Research Paper

ANTIOXIDANT AND ANTICANCER STUDY OF *AGERATUM CONYZOIDES* AQUEOUS EXTRACTS

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Abstract

Ageratum conyzoides, an annual herb, has been used in folklore for managing of a wide range of diseases including cancer. However, the safety and effectiveness of this medicinal plant is poorly evaluated. The intention of this study was to evaluate the *in vitro* antioxidant and anticancer activities of aqueous leaf, flower, stem and whole plant extracts of *A. conyzoides* as well as the qualitative phytochemical constituents. The DPPH, Folin-Ciocalteau and glutathione assays were used to evaluate the plant's antioxidant potential. The *in vitro* 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was exploited for determination of *in vitro* anticancer activity against four selected human cell lines: leukemic (Jurkat), prostate (LNCap), breast (MCF-7), and normal prostate (PNT2). Curcumin was used as standard anticancer compound. The selectivity index (SI) for each extract was also estimated. The extracts scavenged DPPH in a dose dependent manner compared to the positive control, butylated hydroxy toluene (BHT). The leaf extract was the strongest scavenger of DPPH free radical, EC_{50} value of 0.091 ± 0.024 mg/ml, and also recorded the highest total phenol content of 1678.86 ± 40.67 mg/g GAE. However, glutathione was not detected in any of the crude extracts. The cytotoxicity exhibited by the aqueous leaf extract was weak on Jurkat cells with IC_{50} value of 408.15 ± 23.25 µg/ml, and inactive to the remaining cancer cell lines. Moreover, the other extracts showed weak or no activities on the cancer cell lines. All extracts were not cytotoxic to the normal prostate (PNT2) cell line. The leaf extract was selective against Jurkat cell lines with SI value of 2.5, a little higher than curcumin (SI = 2.3). The presence of tannins, saponins and terpenoids were concentrated in the leaf and flower extracts of the plant. The present investigations suggest that *Ageratum conyzoides* aqueous extracts possess remarkable antioxidant effects, and weak anticancer properties. The phytochemical compounds detected could be responsible for these properties. Further studies should be conducted to unravel the active chemical principle(s).
Key words: *Ageratum conyzoides; cytotoxicity; MTT; DPPH; selectivity index*.

List of Abbreviations: DPPH, 2, 2-diphenyl-1-picrylhydrazyl radical; BHT, butylated hydroxyl toluene; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OPT, O-phthaldehyde.

INTRODUCTION

Over the past two decades, medicinal plant products have gained attention as natures cure for human cancer, which is a major reason for human mortality globally [1], especially with a growing number of aging populations in the world. In 2012, 41% of the developed antitumor agents were either natural products or modified natural products [2]. Some of these have been correlated with antioxidant activities [2]. Antioxidants are one of the major plant products that play a role as anticancer agents through acting as reducing agents, hydrogen donators, and singlet oxygen and nitrogen quenchers that suppress the naturally produced free radicals and delaying oxidative stress-related reactions such as lipid oxidation [3].

Anticancer agents mainly exhibit a preventative or curative role in a damaged system [4]. Under normal conditions, the cells in which the DNA or other components are irreversibly damaged by various causes, undergo programed cell death (apoptosis), which is a self-destructive pathway according to the genetically encoded cell death-signal [4]. However, cancer cells, which are already irreversibly developed, obtain the capability to evade apoptosis by various ways [5]. The aim of anticancer agents is to trigger the apoptosis signaling system in these cancer cells which controls their proliferation [5].

Current research therefore seeks for novel natural products from most known medicinal plant sources that exhibit anticancer activities based on ethnomedicinal or ethnobotanical studies which reveal sacred prescriptions and folkloric beliefs inherited through generations. Curcumin, a historically acknowledged component of the Ayurvedic, Unani, and Siddha medicine, and indigenous to Southern and Southeastern tropical Asia, has extensively been reported to having promising anticancer activities [6]. As the major chemical component of turmeric (*Curcuma longa*), *in vitro* cell culture and *in vivo* animal studies have suggested its effectiveness to treating numerous types of cancers: breast, colon, kidney, liver, leukemia, prostate, rhabdomyosarcoma, and melanoma [7]. It has been reported to be cytotoxic to Jurkat cells and induce a caspase mediated apoptosis in these cells [8].

The Asteraceae family of plants has been employed for diverse beneficial purposes due to its wide distribution globally[9]. *Ageratum conyzoides* L. is a member of this family and is native to Central America, the Caribbean, Florida (USA), Southeast Asia, South China, India, Ghana (West Africa), Australia and South America [10]. It has been used in alternative medicine for the treatment of epilepsy, wounds, and also as an insect repellent [11]. The leaves are applied to burns, cuts and sore and throat infections [11]. Many different bioactive principles isolated and identified in *A. conyzoides* include chromenes, 1,2-benzopirone, 1,2-desifropirrolizidinic and licopsamine[12-14]. To the best of our knowledge, no studies have evaluated and published the *in vitro* antioxidant and anticancer activities of the plant using crude aqueous extracts. The focus of this research was to access the *in vitro* antioxidant and anticancer activities of crude aqueous extracts of the leaf, stem, flower and whole plant of *Ageratum conyzoides*. Qualitative phytochemical constituents of the crude extracts were also investigated.

MATERIALS AND METHOD
Cell lines and reagents
Human leukaemia-immortalized T lymphocyte (Jurkat), human androgen-sensitive prostate carcinoma (LNCap), human hormone-responsive breast carcinoma (MCF7) and normal prostate (PNT2) cell lines were obtained from RIKEN BioResource Center Cell Bank (Japan).

Curcumin (Sigma-Aldrich, Illinois, USA), was dissolved in dimethyl sulphoxide (DMSO) (Sigma-Aldrich, Illinois, USA). The 2,2- diphenyl-1-picryl hydrazyl (DPPH) free radical reagent (Sigma-Aldrich, Illinois, USA) and butylated hydroxytoluene (BHT) (Sigma-Aldrich, Illinois, USA) were dissolved in methanol (Sigma-Aldrich, Illinois, USA). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye (Sigma-Aldrich, Illinois, USA) was dissolved in phosphate buffered saline (PBS) (Sigma-Aldrich, Illinois, USA). Concentrated stock solutions were stored at 20°C until use.

Cell culture
The cell lines were cultured as described with slight modifications [15]. The Jurkat, LNCap, and PNT2 cells were cultured in RPMI 1640 medium (Sigma-Aldrich, Illinois, USA). MCF7 cell line was cultured in DMEM medium (Sigma-Aldrich, Illinois, USA). All culture media were supplemented with 1% penicillin streptomycin L-glutamine (PSG) (Sigma-Aldrich, Illinois, USA) and 10% fetal bovine serum (FBS) (Sigma-Aldrich, Illinois, USA). The cells were maintained in a humidified incubator with 5% CO_2 concentration at 37°C and sub-cultured on reaching about 90% confluency.

Plant material
Healthy plants of *A. Conyzoides* were handpicked from the main campus of KNUST, Kumasi in October, 2013. The plant was authenticated at the Department of Pharmacognosy, KNUST, Kumasi, Ghana by Dr. George Sam (taxonomist) and a voucher specimen (KNUST/HMI/2014/ WP005) was deposited in the herbarium for reference purpose. The plant was manually separated into whole plant and parts-stem, leaves and flower. These were then washed (three times) under running water, air-dried for two weeks, pulverized, and stored in air-tight   containers.

Extraction
One hundred grams each of pulverized stem, whole plant and leaf as well as 50g of flower samples were decocted in distilled water by heating on a hot plate at 80 ºC for one hourand the mixture filtered by centrifugation. Subsequently, the water portion was frozen and later lyophilized using a vacuum freeze dryer (Labconco, England) to obtain the various crude extracts.

In vitro antioxidant activity
DPPH scavenging activity
The effects of crude extracts of *A. conyzoides* on the scavenging activity of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) were determined as earlier described [16] with slight modifications. The extracts were serially diluted in distilled water to obtain a concentration range of 0-1.25 mg/ml. The reaction mixture was made up of $100\mu L$ of 0.5 mM 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH), and 100 μL of each concentration of the test extract. Butylated hydroxytoluene (BHT) was used as standard compound at a concentration range of 0 - 0.14 mg/ml in methanol. The solvents, methanol and distilled water were used as blanks. Triplicate experiments were

performed. Negative control experiments were also included. The plates were covered, shaken gently and kept in the dark for 20 minutes after which the absorbance was read on a Tecan-PC infinite M200 Pro Plate reader (Austria), at the absorbance wavelength of 517 nm. Percentage DPPH scavenging activity was determined by;

$$\% \text{ Scavenging Activity} = [(OD_{Control} - OD_{TestSample})/OD_{Control}] \times 100 \quad (1)$$

Where $OD_{Control}$ is the mean absorbance of the wells containing negative control; $OD_{TestSample}$ is the mean absorbance of the wells with test sample or standard compound (BHT) that scavenged DPPH free radical.

The mean percentage scavenging (antioxidant) activity for the triplicate experiment was plotted against sample concentrations to obtain effective concentrations at 50% (EC_{50}) values. The latter is the test sample concentration required to scavenge 50% of DPPH radicals and was determined using nonlinear regression analysis.

Total phenolic content determination

The total phenolic content of crude extracts was determined as earlier described with slight modifications [17]. Two-fold serial dilutions were carried out on the standard compound (gallic acid), and on each crude extract to obtain concentration ranges of 0.156-5 mg/ml and 2.5-10 mg/mL, respectively. Absolute ethanol was used as blank for the gallic acid samples. Volumes of 10µL of each dilution were aliquoted into eppendorf tubes followed by the addition of 790 µL of distilled water and 50 µL of Folin-Ciocalteau reagent. The tubes were incubated in the dark at room temperature for 8 minutes, subsequently 150 µL of 7% sodium carbonate solution was added to each tube and incubation was continued for 2 hours in the dark at room temperature. Two hundred microlitres of each reaction mixture was aliquoted into wells on a 96-well plate in triplicate. Absorbance was read at 750 nm using microplate spectrophotometer. The total phenolic content of each crude extract of *A. conyzoides* was calculated from the regression equation of the gallic acid calibration curve ($y = 0.0134x - 0.0003$, $R^2 = 0.9986$) and expressed as gallic acid equivalents (GAE).

Quantification of glutathione

The procedure described earlier by [16] was adopted for the determination of the amount of glutathione in each crude extract of *A. conyzoides* with slight modification. The extracts and GSH standard were serially diluted (2-fold) in distilled water and methanol, respectively, to obtain concentration ranges of 2.5–10 mg/ml and 0.003125-0.1 mM, respectively. The reaction mixture was made up of 180 µL of the GSH buffer of pH 8.0, 10 µL extract or GSH standard dilutions and 10 µL of 0.0075mM of O-phthaldehyde (OPT). Absolute methanol was used as blank for the GSH standard. Triplicate experiment was performed. The reaction mixture was incubated in the dark at room temperature for 15 minutes and fluorescence read at 412 nm using microplate spectrophotometer. The amount of glutathione in each extract was calculated from the regression equation from the GSH standard calibration curve ($y = 236471x + 3573.6$, $R^2 = 0.9876$).

In vitro cytotoxicity (MTT) assay

The *in vitro* cytotoxicity of the crude extracts was performed on Jurkat, LNCap, MCF-7 and PNT2 cell lines as described [18]. Cells in exponential growth were seeded into 96-well plates at a concentration of 10^4 cells/well. The cells were then treated with various concentrations of the crude extracts of *A. conyzoides* at a concentration range of 0–1000

μg/ml using distilled water as the solvent. Negative control (untreated) and positive control (curcumin) experiments were included. For curcumin, a concentration range of 0-100 μg/ml was used for cell treatment and DMSO (1%) was used as solvent. Culture medium was used as blank. A colour control plate was also setup for each test extract.

After 72 h of incubation at 37 ^{0}C, under 5% CO_2, in humidified atmosphere, 20 μL of 2.5 mg/mL of MTT was added to each well and the plates were kept in the dark for 4 h. Subsequently, 150 μL of acidified isopropanol was added to stop the reaction and also solubilize the formazan crystals formed. Absorbance readings were taken at 570 nm on a microplate reader after overnight incubation of the plates in the dark. Triplicate experiments were performed. Dose response curves were plotted as percentages of cell viability against concentration.

% Cell Viability = [(ODT_0-ODT_1)/(ODU_0-ODU_1)] × 100(2)

where ODT_0 is the average absorbance of wells treated with test extracts or curcumin (standard compound) for all cell lines; ODT_1 is the average absorbance of wells with curcumin or test extract control; ODU_0 is the average absorbance of wells with untreated cells (negative control) for all cell lines; ODU_1 is the average absorbance of wells containing blank (culture media only).

The inhibition concentration at 50% (IC_{50}) values, that is, concentration of test sample inducing 50% inhibition on all cell lines were determined from dose response curves by nonlinear regression analysis.

The selectivity index (SI), a measure of cytotoxic selectivity, was also calculated for the test samples. This was calculated as follows;

Selectivity index (SI) = <u>IC_{50} of test sample on normal prostate (PNT2) cells</u>(3)
                        IC_{50} of test sample on cancerous cell lines

where cancerous cell lines used were Jurkat, LNCap and MCF-7.

Samples with an SI greater than 2 were considered to have a good selectivity towards cancer cells [19].

Phytochemical analysis
The crude extracts were screened for the presence of alkaloids, tannins, terpenoids, saponins and flavonoids according to the methods described with slight modifications [20]. Briefly, the methods were as follows;

Terpenoids
One millilitre of absolute chloroform was added to 10 mg of each extract and standard, urosolic acid, and 1 ml of 0.1M sulphuric acid was subsequently added. A reddish brown colour at the interface was indicative of the presence of terpenoids.

Saponins
One millilitre of distilled water was added to 10 mg of each plant extract and shaken vigorously for 1 minute. A stable persistent froth indicated the presence of saponins.

Tannins
Ten milligrams of each extract and standard, gallic acid, was boiled with 2 ml of distilled water. The boiled extracts were centrifuge to obtain supernatant to which three drops of 0.1% $FeCl_3$ was added to each supernatant. A blue black colouration indicated the presence of tannins.

Alkaloids
Ten milligrams of standard, quinidine, and crude plant extracts were dissolved in 2 ml of acid alcohol (v/v). The solution was boiled for three minutes and centrifuged to

obtain supernatant. One millilitre of dilute ammonia was added to the supernatant. Subsequently, 2 ml of absolute chloroform was added and shaken gently to extract alkaloidal base. The chloroform fraction was then extracted with 2 ml of acetic acid. After adding four drops of Draggendorf's reagent to each extract and standard, a reddish brown precipitate indicated the presence of tannins.

Flavonoids

Two millilitres of dilute ammonia was added to 2 ml portions of aqueous supernatant of each plant extracts and standard, quercetin. Subsequently, 1ml of 0.1M sulphuric acid was added to the mixture. A yellow colouration disappeared on standing for 5 min indicates the presence of flavonoids.

Statistical analysis

Microsoft Excel for Mac Version 14.4.7 was used for the calculation of mean and S.D values of triplicate experiments and plotting of the DPPH dose response graphs. Mean EC_{50} and IC_{50} values were compared by one way ANOVA using GraphPad Prism 6.1 (San Diego, USA) and values with $P < 0.05$ were considered statistically significant. GraphPad Prism 6.1 (San Diego, USA) was also used in plotting the total phenol graph.

RESULTS
In vitro antioxidant activity
DPPH scavenging activity

All crude extracts scavenged DPPH free radical in a dose dependent pattern compared to the positive control (BHT), that is, increasing the concentration of the extract between 0-1.25 mg/ml greatly increased DPPH free radicals cavenging activity. Among the aqueous extracts, the leaf exhibited the strongest antiradical activity while the stem extract was the weakest compared to BHT (p = 0.0003) (Figure 1). The EC_{50} value of aqueous leaf extract was calculated to be approximately 0.09 ± 0.02 mg/ml.

Total phenolic content determination

The results of total phenolic content was calculated in terms of gallic acid equivalent (GAE) in mg/g crude extracts of *A. conyzoides*. The aqueous leaf extract had the highest amount of total phenols with value of 1678.86 ± 40.67 mg/g plant extract (in GAE) (p < 0.0001), and the order decreased as flower > whole plant > stem (Figure 2).

Determination of the tripeptideglutathione

The OPT method was used for the determination of the tripeptide glutathione in the crude extracts. However, glutathione was not detected in any of the test extracts.

In vitro cytotoxicity

The cytotoxic effect of the crude extracts of *A. conyzoides* was measured on all four cell lines; leukemic (Jurkat), prostate (LNCap), breast (MCF-7) and normal human prostate (PNT2) using the *in vitro* MTT assay. Table 1 shows that the leaf, flower and whole plant were weakly cytotoxic at a working concentration of 0-1000 μg/ml (p < 0.0001) compared to the standard compound, curcumin. Among them, the leaf extract exhibited the best cytotoxicity (IC_{50} = 408.15±23.25 μg/ml) in the leukemic cell lines. However,the leaf extract showed no cytotoxic effect (IC_{50} > 1000 μg/ml) to both LNCap and MCF-7 cells. Similar pattern was observed for the aqueous flower and whole plant extracts, but not for the stem extract (Table 1). The crude extracts were further tested on human normal prostate cell line (PNT2) to calculate their selectivity index (SI) values (Tables 1). The aqueous leaf extract exhibited the highest cytotoxic selectivity on Jurkat cell lines (SI=2.5), a little better, when compared to curcumin (SI=2.3). However, none of the

crude extracts as well as curcumin was selective (SI≤1) against the other cancer cell lines (that is, LNCap and MCF-7).

PHYTOCHEMICAL ANALYSIS

Comparing the aqueous crude extracts, the presence of terpenoids, tannins and saponins did not differ from each other (Table 2). Terpenoids, tannins and saponins were found to be most abundant in the leaf and flower extracts of *Ageratum conyzoides*. However, the presence of flavonoids and alkaloids could not be detected in any of the extracts.
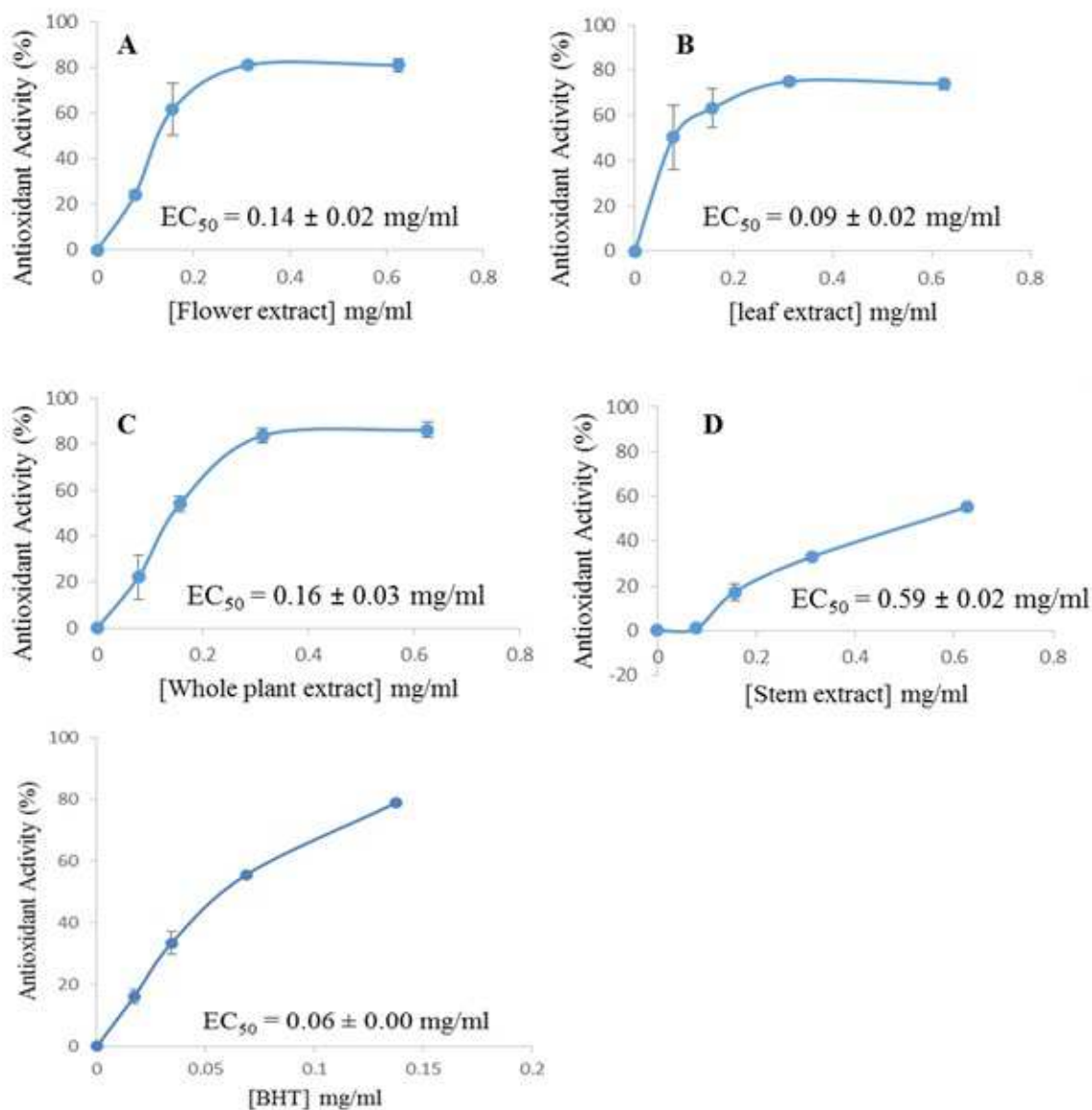

Figure 1. DPPH scavenging activity of water extracts of *A. conyzoides* and positive control, BHT. A concentration dependent experiment of DPPH free radical scavenging activity was evaluated after treatment with flower (A), leaf (B), whole plant (C), and stem (D) extracts (0 - 1.25 mg/ml) and BHT (E) (0 – 0.14 mg/ml). Each point represents mean ± SD of triplicate experiment.
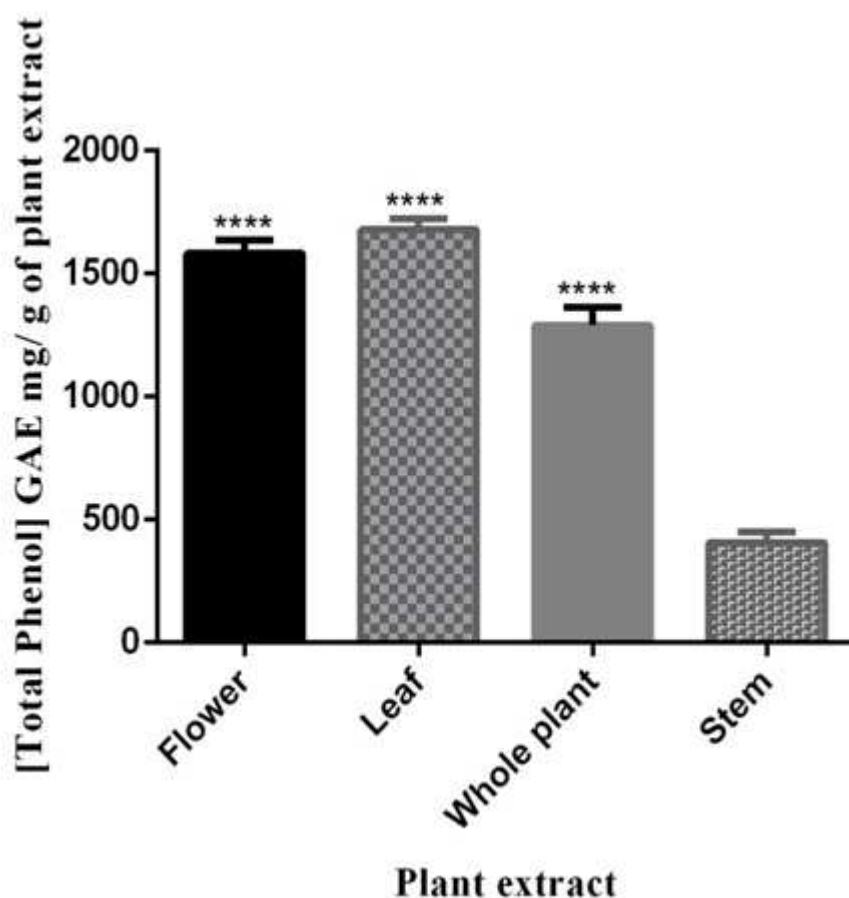
Figure 2. A graph of the concentration of total phenol in terms of gallic acid equivalence of aqueous extracts of *A. conyzoides.* The results are represented as mean ± SD of triplicate experiments. **** represent p < 0.0001 of flower, leaf and whole plant extracts versus stem extract.

Table 1. Cytotoxicity of water extracts of *A. conyzoides* to cancer cell lines

	Jurkat	LNCap	MCF-7
Flower	642.3±19.8(1.6)	>1000 (1)	>1000 (1)
Stem	>1000 (1)	>1000 (1)	>1000 (1)
Whole plant	608.2 ± 18.0 (1.6)	>1000 (1)	>1000 (1)
Leaf	408.2±23.3 (2.5)	>1000 (1)	>1000 (1)
p-value	< 0.0001[a]	< 0.0001[a]	< 0.0001[a]
Curcumin	0.002 ± 0 (2.3)	0.004 ± 0 (1.0)	0.01 ± 0 (0.3)

Values are means ± standard deviation, n=3, IC_{50} values for test samples are in μg/ml. SI values are in parenthesis, (P < 0.0001[a] represent significant differences in cytotoxicity of the different extracts compared to curcumin on all cell lines)

Table 2. Phytochemical constituents of water extracts of *A. conyzoides*

Phytochemical	Leaf	Flower	Whole plant	Stem
Terpenoids	+++	+++	++	+
Alkaloids	-	-	-	-
Tannins	+++	+	+++	+
saponins	+	+++	+	+
Flavonoids	-	-	-	-

(-) Absent; (+) Low concentration; (++) Moderate concentration; (+++) High concentration (Urosolic acid, quinidine, gallic acid and quercetin were standard compounds used for terpenoids, alkaloids, tannins and quercetin, respectively. All standards were scored as +++).

DISCUSSION

The search for novel natural antioxidants continues as geared by safety considerations, public's perception and risk reduction of chronic diseases by consumption of edible fruits and vegetables [21]. Antioxidants can protect the body by preventing the formation of free radicals through interruption of free radicals attack, or by scavenging the reactive metabolites or converting them to less reactive molecules [22].

Active compounds such as flavonoids, diterpenoids, triterpenoids, and alkaloids have been shown to be responsible for the cancer preventive effects of plants [23]. Also, several mechanisms of action responsible for the cancer preventive effects have been proposed. These include inhibition of mutagenesis by inhibiting metabolism, inhibition of DNA adduct formation, free-radical scavenging, and effects on cell proliferation and tumour growth through apoptosis signalling [24]. In the present study, the *in vitro* antioxidant and anticancer activities as well as phytochemical constituents of *Ageratum conyzoides*, using crude aqueous extracts of the plant, and its parts (leaves, flowers, and stem) were reported for the first time. Polyphenolic phytochemicals including flavonoids and phenols have been reported in *A. conyzoides* which has been suggested to contribute to its promising cytotoxic and antioxidant activity [25-27]. An earlier study by [10], reported that the water fraction of 95% ethanol leaf extract of *A. conyzoides* had the highest percentage DPPH scavenging activityfrom preliminary investigations. All the extracts tested scavenged the DPPH radical in a concentration dependent manner, with the leaf extract possessing the strongest DPPH scavenging potential while the weakest effect was observed in the stem extract. The presence of phenolic compounds was also observed in all extracts, with the leaf extract recording the highest content while the stem extract recorded the lowest. Studies conducted earlier on reported a high correlation between DPPH radical scavenging potential and total polyphenolics [28,29].This trend was observed for the present antioxidant investigations in all extracts. Moreover, to the best of our knowledge, the presence of the tripeptide glutathione has never been investigated in the crude extracts of *A. conyzoides*. However, glutathione was not present

in any of the water extracts investigated. This suggests that the presence of phenolic compounds significantly contributed to the observed total antioxidant activity.

Studies by [10] suggested that flavonoids may be responsible for the anticancer activity of *A. conyzoides*. Moreover, the same study reported that the water fraction of 95% ethanol leaf extract had no significant inhibitory effects on the various cancer cell lines tested. Results from this study demonstrates that the leaf extract of *A. con*yzoides had a better cytotoxic effect on leukemic (Jurkat) cell lines compared to the other extracts, despite the fact that this effect was weak. Interestingly, the cytotoxic effect on normal prostate cells (PNT2) by the crude extracts was very weak (IC_{50} values > 1000 µg/ml). This indicate that the water extracts were not cytotoxic to the normal cells. It is important for an anticancer agent to exhibit cytotoxicity but such activity should be specific for cancer cells only [30]. Only, the leaf extract was selective towards the Jurkat cell lines (SI>2), a little better than the standard compared curcumin. However, none of the water extracts as well as curcumin were selective against the LNCap and MCF-7 cell lines. High cytotoxic selectivity between cancer and normal cell lines increase the prospects that this plant contain compound(s) which could serve as leads for novel anticancer drugs.

Studies by [31] revealed the presence of alkaloids, flavonoids, tannins, saponins and cyanic acid in the pulverized samples of leaf, stem, flower and root of *A. conyzoides* and these secondary metabolites were concentrated in the leaf and flower samples. In our present investigations, the presence of tannins, terpenoids and saponins but not alkaloids and flavonoids were detected. These as well as phenolic compounds could partly be attributed to the observed antioxidant and anticancer activities.

CONCLUSION
The results of the present investigations has showed that aqueous leaf extract of *A. Conyzoides* possesses better antioxidant potential as compared to its anticancer property. However, further work is needed to unravel the active chemical principles responsible for the observed properties and also to ascertain their molecular mechanism of actions.

COMPETING INTERESTS
The authors declare none.

AUTHORS' CONTRIBUTION
FA: experimental design, crude extracts creation, data and statistical analyses, carried out some of the *in vitro* assays and drafted the manuscript; CL conceived of the study, and participated in its design, statistical analysis and writing of manuscript, RAO participated in the design of study, coordination, data analysis and helped to draft the manuscript, FKNA participated in the design of study, coordination, and helped draft the manuscript, IT performed the cell culture experiments, some of the *in vitro* assays and data analysis. All authors read and approved the final manuscript.

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