ANTIOXIDANT AND ANTI-PROLIFERATIVE EFFECTS OF AN ETHYL ACETATE FRACTION OF THE HYDRO-ETHANOLIC EXTRACT OF SYNEDRELLA NODIFLORA (L) GAERTN

PATRICK AMOATENG1, EMELIA OPPONG BEKOE2, SETH KWABENA AMPONSAH3, REGINA APPIAH-OPONG4, DORCAS OSEI-SAFO5, BENOIT BANGA N’GUSSAN3, M. V. N. L. CHAITANYA6, RAMAN RAJESHKUMAR7, S. P. DHANABAL6

1Department of Pharmacology and Toxicology, School of Pharmacy, College of Health Sciences, University of Ghana, 2Department of Pharmacognosy and Herbal Medicine, School of Pharmacy, College of Health Sciences, University of Ghana, 3Department of Medical Pharmacology, UG Medical School, College of Health Sciences, University of Ghana, 4Department of Clinical Pathology, Noguchi Memorial Institute for Medical Research, College of Health Sciences, University of Ghana, 5Department of Chemistry, School of Physical and Mathematical Sciences, College of Basic and Applied Sciences, University of Ghana, P. O. Box LG 56, Legon, Accra, Ghana, 6Department of Pharmacognosy and Phytopharmacy, JSS College of Pharmacy (A constituent College of JSS University, Mysore), Rock lands, Ootacamund 643001, Tamilnadu, India, 7Department of Pharmaceutical Biotechnology, JSS College of Pharmacy (A constituent College of JSS University, Mysore), Rock lands, Ootacamund 643001, Tamilnadu, India

Email: pamoateng@ug.edu.gh

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ABSTRACT

Objective: Synedrella nodiflora is traditionally used in the treatment of several ailments. Pharmacologically, this plant has anticonvulsant, sedative, anti-nociceptive and anti-proliferative effects. This study further investigated S. nodiflora for its antioxidant and in vitro inhibition of cancerous cell lines.

Methods: Phytochemical assays, and the DPPH radical scavenging method were employed in preliminary screening for antioxidant activities of the crude hydro-ethanolic extract (SNE) and resulting fractions. The potent ethyl acetate fraction (EAF), was further investigated for total phenol and flavonoid contents, reducing power, lipid peroxidation potential, and cytotoxic effects on human breast cancer (MCF-7), leukemic (Jurkat), and normal liver (Chang’s liver) cell lines.

Results: The extract contained phenols, flavonoids, tannins, glycosides, sterols, terpenoids, and alkaloids. It scavenged for DPPH with an IC50 of 114 µg/ml, whereas that of EAF was 89 µg/ml. EAF prevented peroxidation of egg lecithin at an IC50 of 24.01±0.08 µg/ml. These IC50 are four and three times lower than the reference standards. EAF produced anti-proliferative effects against MCF-7, and Jurkat cell lines with IC50 of 205.2 and 170.9 µg/ml respectively. EAF had a high IC50 of 252.2 µg/ml against Chang’s liver cells. At 0.1 mg/ml EAF had similar total flavonoid content to SNE, but a significantly higher total phenol content.

Conclusion: The ethyl acetate fraction of S. nodiflora, exhibited the most potent antioxidant activity. It inhibited the proliferation of breast and leukemic cancer cell lines, while having weak cytotoxic effect on normal liver cells. These can be explored for further drug development.

Keywords: Synedrella nodiflora, Antioxidant, Anti-proliferative, Ethyl acetate fraction, Cancer

INTRODUCTION

Synedrella nodiflora (L.) Gaertn family Asteraceae is a common weed found growing along the banks of rivers, streams and roadsides [1]. This plant is an erect branched ephemeral herb that may grow to about 30-80 cm high. It originated from tropical America, but has currently spread all over the world. The leaves appear in opposite pairs, and are about 4-9 cm in length. The leaves are elliptic to ovate, with three prominent veins, and nearly toothed margins. The leaf grows in small bunches of about 2-8 inflorescences at nodes and tips. Each inflorescence consists of several erect bracts 3-5 mm long surrounding 5-6 marginal ray florets and 10-20 central disc florets, each 3-4 mm long with a yellow petal [2]. In Ghana, the whole plant is boiled, and the aqueous extract drunk for the treatment of neurological conditions such as epilepsy, whiles the leaves are used for threatened abortion, hiccup, laxative and as feed for livestock [1].

The crude hydro-ethanolic extract of this whole plant has been found to possess anticonvulsant [3], sedative [4], antioxidant [5], anti-nociceptive properties [6, 7] and anti-proliferative effects on onion root and shoot apical meristem and wister rat bone marrow cells [8]. Hence, this study seeks to investigate further the antioxidant potential and anti-proliferative activities of various fractions of the hydro-ethanolic extract of this plant.

Oxidative stress exists when there is an imbalance between the production of free radicals and reactive metabolites, and their elimination by protective mechanisms involving antioxidant agents in the body [9]. Numerous findings have revealed that persistent oxidative stress can lead to chronic inflammation, which in turn mediates chronic diseases including cancer, diabetes, cardiovascular, neurological and pulmonary diseases [10]. Cancer is also currently one of the most severe health challenges worldwide [11], and reactive oxygen species in different phases of tumorigenesis has been strongly implicated [12, 13]. The use of antioxidants for cancer prevention, and therapy has been investigated, and several antioxidants such as curcumin, vanillin and tannic acid found in food products possess anticancer properties [14, 15]. Other antioxidants such as resveratrol, obtained from grapes and other food products, protect cells from oxidative damage, and programmed cell death [16, 17]. Quite a significant number of plants extracts with both antioxidant and anti-proliferative activities have been evaluated to have potential anti-cancer properties [18-21]. In view of this, Synedrella nodiflora which has already been shown to be effective in the treatment of a neurological condition such as epilepsy, and having both anti-oxidant and cell inhibitory activity was further investigated for its in vitro anti-oxidant and inhibitory effects on cancer cells.

MATERIALS AND METHODS

Chemicals and reagents

RPMI 1640 (Roswell Park Memorial Institute medium), DMEM (Dulbecco’s Modified Eagle’s medium) and EEME (Eagle’s minimal essential medium) obtained from Sigma-Aldrich, (Illinois, USA) was
used for the various cell line culture. Curcumin (Sigma-Aldrich, Illinois, USA), 2, 5-diphenyl-1-picyl hydradyl (DPPH) free radical reagent and butylated hydroxytoluene (BHT) (Sigma-Aldrich Illinois, USA), 3(4, 5-dimethylthiazol-2-y)1, 5-diphenyltetrazolium bromide (MTT) dye (Sigma-Aldrich, Illinois, USA), while all solvents used including (e. g. methanol, chloroform, ethyl acetate, acetone, n- hexane, petroleum ether, glacial acetic acid) were of HPLC grade, and all other reagents were obtained from standard suppliers.

Plant collection and extraction
Samples of the plant were collected from the Botanical Gardens, University of Ghana, Accra, in August 2012. They were identified and authenticated at the Ghana National Herbarium (GHNHN) Department of Plant, Environmental Science, University of Ghana, Accra where a voucher specimen (PA01/UJS/FG/012) was kept. The whole plants were collected, air-dried for fourteen days and powdered. Three hundred (300 g) of pulverized plant material was cold-macerated with 70 % v/v of ethanol in water. The hydro-ethanolic extract was then evaporated using a rotary evaporator (Buchi Rotavapor® R-300, Flawil, Switzerland) under reduced pressure to remove ethanol. The aqueous portion was frozen at -20°C, and lyophilized (Bench-top Freeze Dryer, Labfrees Instruments Co., Ltd, Beijing, China). The percentage yield of the dried sample (SNE) was calculated before it was kept in a desiccator.

Phytochemistry of crude extract
The crude extract was tested qualitatively for the presence of saponins, flavanoids, tannins, alkaloids, glycosides, sterols, terpenoids coumarins, and anthraquinones according to published methods in and Sowwora, 1993, and Evans, 2009 [22, 23].

Fractionation
The weight of 50 g of dried crude 70 % v/v hydro-ethanolic extract of S. nodiflora (SNE) was suspended in 500 ml of 70% ethanol (v/v) and successively partitioned with an equal volume of petroleum ether, n-hexane, dichloromethane, ethyl acetate, acetone, and glacial acetic acid to obtain a petroleum ether (PEF), n-hexane (NHF), dichloromethane (DCM), ethyl acetate (EAF), acetone (ACF), glacial acetic acid (AAF), and the remaining aqueous (AOF) fractions respectively. Each fraction was concentrated at 40°C, using a rotary evaporator, and lyophilised. Extract yields were recorded.

Total phenol assay
The total phenolic content of the crude extract (SNE) was assayed by the Folin Ciocalteu’s method, as published by Amoateng et al., 2011 [5] with slight modifications. An aliquot (10 µl) of the extract (20 mg/ml) was placed into a 96-well plate, and 50 µl of 2 %, w/v aluminum chloride solution was added. The mixture was incubated at room temperature for 10 min. Absorbance was then taken after incubation, at a wavelength of 760 nm. Catechin was used as control. Two-fold serial dilutions were made for both the extract and the control to obtain initial concentrations of 0.1, 0.3, 1, 3 mg/ml for the extract and 0.1, 0.2, 0.4, 0.6, 0.8, 1 mg/ml for catechin respectively. Triplicate experiments were performed.

Lipid peroxidation (LPO) assay of the ethyl acetate fraction (EAF)
To prepare egg lecithin, three eggs were obtained from ZamZam store, 10A Bombay Castle, Ooty-1, India. The egg yolks were separated from the albumin, and mixed with the aid of a glass rod. A volume of 100 ml acetone was added, mixed and allowed to stand for about 5 min. A yellowish supernatant which formed was discarded. This was done successively until the entire yellowish colour of the egg yolk mixture was bleached and the supernatant was colourless. The creamy mixture was poured into a glass dish and dried in an oven at 37°C. The dried white powdered precipitate (egg lecithin) was collected and stored in a desiccator until use. Test samples of 100 µl aliquots of different concentrations (1.14-833.33 µg/ml) and rutin (0.0011-0.833 µg/ml) were added to 1 ml of egg lecithin in phosphate buffer, while control was without test sample. Lipid peroxidation was induced by adding 10 µl FeCl3 (400 mMol) and 10 µl L-ascorbic acid (200 mMol). After incubation for 1 h at 37°C, the reaction was stopped by adding 1 ml of 15 % trichloroacetic (TCA) and 1 ml of 0.375 % thiorbarbituric acid (TBA) in 20% acetic acid and the reaction mixture was boiled for 15 min then cooled and centrifuged. The absorbance of the supernatant was measured at a wavelength of 532 nm.

Cell culture
All the cell lines were cultured as previously described by Ham et al., 2012 [26]. The Jurkat cell lines were cultured in RPMI 1640, the MCF-7 in a DMEM medium and the Chang liver cell lines in EMMEM medium. All culture media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin L-glutamine (PSSG) (Sigma-Aldrich, Illinois, USA). The cells were maintained in a humidified incubator with 5 % CO2 at 37°C. The cells were harvested and plated either for cytotoxicity tests or subcultures when they have reached about 80 % confluence. The DPPH reagent and BHT were dissolved in methanol to a concentration of 1 mg/ml
and MTT dye was dissolved in phosphate-buffered saline (PBS) to the same concentration.

**In vitro** antiproliferative activity of ethyl acetate fraction

**Cytotoxic (MTT) assay**

The tetrazolium-based colorimetric assay (MTT) was employed to determine the effect of the ethyl acetate fraction of *S. nodiflora* (EAF) on the cell growth and viability of cancerous and normal cell lines as described previously by Approy et al, 2015 [27]. The antiproliferative effects of this fraction on leukemia (Jurkat), breast cancer (MCF-7) and normal liver (Chang’s liver) cell lines were recorded. The cells were plated at 1.0×10³ cells per well in 96-well plates and incubated for 24 h. The cells were then incubated with EAF (6.25, 125, 250, 500 and 1000 μg/ml) or curcumin (1.15, 2.3, 4.6, 9.2, and 18.4 μg/ml) for 72 h. Curcumin was dissolved in DMSO. After incubation, 20 μl of MTT solution (2.5 mg/ml) was then added to each well and the plates were further incubated for 4 h. Insoluble purple formazan crystals formed were dissolved by adding 150 μl of acidic isopropanol and incubating the plates overnight. The absorbance was taken at a wavelength of 570 nm using a microplate reader. The percentage of cell viability was calculated from the formula

\[
\% \text{ Cell viability} = \frac{A_0 - A_1}{A_0 - A} \times 100
\]

Where \(A_0\) = Mean absorbance of control wells; \(A_1\) = Mean absorbance in test wells and \(A\) = Mean absorbance of blank wells.

The concentration of the ethyl acetate fraction causing 50% inhibition of the cancer cell growth was considered as IC₅₀. This was obtained from a plot of percentage cell viability versus concentration of sample tested.

**Statistical analysis**

The IC₅₀ (concentration responsible for 50% of the maximal effect) and inhibitory effects of drugs were analyzed by using an iterative computer least squares method, GraphPad Prism for Windows version 5.0 (GraphPad Software, San Diego, CA, USA) with the following nonlinear regression (four-parameter logistic equation).

\[
Y = \frac{a + (a - b)}{1 + 10^{(\log ED_{50} - X \times \text{Hill Slope})}}
\]

Where \(X\) is the logarithm of concentration. \(Y\) is the response and \(a\) and \(b\) with a sigmoid shape.

The fitted midpoints (IC₅₀) of the curves were compared statistically using the *F* test [28, 29]. *P*<0.05 was considered statistically significant in all analyses (one or two-way ANOVA followed by an appropriate post hoc test). The graphs were plotted using Sigma Plot for Windows Version 11.0 (Systat Software Inc., Germany).

**RESULTS**

**Phytochemical analysis**

The 70% hydro-ethanolic extract of *S. nodiflora* (SNE) showed the presence of the following compounds: flavonoids, tannins, saponins, alkaloids, cardiac glycosides, coumarins, triterpenes, steroids, anthraquinones and phenols. Similar constituents were also observed in the ethyl acetate fraction (EAF).

**Fractionation of 70% hydro-ethanolic extract of *S. nodiflora* (SNE)**

The successive fractionations of the 70% hydro-ethanolic extract of *S. nodiflora* resulted in the extract yields: dichloromethane (DCMF) (0.32%), 70% hydro-ethanolic extract (1.15%), n-hexane (NHF) (0.68%), ethyl acetate (EAF) (2.00%), petroleum ether (PEF) (2.79%), acetone (ACF) (14.74%), and aqueous (AQF) (78.82%). The highest amount of constituents remained in the aqueous fraction.

**DPPH scavenging effect of the extract and fractions of 70% hydro-ethanolic extract of *S. nodiflora* (SNE)**

DPPH analysis showed that SNE exhibited a concentration-dependent free radical scavenging activity (fig. 1), with an IC₅₀ of 114.3±0.01 μg/ml in the DPPH scavenging assay (table 1). This was significantly lower than ascorbic acid (AA). Table 1 shows that the fractions with significant DPPH scavenging effects (with regards to the IC₅₀ values) were EAF and DCMF. EAF was four times lower in activity than ascorbic acid.

![Fig. 1](image-url)

**Table 1: IC₅₀ (μg/ml) values of DPPH free radical scavenging assay of crude extract and fractions of *S. nodiflora***

|          | SNE   | AqF     | AAF     | ACF     | EAF     | DCMF    | NHF     | PEF     | AA      |
|----------|-------|---------|---------|---------|---------|---------|---------|---------|---------|
| IC₅₀ (μg/ml)±SEM | 114.3±0.01 | 118.9±0.03 | 26.7±0.29 | 42.0±0.01 | 8.9±0.02 | 23.5±0.03 | 904.9±0.20 | 351.4±0.06 | 2.1±0.02 |

n=3, data given is mean ±SEM
Fig. 2: (A) Calibration curve of gallic acid (0.1-1.0 mg/ml) and (B) total phenolic substances present in various concentrations of SNE (0.1-3 mg/ml) expressed as gallic acid equivalent. Each point in A and columns in B represent the mean±SEM (n=3).

Fig. 3: (A) Total phenolic content of EAF (0.125-1 mg/ml) expressed as gallic acid equivalent and (B) total flavonoids content of EAF (0.125-1 mg/ml) expressed as catechin equivalents. Each point in A and B represent the mean±SEM (n=3).

Total phenolic content and total flavonoid content of 70 % hydro-ethanolic extract of S. nodiflora (SNE) and the ethyl acetate fraction (EAF)

The total flavonoid and phenol contents of SNE at a concentration of 1 mg/ml was 0.9 mg/CE/ml and 0.03 mg/GAE/ml respectively (fig. 1B and 2B). EAF, which was the fraction having shown the most potent ability to scavenge for DPPH, was further assessed for its total phenolic and flavonoid content. The fraction EAF showed a concentration-dependent increase in both total phenol (fig. 3A) and total flavonoid contents (fig. 3B). EAF at an approximate concentration of 0.1 mg/ml, had a total phenol content of 0.07 mg/GAE/ml (fig. 3A). The flavonoid content of the same concentration of EAF was 0.9 mg/CE/ml.

Reducing power and lipid peroxidation effects of EAF

Both BHT (the reference antioxidant) and the EAF exhibited a concentration-dependent reduction of Fe\(^{3+}\) to Fe\(^{2+}\), resulting in a concentration-dependent increase in absorbance (fig. 4). BHT was found to be about 1000 fold more potent than EAF (table 2). Also, both rutin and EAF exhibited concentration-dependent inhibition of lipid peroxidation, as seen in fig. 5, however as seen with the IC\(_{50}\) values, EAF is about 3-fold less potent than rutin (table 2).

| Table 2: IC\(_{50}\) values (µg/ml) of EAF and reference antioxidant drugs in reducing power and lipid peroxidation assays |
|---------------------------------------------------------------|
| **Reducing power** | **Lipid peroxidation** |
|---------------------|------------------------|
| EAF                 | 151.1±0.04             | 24.01±0.08 |
| BHT                 | 0.15±0.02              | -          |
| Rutin               | -                      | 8.38±0.04  |

n=3, data given is mean±SEM
Fig. 4: Reducing power effects of EAF (1.14-833.33 µg/ml) and BHT (0.001-10 µg/ml). Each point represents the mean±SEM (n = 3)

Fig. 5: Effects of the inhibition of lipid peroxidation by EAF (3.906-1000 µg/ml) and rutin (3.125-100 µg/ml). Each data point represents the mean±SEM (n = 5)

Cytotoxicity of ethyl acetate fraction (EAF) on cell lines

Both curcumin (the reference compound) and EAF exhibited concentration-dependent inhibition of the cell growth of Chang’s liver (Fig. 6), Jurkat (Fig. 7), and MCF-7 (Fig. 8) cell lines. The rank order of potency, as defined by the IC50 (Table 3), showed that EAF was about 114-fold less cytotoxic to normal human (Chang’s liver) cells than curcumin. The EAF also showed 187-fold less cytotoxicity than curcumin on the Jurkat cells and 57-fold less than curcumin on the MCF-7 cells (Table 3).

Table 3: IC50 (µg/ml) values of EAF and curcumin on MCF-7, Chang’s liver and Jurkat cell lines

|          | MCF-7    | Chang’s liver | Jurkat    |
|----------|----------|---------------|-----------|
| EAF      | 205.2±0.14 | 252.2±0.14 | 170.9±0.02 |
| Curcumin | 3.56±0.06 | 2.20±0.03 | 0.91±0.05 |

n=3, data given is mean±SEM

Fig. 6: Effects of EAF (62.5-1000 µg/ml), and curcumin (1.15-18.4 µg/ml) on the cell growth of chang liver cells. Each data point represents the mean±SEM (n = 3)
Fig. 7: Effects of EAF (62.5-1000 μg/ml), and curcumin (1.15-18.4 μg/ml) on the cell growth of Jurkat cells. Each data point represents the mean±SEM (n = 3)

Fig. 8: Effects of EAF (62.5-1000 μg/ml), and curcumin (1.15-18.4 μg/ml) on the cell growth of MCF-7 cells. Each data point represents the mean±SEM (n = 3)

DISCUSSION

This study demonstrates the free radical scavenging, as well as the anti-lipid peroxidative effects of the hydro-ethanolic extract, and its solvent-extracted fractions in vitro. This result further supports the basic antioxidant activities of *S. nodiflora* which has already been published [5]. Antioxidants protect the body against various reactive oxygen species [30], which otherwise are linked to the etiology of several diseases. The phytochemistry of *S. nodiflora* revealed the presence of flavonoids, tannins, saponins, alkaloids, cardiac glycosides, coumarins, triterpenes, sterols, anthraquinones and phenols. This confirms earlier findings on the phytoconstituents present in similar extracts of the plant [31]. The phytochemicals of first-time identification for this extract are the cardiac glycosides and coumarins. Many of the secondary metabolites present in this plant have been found to possess antioxidant properties [32]. Many alkaloids, and polyphenols such as tannins and flavonoids, are known to be potent free radical scavengers [33-36]. Polyphenols are electron-rich compounds and can go into electron-donation reactions with oxidizing agents to form stable species [37], and thus inhibit or delay the oxidation of different biomolecules [25, 38]. Phenolic antioxidants are potent free radical terminators and this is thought to be due to the ability to donate hydrogen to free radicals, and their presence in *S. nodiflora* is a good marker of potential antioxidant activity [39, 40].

The DPPH test is valid for the primary characterization of the scavenging potential of compounds [41]. *S. nodifolia* extract’s ability to scavenge for DPPH concentration-dependently confirms earlier reports [5]. Fractionation showed an increase in this activity for the acetone, glacial acetic acid, dichloromethane and ethyl acetate fractions. These fractions most likely retained polar phenolic compounds that may possess antioxidant activities [42, 43]. However, the most potent fraction was the ethyl acetate fraction (EAF) with IC₅₀ of 8.9±0.02 μg/ml. This is about 4 times lower in activity than ascorbic acid. This suggests the need for further optimization, to isolate the antioxidant component from this fraction. Hence it was selected for further assessment for reducing power, lipid peroxidation and inhibition of the cancerous cells. At the concentration of 0.1 mg/ml, the total phenolic content per mg GAE/ml of the EAF was about ten times higher than that of the extract SNE; but the total flavonoid content was similar. This suggests that the antioxidant activity may likely be due to a non-flavonoid component of the extract. This implies that EAF, and other fractions may need to be optimized to determine the most potent fractions with regards to biological activities also.

Reducing power reflects the ability of an agent to convert iron (Fe²⁺) to the ferric (Fe³⁺) state. In principle, an agent having this reduction potential will react with potassium ferrocyanide (Fe³⁺) to form potassium ferrocyanide (Fe²⁺), which then reacts with ferric chloride to form ferric-ferrous complex [44]. Physiologically, iron in the ferric state reacts with peroxides to form free radicals that initiates further oxidation of physiological molecules. Lipid peroxidation has been implicated in several diseases, conditions and ageing; atherosclerosis, cataract, rheumatoid arthritis, and neurodegenerative disorders [45]. Antioxidants that inhibit lipid peroxidation may thus have a potential role in the prevention of such diseases. Four main mechanisms associated with antioxidant defense against lipid peroxidation include: prevention of the formation of active oxidizing agents, scavenging quenching and removal of active oxidants, repair of damage and excretion of products of toxic oxidation, and adaptive responses [45]. EAF inhibited the peroxidation of egg lecithin in a concentration-dependent order which may be attributed to the presence of antioxidant phytoconstituents.

EAF produced anti-proliferative activity against breast cancer (MCF-7), and leukemia (Jurkat) cell lines. The MCF-7 cell lines are known
to possess progesterone and estrogen receptors and provides a proliferative response to estrogen [46-48]. Jurkat cells are immortalized line of T-lymphocyte cells which are used to determine the mechanism of the susceptibility of leukemic cancers to drugs [49]. Thus the ability of EAF to inhibit these cell lines suggests that it may have a potential use in breast and leukemic cancers. Several findings for example, have proven that phytoconstituents such as flavonoids have significant anticancer properties [50].

In this study, the extract had the highest cytotoxic effect on the breast cancer cell line. A recent report showed that the aqueous extract from S. nodiflora was able to inhibit the proliferation of onion root and shoot apical meristem and Wister rat bone marrow cells [8]. EAF had a very high IC50 (25.2±0.14 μg mL) hence it is less inhibitory to the human hepatocyte-derived cell line (Chang Liver cell) in comparison to curcumin [15]. This suggests that EAF has some selective killing potential on cancerous cells, while leaving the normal cells and this may warrant further investigation.

CONCLUSION

In summary, the ethyl acetate fraction from the hydro-ethanolic extract of the whole plant of Synedrella nodiflora possesses potent antioxidant activities and anti-proliferative activities against MCF-7, Jurkat, and Chang liver cells. These results may warrant further investigation for potential anti-cancer drug development.

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AUTHORS CONTRIBUTIONS

PA, EOB, RAO, and SPD participated sufficiently in the intellectual content, conception and design of this work. PA, RAO, MVLNC and RR performed the experiment, analysis and interpretation. PA and EOB drafted the initial manuscript and RAO, BBN and DOS made inputs and corrections. All authors read and made corrections to the finalised manuscript before submission.

CONFLICT OF INTERESTS

None

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