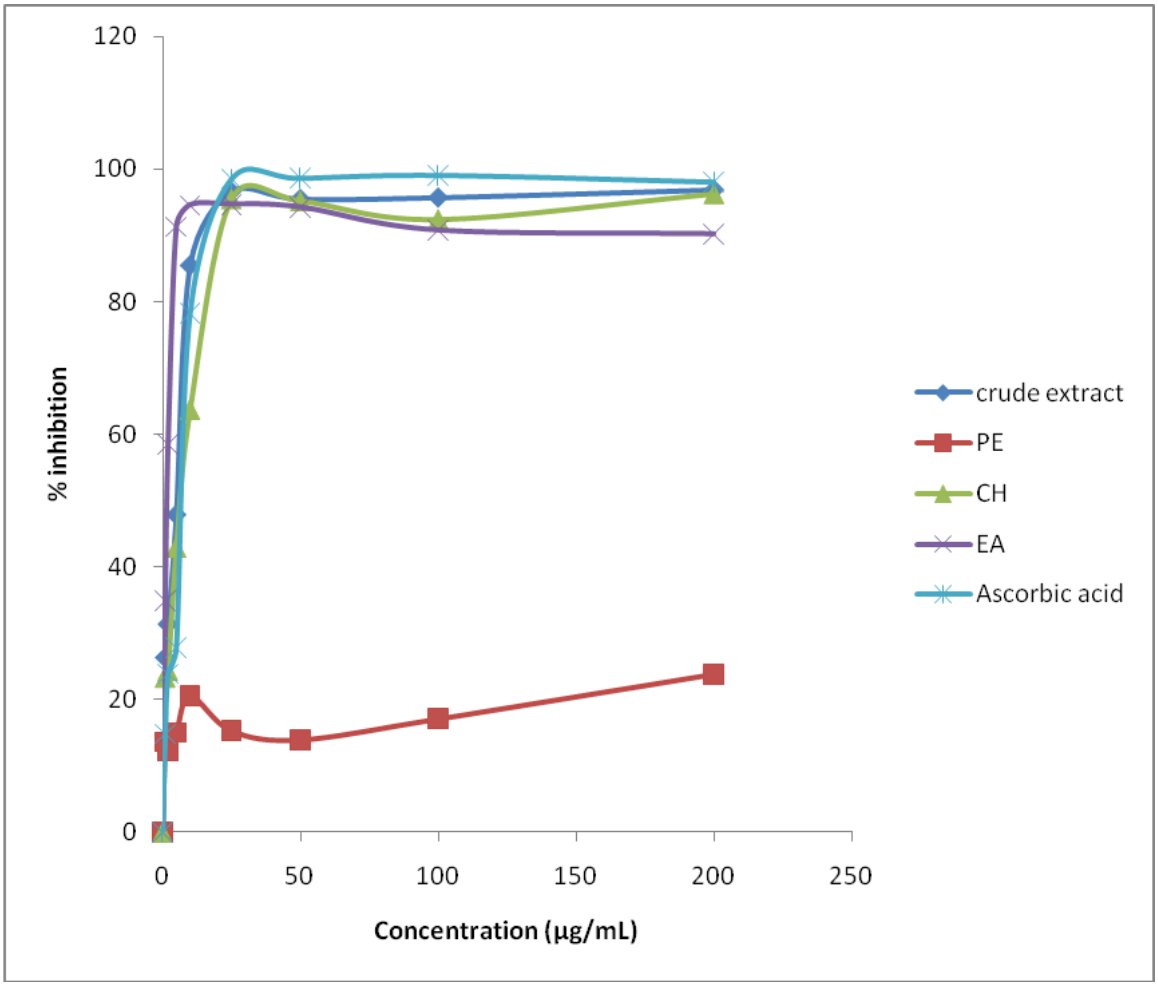

Figure 2: DPPH radical scavenging activity of crude extract and fractions of *T. cacao* seed compared with standard (ascorbic acid).

Table 1: Evaluation of IC_{50} values of *T. cacao* seed extracts

Sample	IC50 value (µg/ml)
Ascorbic acid	8.51 ± 0.06^{a}
Methanol extract	23.14 ± 0.11^{b}
Petroleum ether fraction	623.44 ± 0.08^{c}
Chloroform fraction	23.62 ± 0.04^{b}
Ethyl acetate fraction	5.00 ± 0.12^{a}

Data represent mean ± Standard Deviation of triplicate analysis.

Different lowercase letters within column indicate significant difference at $p < 0.05$

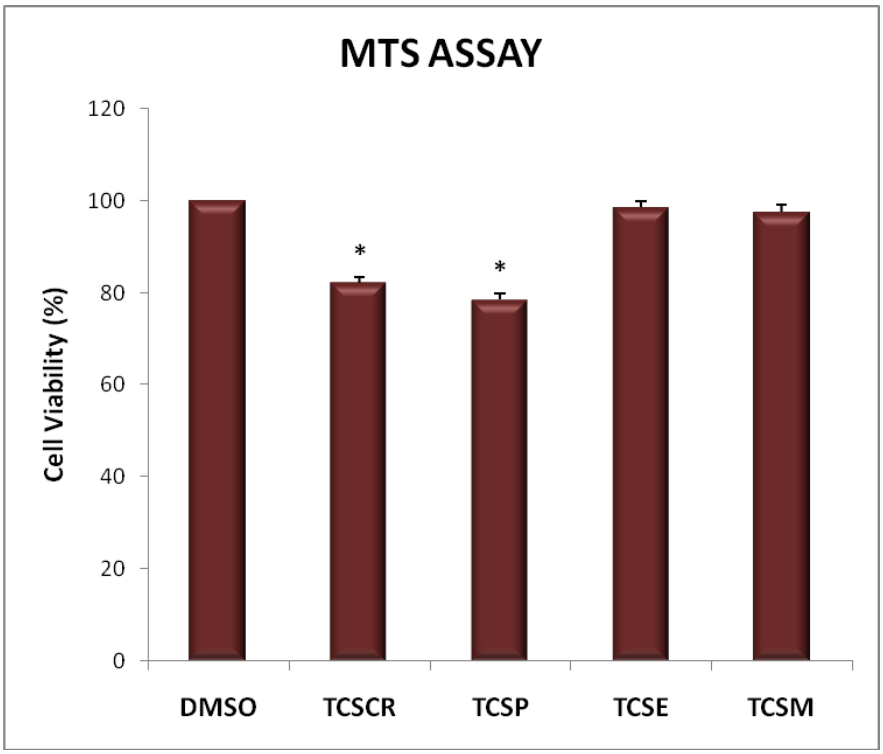

Figure 3: Comparative cell viability (Mitochondrial activity) of *Theobroma cacao* seed extract.
TCSCR: Crude methanol extract of *Theobroma cacao* seed
TCSP: Petroleum ether fraction of *Theobroma cacao* seed extract
TCSE: Ethyl acetate fraction of *Theobroma cacao* seed extract
TCSM: Aqueous methanol fraction of *Theobroma cacao* seed extract
***** = Significant difference at $P < 0.05$

DISCUSSION

Phenolic and polyphenolic compounds are ubiquitous in plants. They are a major class of phytochemicals with interesting functional properties that are of potential benefit to human health. Numerous studies have linked the antioxidant properties of plant extracts to their phenolic compounds.[23-25] The higher value of the phenolic and flavonoid contents in the ethyl acetate fraction compared to the other fractions may be due to the polar nature of these components. Phenolic compounds are generally polar and solvents appear to play a significant role in their extraction so that polar solvents tend to contain more of these components compared to the less polar or non polar solvents.

Comparison of the various extracts reveals that the higher the total phenolic and flavonoid content, the lower the IC_{50} value obtained in the DPPH assay. This therefore suggests a direct relationship between cocoa seed polyphenolics and antioxidant activity. Our observations corroborate the findings of other studies which have shown that cocoa has good antioxidant properties, which may be linked to its high polyphenolic content.[26-28]

Tetrazolium salt reduction is a widely used, easy and indirect measure of cell viability. Assays commonly available to assess cell number, cell viability and cytotoxicity include the metabolic MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], MTT [3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide], WST-1 [4-(3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio)-1,3-benzene disulfonate] and XTT [sodium 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] assay.[29-32] The MTS assay is based on the intracellular reduction of the yellow tetrazolium salt (MTS) solution by mitochondrial dehydrogenase to a purple formazan product. The absorbance of the reduction product is measured spectrophotometrically.

In this study, we observed about 17.90% reduction in MCF-7 cell viability after 48 h exposure to *T. cacao* extract (50 µg/mL) compared to the control. Evaluation of the cytotoxic effect of the various fractions (TCSP, TCSE and TCSM) at 50 µg/mL shows the petroleum ether fraction (TCSP) was most cytotoxic. The mean cell

viability in the TCSP treated group was 78.50% (representing about 22.00% reduction in cell viability compared to the control (DMSO) group). This effect was found to be significant ($P < 0.05$) in comparison to the control. While the ethyl acetate (TCSE) and methanol fraction (TCSM) were barely cytotoxic against MCF-7 (1.50% and 2.5% reduction in cell viability for TCSE and TCSM respectively) compared to the control. The present observation shows that the cytotoxic effect of *T. cacao* seed extract resides in its non-polar constituents. However, studies have demonstrated different impressions between MTS assays and other assays involving measurement of cell number, cell viability and proliferation e.g. DNA labeling, live-dead staining and flow cytometric analysis.[33-34] Cell cycle arrest does not necessarily result in metabolic dysfunction and in some cases disturbances of cell metabolic processes can lead to erroneous readout of cell response to treatment.[35] Therefore, perturbation in cellular processes as measured by the MTS assay may not directly translate to antiproliferative effect. Hence, the present study underscores the need for further studies such as flow cytometry, live-dead staining, DNA labeling and/or online monitoring of metabolic activities (adhesion, respiration and cellular acidification) to confirm cell cycle arrest. Chromatographic separation of the fractions and testing of the individual component for their cytotoxic effect is also highly recommended.

CONCLUSION

The results of this study demonstrated that *Theobroma cacao* seed has good free radical scavenging activity which is a measure of its antioxidant capacity with the activity residing more in the ethyl acetate fraction. The study has also shown that *T. cacao* contains high concentration of phenolic compounds and that its antioxidant activity may be attributed to the presence of these valuable constituents. *T. cacao* seed also demonstrated potential cytotoxic activity against breast cancer cell which is subject to further investigations.

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