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RESEARCH ARTICLE

PHYTOCHEMICAL SCREENING, ANTIOXIDANT PROPERTY AND ANTICANCER POTENTIAL AGAINST MCF-7 CELL LINES OF *VERNONIA CINEREA* LEAVES

***Varsha, V., Dr. Suresh, S. N. and Prejeena, V.**

Department of Biotechnology, Sree Narayana Guru College, K. G. Chavadi, Tamil Nadu- 641105

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ABSTRACT

To screen the phytochemicals present in petroleum ether, ethanol and aqueous extracts of leaves of *Vernonia cinerea* and to evaluate its antioxidant potential and anticancer activity against breast cancer cell lines. Extracts were prepared by allowing the leaf powder to react with respective solvents with continuous agitation; it is then filtered and condensed. The extracts are then screened for its phytocompounds by preliminary screening methods. Antioxidant activity was detected by DPPH, FRAP and Total Antioxidant Activity Assays Ethanol extract shown the presence of more phytochemicals. Petroleum ether, ethanol and aqueous extracts exhibited radical scavenging property at different concentrations of 10, 20, 40, 60, 80 and 100µg/mL. The extracts were tested for their anticancer potential against MCF7 breast cancer cell lines and IC50 values were calculated. Ethanol and Aqueous extracts has shown anti- cancer property. The leaves of V.cinerea thus found to possess antioxidant and anticancer properties.

INTRODUCTION

Nature has been gifted with valuable sources of medicinal agents. From ancient times medicinal plants are considered to be important source of chemical substances with potential therapeutic effects (Chandra, 2013). Traditional medicinal system should play a greater role in modern health care system. Natural medicines are more acceptable to human body with fewer side effects when compared with modern synthetic drug system. Recent scientific studies have revealed the existence of good correlation between the traditional or ancient application of some of the plants further strengthens the search for bioactive plant compounds (Egharevba and Kunle, 2010). Plants have the ability to synthesize secondary metabolites, which were found to be source of certain phytochemicals that could be used as intermediates for novel drug production. Plants are fundamental source for all other living organisms. Research in medicinal plants to reveal the chemical constituents help in novel therapeutic drug development. Many pharmaceutical industries are depended on plant source for production of medicinal compounds (Paul John *et al*., 2012). Bioactive compounds synthesized by plants possessing certain biological properties helps in the well being of human health. Plant phytoconstituents protects against various diseases. Major Phytocompounds present in plants are alkaloids, flavonoids, terpenoids and saponins. Detailed research has to be done to identify new plant compounds which could remediate various diseases (Milne *et al*, 1993). Antioxidant compounds present in medicinal plants plays major role in protection of health and reduce the risk of chronic disorders like cardiac diseases and cancer. Antioxidant compounds scavenge free radicals like peroxides, hydroperoxide or lipid peroxides and inhibit these mechanisms that lead to degenerative diseases (Tyagi *et al*, 2010). The extent of damage caused by free radicals might be mitigated through antioxidant rich medicinal plants. There are various methods to monitor the antioxidant activity of plants. Recently oxygen radical absorbance capacity assays and enhanced chemiluminescence assays have been used in the evaluation of antioxidant capacity. Cancer is a serious disease occurring more commonly in today's world. Cancer may occur at any age in any part of our body and is considered to be the second largest cause of mortality. In 2012 over 14.1 million cancer cases has been reported globally which causes about 14.6 % human deaths. Breast cancer is the main cause of mortality, which contributes one of the most common malignancies. In 2010, over 1.5 million women worldwide were estimated to be diagnosed with breast cancer (Nuria *et al*., 2013). Cancer can be characterized by uncontrollable multiplication of normal cells which turns to tumours thereby invade nearby cells or other body parts. Not all tumours are cancerous; some tumours do not spread to other parts and are not always dangerous. In developing world, rates of cancer patients are increasing due to life style changes and certain environmental conditions. Present treatment options like chemotherapy and radiotherapy available for cancer patients possess serious side effects and are found to have limited survival period. Hence an alternative natural product has to be developed for cancer treatment, prevention and well-being of patients. Medicinal plants are magical boxes with various compounds that are biologically active against various diseases. Certain medicinal plants are found to be having active phytocompounds that can treat or

Corresponding author: Varsha, V.,
Department of Biotechnology, Sree Narayana Guru College, K. G. Chavadi, Tamil Nadu- 641105.

prevent the growth of cancer cells. Plant secondary metabolites synthesized by medicinal plants possess the activity to act as free radical scavengers. Such compounds need to be explored by further studies and can be purified for drug development (David *et al*., 2015). *Vernonia cinerea* is an annual herb with flat topped arrays of numerous flower heads, each with pinkish ray florets and grows up to 120cm height. The plant possesses great medicinal value in diverse traditional usage in different nations. The whole plant is used in decoction or infusion to treat fever and eye infections. It has been used as remedy for spasms of the urinary bladder and strangury, also often combined with quinine to treat malaria. Seeds are used as a source for alexipharmic and anthelmintic drugs (Gunjan *et al*., 2011). The whole plant is used for kidney disorders, diarrhoea, eczema and menstrual pains. Juice of this plant is given to children to treat bed- wetting. Recently research is going on this plant as an aid to give up cigarette smoking. Natural products provide abundant opportunities for the formulation of novel drug. The compounds in plants are part of phytomedicines that can be derived from leaves, stem, bark, root, flowers and seeds. Analysis of these compounds provides insight into the biological activity of plants (Suresh *et al*., 2015). Thus the present study was done to screen for the available biocompounds present in petroleum ether, ethanol and aqueous extracts of *Vernonia cinerea* and to further analyse its antioxidant and anticancer property against MCF-7 breast cancer cell line.

MATERIALS AND METHODS

Collection of plant material

Fresh leaves of *Vernonia cinerea* were collected from different areas of Kerala. It was authenticated from Botanical Survey of India, Coimbatore.

Preparation of plant extract

Fresh leaves of *Vernonia cinerea* was washed thoroughly, shade dried and powdered. The plant powder was then kept in contact with petroleum ether, ethanol and distilled water separately in a stoppered container for a defined period with continuous agitation. The extract is then filtered, condensed and stored.

Phytochemical Screening

Test for Phlobtannins

To each plant extract 1% hydrochloric acid solution was added and boiled in water bath. Formation of red colour precipitate indicates the presence of phlobtannins (Abdul Wadood *et al.*, 2013).

Test for alkaloids

Dragendroff's test

Each extract was treated with Dragendroff's reagent. Formation of red precipitate indicates positive result.

Mayer's test: Each extract was treated with 2ml of Mayer's reagent. Formation of yellow coloured precipitate indicates the presence of alkaloids.

Test for proteins (ninhydrin test)

Each extract was treated with 2ml of 0.2% ninhydrin solution. Presence of violet colouration indicates amino acids and proteins.

Test for carbohydrates

Fehling's Test

Equal volume of Fehling A and Fehling B was mixed, 2ml of this solution was added to each extract and boiled. Formation of red brick precipitate at the bottom of the test tube indicates the presence of carbohydrates.

Benedict's Test

2ml of Benedict's solution was added to each extract and boiled. Formation of reddish brown precipitate indicates the presence of carbohydrates.

Iodine test

2ml of iodine solution was treated with each extract. Dark blue or purple coloration indicates the presence of carbohydrates.

Test for phenols

2ml of 2% ferric chloride solution was added to each extract. Blue green or purple coloration indicates the presence of phenols.

Test for flavonoids

Alkaline reagent test

Each extract was mixed with 2ml of 2% NaOH solution. Formation of intense yellow colouration turned colourless on addition of few drops of dilute acid indicates the presence of flavonoids.

Test for saponins

Foam test

Each extract was mixed with 5ml of distilled water and shaken vigorously. Formation of stable foam indicates the presence of saponins.

Froth test

Each extract was diluted with 20ml of distilled water and shaken for 15 minutes. Formation of 1cm layer of foam indicates the presence of saponins.

Test for glycosides

Liebermann's test

Each extract was mixed with 2ml of chloroform and 2ml of acetic acid. The mixture was cooled in ice. Concentrated sulphuric acid was carefully added. A colour change from violet to blue to green indicates the presence of glycosides.

Salkowski's test

2ml of chloroform was mixed with each extract. 2ml of concentrated sulphuric acid was added and shaken gently. Reddish brown colour indicates the presence of glycosides.

Test for steroids

Extracts was mixed with 2ml of chloroform and concentrated sulphuric acid. A red colour formed at the chloroform layer indicates the presence of steroids.

Test for terpenoids

2ml of chloroform was added to each extract and mixed well. 3 ml of concentrated sulphuric acid was added to each tube. Formation of reddish brown colour indicates the presence of terpenoids.

Determination of Antioxidant Activity

DPPH (1, 1- diphenyl-2-picrylhydrazyl) Radical scavenging activity

The hydrogen donating ability of *Vernonia cinerea* was examined in the presence of DPPH stable radical. One millilitre of 0.3 mM DPPH methanol solution was added to 1 mL extract (1000 µg/mL) at different concentration and allowed to react at room temperature. After 30 minutes the absorbance values were measured at 517 nm. Methanol solution was used as a blank and DPPH solution (1.0 mL, 0.3 mM) with 1 mL methanol served as negative control. Ascorbic acid (1000 µg/mL) was taken as the positive control. The capability to scavenge the DPPH radical was calculated using the following equation

$$\% \text{ inhibition} = \frac{A_{control} - A_{test}}{A_{control}} X\ 100$$

Where '$A_{control}$' was the absorbance of the control reaction and 'A_{test}' was the absorbance in the presence of the extract/standard. The mean values were obtained from triplicate analysis. The antioxidant activity of the extract was expressed as IC_{50}.

Ferric Reducing Antioxidant Power (FRAP)

FRAP reagent was prepared by mixing 25 ml of acetate buffer (500 mM/l) with 2.5 ml of tripyridyltriazine (TPTZ) (10 mM/l) and 2.5ml of ferric chloride (20 mM/l) solution. The reaction mixture contained 300 µl of freshly prepared FRAP reagent warmed to 37^{o} C, added to 10 µl of test along with 30 µl of water. Absorbance of this solution was taken at 593 nm, just after 4 min from the time of addition of FRAP reagent. An increase in absorbance indicated enhanced reducing potential (Iris *et al*, 1996). Quantitative calculation for each sample was done using an equation obtained from the standard curve of Fe^{++}-TPTZ.

Absorbance = 0.274 x µM of Fe^{++} + 0.114 [R^2 = 0.974]

Cell line

The human breast cancer cell line (MCF 7) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS). The cells were maintained at 37^{0}C, 5% CO2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

Cell treatment procedure

The monolayer cells were detached with trypsin-ethylene diamine tetra acetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium containing 5% FBS to give final density of 1x10^{5} cells/ml. One hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37^{0}C, 5% CO_2, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentrations of the test samples. They were initially dissolved in neat dimethylsulfoxide (DMSO) and an aliquot of the sample solution was diluted twice the desired final maximum test concentration with serum free medium. Additional four serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, resulting in the required final sample concentrations. Following sample addition, the plates were incubated for an additional 48 h at 37^{0}C, 5% CO_2, 95% air and 100% relative humidity. The medium without samples served as control and triplicates was maintained for all concentrations.

MTT assay

3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. After 48 h of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37^{0}C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The percentage cell viability was then calculated with respect to control as follows

% Cell viability = [A] Test / [A]control x 100
% Cell inhibition = 100 - [A] Test / [A]control x 100

RESULTS

Phytochemical Screening of *Vernonia cinerea*

The phytochemical screening of petroleum ether, ethanol and aqueous extracts of *Vernonia cinerea* revealed the presence of certain phytocompounds which is summarized in table 1. Petroleum ether extract shown the presence of alkaloids, tannins, saponins and glycosides. Alkaloids, phenols, tannins,

steroids, glycosides, flavonoids, carbohydrates and terpenoids were present in ethanolic extracts of *V. cinerea*. Alkaloids, phenols, saponins and phlobtannins are the compounds that were found to be present in aqueous leaf extracts.

Table 1: Phytochemical Screening of *Vernonia cinerea*

	Petroleum Ether	Ethanol	Aqueous
Alkaloids	+	+	+
Phenols	_	+	+
Tannins	+	+	_
Saponins	+	_	+
Steroids	_	+	_
Glycosides	+	+	_
Flavonoids	_	+	_
Carbohydrates	_	+	_
Proteins	_	_	_
Phlobtannins	_	_	+
Terpenoids	_	+	_

+ = presence of compound, - = absence of compound

Antioxidant Activity of *Vernonia cinerea*

DPPH Assay

Vernonia cinerea leaf extracts were checked for its radical scavenging activity by DPPH assay. The percentage of inhibition was calculated and graphs were plotted. The percentage inhibition of DPPH assay (Table 2) shows that ethanolic extract has more antioxidant capacity with an IC50 value of 56.96µg/mL. Ascorbic acid was used as the standard and the IC50 value was 30.12µg/mL (Graph 1&2).

FRAP Assay

Petroleum ether, ethanol and aqueous extracts of *Vernonia cinerea* leaves expressed ferric reducing ability with IC50 values of 39.56µg/mL, 49.15µg/mL and 50.19µg/mL. Standard used was Ascorbic acid and the IC50 value of 14.13µg/mL was observed (Table 3). When compared with standard, petroleum ether extract shown increased reducing capacity (Graph 3&4).

Total Antioxidant Activity

The total antioxidant activity was measured by using Quercetin as standard, where percentage inhibition of aqueous extracts of *V. cinerea* leaves showed high radical scavenging activity. The IC50 value of aqueous extract was 61.21µg/mL and that of standard was 44.31µg/mL (Table 4). Percentage of inhibition for the extracts were graphically represented (Graph 5&6).

Anticancer Activity of *Vernonia cinerea*

Petroleum ether, Ethanol and Aqueous extracts of *V. cinerea* leaves was screened for its anticancer activity against MCF-7 breast cancer cell lines. The inhibition was evaluated by MTT assay. Extracts showed increased inhibition with increasing concentration (Table 5). Petroleum ether doesn't exhibit much activity against the cell lines (Graph 7). Ethanol and aqueous extracts were found to have anticancer property (Graph 8&9) and the IC50 values were calculated. IC50 values for ethanol and aqueous extracts are 251.75µg/mL and 275.10µg/mL. The regression value obtained was 0.99µg/mL.

Table 2. Percentage inhibition of DPPH Assay

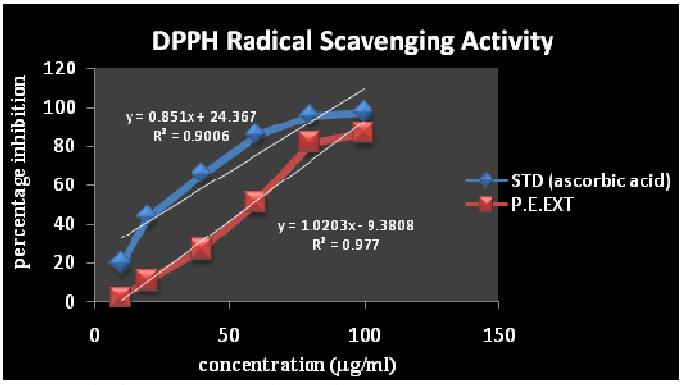
S.NO	Concentration	Petroleum Ether	Ethanol	Aqueous	Standard(Ascorbic Acid)
		% of Inhibition	% of Inhibition	% of Inhibition	% of Inhibition
1	10	2	3	4	20
2	20	11	20	2	44
3	40	27	25	28	66
4	60	51	53	46	86
5	80	82	76	68	96
6	100	87	92	94	98
	IC50 Value	58.21 µg/mL	56.96 µg/mL	61.00 µg/mL	30.12 µg/mL

Table 3. Percentage inhibition of FRAP Assay

S.NO	Concentration	Petroleum Ether	Ethanol	Aqueous	Standard(Ascorbic Acid)
		% of Inhibition	% of Inhibition	% of Inhibition	% of Inhibition
1	10	72	84	87	68
2	20	65	67	81	41
3	40	56	55	71	20
4	60	31	37	31	15
5	80	15	32	15	3
6	100	2	14	6	1
	IC50 Value	39.56 µg/mL	49.15 µg/mL	50.19 µg/mL	14.13 µg/mL

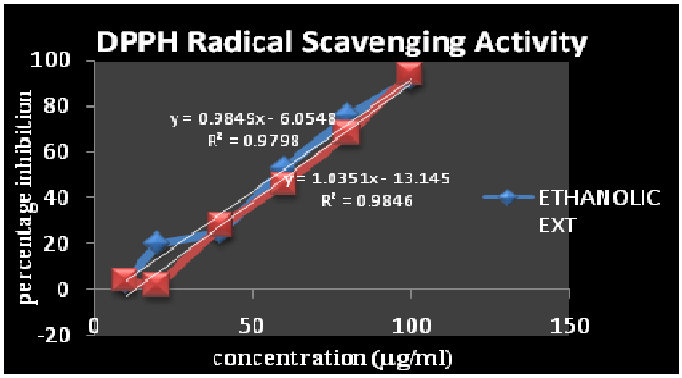
Table 4. Percentage inhibition of Total Antioxidant Activity

S.NO	Concentration	Petroleum Ether	Ethanol	Aqueous	Standard(Quercetin)
		% of Inhibition	% of Inhibition	% of Inhibition	% of Inhibition
1	10	85	26	57	40
2	20	72	12	38	10
3	40	65	10	25	32
4	60	21	33	34	41
5	80	4	53	21	47
6	100	11	66	8	62
	IC50 Value	81.55 µg/mL	81.71 µg/mL	61.21 µg/mL	44.31 µg/mL



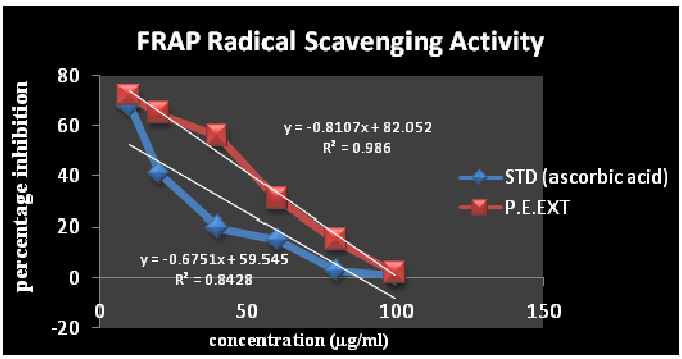
STD- Standard, P.E.EXT- Petroleum ether extract

Graph 1. DPPH radical scavenging activity of Ascorbic acid (standard) and Petroleum ether extract of V. cinerea leaves.



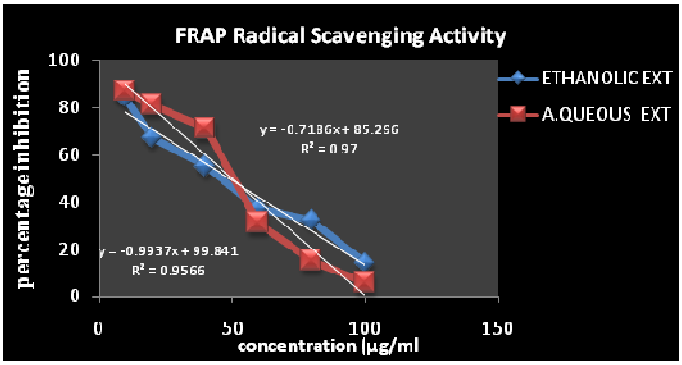
ETHANOLIC EXT- Ethanolic Extract, AQUEOUS EXT.-Aqueous Extract

Graph 2. DPPH Radical Scavenging activity of Ethanolic and aqueous extracts of *Vernonia cinerea*



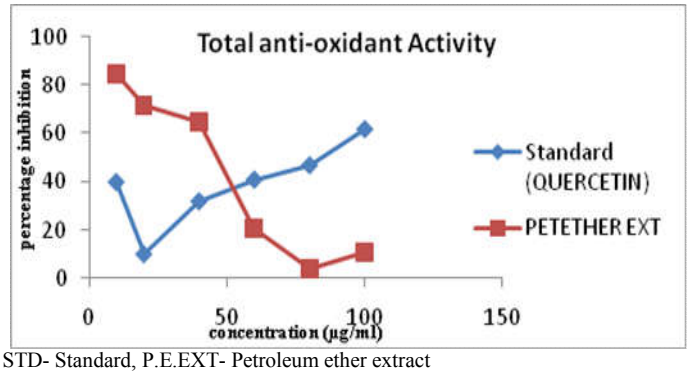
STD- Standard, P.E.EXT- Petroleum ether extract

Graph 3. FRAP radical scavenging activity of standard and petroleum ether extract of *Vernonia cinerea*



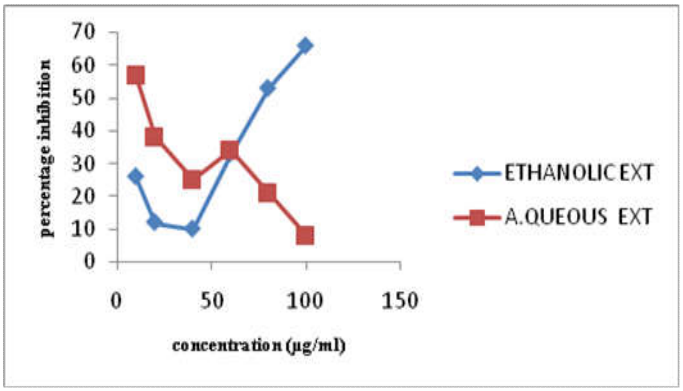
ETHANOLIC EXT- Ethanolic Extract, AQUEOUS EXT.-Aqueous Extract

Graph 4. FRAP radical scavenging activity of ethanol and aqueous extracts of *Vernonia cinerea*



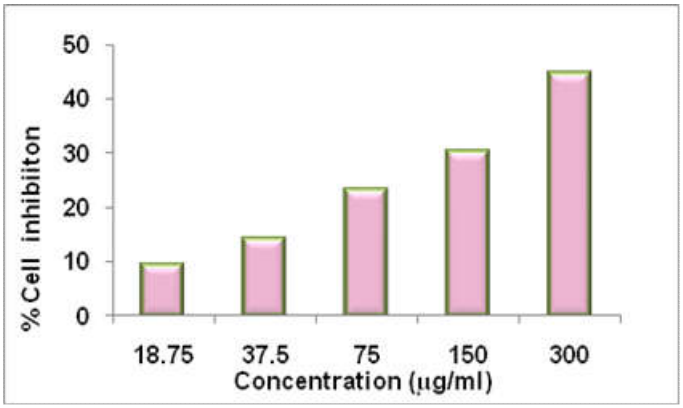
STD- Standard, P.E.EXT- Petroleum ether extract

Graph 5. Total Antioxidant Activity of Standard and petroleum ether extract of *Vernonia cinerea*

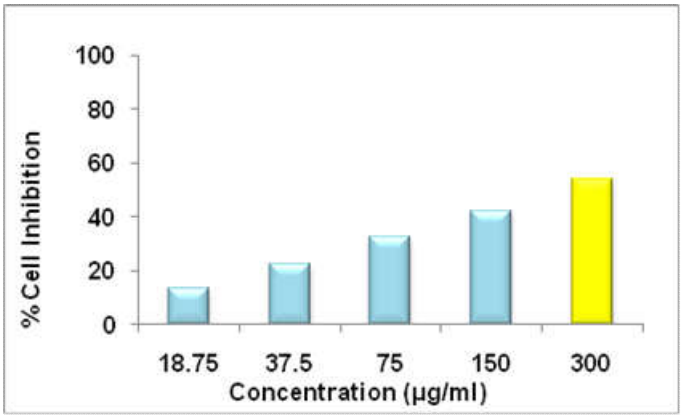


ETHANOLIC EXT- Ethanolic Extract, AQUEOUS EXT.-Aqueous Extract

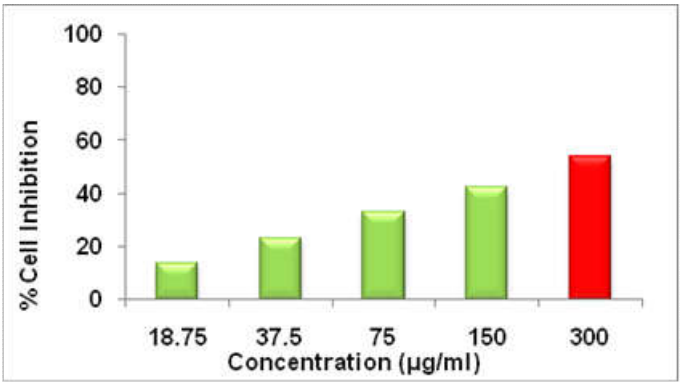
Graph 6. Total Antioxidant Activity of ethanol and aqueous extract of *Vernonia cinerea*.



Graph 7. Activity of Petroleum ether extract against MCF-7



Graph 8. Activity of Ethanol extract against MCF-7



Graph 9. Activity of Aqueous extract against MCF-7

Table 5. Percentage of inhibition against MCF-7 cancer cell lines

S.No	Concentration (µg/mL)	Petroleum ether	Ethanol	Aqueous
		% of Inhibition	% of Inhibition	% of Inhibition
1	18.75	8.961	13.383	12.238
2	37.5	13.975	22.660	20.844
3	75	22.937	32.846	30.477
4	150	29.924	41.966	42.163
5	300	44.413	53.809	51.559
	IC 50(µg/mL)		251.75	275.10
	R^2		0.99	0.99

DISCUSSION

Phytochemical Analysis

Plants are used medicinally and are sources of many potent drugs. Active principles of many drugs found in plants are secondary metabolites. Therefore, basic phytochemical investigation of the extracts for their major phytoconstituents is needed (Ugbabe .G. E *et al*., 2010). Preliminary phytochemical screening was done in this study to identify the compounds present in leaves of *Vernonia cinerea.* The extracts shown the presence of various phytocompounds where the ethanol extract revealed more phytocompounds like alkaloids, phenols, tannins, flavonoids, glycosides, steroids, carbohydrates and terpenoids (Table 1). Phytochemical screening is very important in identifying new sources of therapeutically important compounds like alkaloids, phenolic compounds, terpenoids, flavonoids, saponins, steroids, tannins etc. (Getahun *et al*., 2012). The present study revealed the presence of phytoconstituents present in the petroleum ether, ethanol and aqueous extracts of *V. cinerea* leaves.

Antioxidant Assays

Antioxidants are compounds that protect cells against the damaging effects of reactive oxygen species (ROS). Oxidative stress as a result of imbalance between antioxidants and ROS has been linked to cancer, ageing, artherosclerosis, inflammation and neurodegenerative diseases (Mohammed *et al*., 2012). Antioxidant activity of *Vernonia cinerea* leaves were evaluated by DPPH, FRAP and Total antioxidant activity assays. Petroleum ether, ethanol and aqueous extracts revealed their radical scavenging properties. Percentage of inhibition was calculated and tabulated (Table 2, 3 &4). Natural antioxidants are an interesting alternative in view of their interactions and numerous biological activities. Intensive research activities are currently being carried out on plant antioxidants (Pierre *et al*., 2015). In this study, the extracts exhibited their ability to act against free radicals.

Anticancer Activity

Breast cancer is the second leading cause of deaths among women. Medicinal plant extracts with phytocompounds, which are antioxidants can have important role in cancer prevention (Nuria *et al*., 2013). Anticancer potential of petroleum ether, ethanol and aqueous extracts of *Vernonia cinerea* was detected by MTT assay. The IC50 values were calculated (Table 5) .IC50 value is the measure of effectiveness of a substance in inhibiting a specific biological reaction or function. Cancer cell lines were used as *In vitro* model for the study of human hepatocytes. MCF-7 cells are found to be suitable models to study the intracellular trafficking because of their high degree of morphological and functional differentiation (Gandhiappan *et al*., 2012). In the study the anticancer potential of leaves of *V. cinerea* was checked on MCF-7 breast cancer cell lines. Ethanol and aqueous extracts exhibited anticancer activity.

Conclusion

The present study revealed the presence of phytocompounds in petroleum ether, ethanol and aqueous extracts of *Vernonia cinerea* leaves. Antioxidant activity of the leaves were confirmed by DPPH, FRAP and Total Antioxidant Activity assays. The anticancer potential of *V.cinerea* leaves were observed in ethanol and aqueous extracts. Further studies could be done to check for its potential against other cancer cell lines since the leaves of *Vernonia cinerea* exhibited antioxidant property.

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