PHYTOCHEMICAL SCREENING AND *IN-VITRO* ANTIOXIDANT AND CYTOTOXIC EFFECTS OF *IPOMOEA BILOBA*

ABSTRACT

Ipomoea biloba is a medicinal plant belonging to the Convolvulanceae family. It is an aquatic perennial runner plant used as a medical herb for various diseases such as asthma and rheumatism and dried leaves are used to apply for burns. In the present study, the Methanol extract of *Ipomoea biloba* leaves was experimented to evaluate the phytochemical properties, *invitro* antioxidant activity and *invitro* anticancer assay on MCF7 cell line. . Plant components can be used to extract both hydrophilic and lipophilic compounds. Methanol is an excellent solvent of its polarity. Because methanol is highly volatile, we can remove the solvent by distillation at a low temperature after extraction. The leaves of *Ipomoea biloba* extract used in an analysis which shows the ability to produced estrogen in the form of estradiol via estrogen receptor in the cell cytoplasm. Phytochemical screening showed the presence of carbohydrate, amino acids, alkaloids, saponin, steroids, terpenoids and phenols. The antioxidant activity of Methanol extract indicates the significant antioxidant content and it's compared with standard ascorbic acid. The results of this study highlight the interest of *Ipomoea biloba* extract for the isolation of anticancer molecules.

***Key words*:** Phytochemicals, Antioxidants, *Ipomoea biloba*, Cytotoxicity, Anticancer

INTRODUCTION

Ipomoea biloba is a semi-aquatic tropical plant. *Ipomoea biloba* was mainly located on the west coast of India, bounded by Arabian Sea. The plant growth was extensive on the sand dunes near the shore "cyanodon dactylon" a gramiane member was also found growing luxuriously Arabian Sea (22). In the West Indies, a weak leaf decoction is a remedy for asthma and rheumatism. It is also drunk daily in the last month of pregnancy to promote an easy delivery (2).

Phytochemicals are chemical compounds formed during the plants normal metabolic process. Phytochemicals could also exhibit other bioactivities such as antimutagenic,

anticancer, antioxidant, anticarcinogenic and anti- inflammatory properties (9).These chemicals are often referred to as "secondary metabolites (15).Most of the phytochemicals from plant source such as phenolics and flavoniods have been reported to have positive impact on health and cancer prevention (13). High content of phenolic and flavonoids in medicinal plants have been associated with their antioxidant activities that plays a role in the prevention of the development of age-related disease particularly cause by oxidative stress(3). The plant derived natural antioxidants which care in the form of raw extracts constituents are very efficient to block the process of oxidation by neutralizing free radicals (21). A free radical is an individual molecule with more than one unpaired electrons. Free radicals lead to antioxidant shield ingestion, which can cause cell function interference and oxidative problems for the membranes (1). Antioxidants showed a substantial role in safeguarding the body against reactive oxygen damage (5).

Cancer is a group of diseases that cause cells in the body to change and grow out of control. Cancer begins with a genetic defect. Human's genetic factors, meaning genes, are located within the cell structures called chromosomes. Cancer develops when mutations take place in genes that control a cell's normal functions but which are simply damaged (6). According to the report of the International Agency for Research on Cancer of the World Health Organization published in 2014, the global incidence of cancer has been approximately 14 million new cases and is projected to register 19.3 million in 2025 (12).The most type cancer found is breast cancer. More than 3.1 million US women with a history of breast cancer were alive on January 1, 2014. While some are being undergoing treatment. In 2015, approximately 40,290 women are expected to die from breast cancer (19). Plants have contributed lot of medicinal compounds being used today to treat diseases like cancer, Hormonal imbalances, jaundice, diabetes, inflammation etc., (17). Treatment with medicinal plants is considered very safe as there is no or minimal side effects (11).

MATERIALS AND METHODS

Plant Collection

Ipomoea biloba were collected in and around Salem District, Tamilnadu, India was identified and confirmed. In Tamil it was known as "Hadapan kodi".

Preparation of Plant Extracts

Fresh leaves were collected from the plants, washed and shade dried and powered. The powder was extracted using Methanol solvent by soxhlet apparatus. The residue was filtered and the solvent were evaporated under reduced pressure and stored for further studies. The extract was used for the determination of phytochemical constituents, *In-vitro* antioxidant and for *In-vitro* anticancer studies.

Quantitative Estimation of Phytochemical analysis

By utilizing following standard techniques as shown in **Table 1**, the leaf extracts were tested for presence of bioactive compounds:

Phytochemicals	**Test procedure**	**Observation**
Carbohydrates	Filtrate + Naphthol + Sulphuric acid	Violet colour
Proteins and Amino acids	Filtrate + Conc. Nitric acid	Yellow colour
	Filtrate + 0.2% Ninhydrin reagent	Purble colour
Flavonoids	2 ml extract + few drops of NaOH	Yellow color that clear on adding dil. HCL
Alkaloids	Filtrate + Mayer's reagent	Yellow coloured precipitate
Saponin	Filtrate + 2ml Water	Foam produced
Phytosterols	2 ml extract + 2 ml $CHCl_3$+2 ml H_2S0_4	Golden yellow colour
Glycosides	5 ml extract + 5 ml water shake	Foam produced
Terpenoids	5 ml extract + 2 ml chloroform and 3ml Conc. H_2S0_4	Reddish brown colour
Phenol and Tannins	Extract + 4 drops of $FeCl_3$	Blue-black coloration

The preliminary phytochemical screenings were carried out to identify the useful constituents present in the plant extract by standard method.

DETECTION OF CARBOHYDRATES

A minimum amount of the extract was suspended in 5 ml of distilled water. The suspension was subjected to the following chemical tests.

1. Molisch's test:

The extract was treated with 2-3 drops of 1% alcoholic alpha naphthol and 2 ml of concentrated sulphuric acid was added along the side of the test tube. The formation of purple ring between two layers shows the presence of carbohydrates.

2. Fehling's test:

The extract was treated with Fehling's A and B solution and heated for few minutes. Formation of brick red precipitate shows the presence of reducing sugar.

3. Benedict's test:

The extract was treated with Benedict's reagent and heated for few minutes. Formation of brick red precipitate shows the presence of reducing sugar.

DETECTION OF PROTEINS AND AMINOACIDS

1. Xanthoproteic Test:

The extracts are treated with few drops of conc. Nitric acid. Formation of yellow colour indicates the presence of proteins

2. Ninhydrin test:

About 0.5 mg of extract was taken and 2 drops of freshly prepared 0.2% ninhydrin reagent was added and heated. The appearance of pink or purple color indicates the presence of proteins, peptides or amino acids.

DETECTION OF FLAVONOIDS

The presence of flavonoids was determined by four methods.

1. Alkaline Test:

Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

2. Lead acetate Test:

Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.

3. Shinoda Tests: -

To 2-3 ml extract, few fragments of magnesium metal were added in a test tube, followed by drop wise addition of concentrate HCl. Formation of magenta colour indicated the presence of flavonoids.

4. Sulphuric acid test:

A fraction of the extract was treated with conc. Sulphuric acid and observed for the formation of orange colour.

DETECTION OF ALKALOIDS

A small quantity of the extract was treated with few drops of dilute hydrochloric acid and filtered. The filtrate was tested with alkaloid reagent such as:

1. Mayer's Test:

Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of a yellow coloured precipitate indicates the presence of alkaloids.

2. Wagner's Test:

Filtrates were treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids.

3. Dragendroff's Test:

Filtrates were treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide). Formation of red precipitate indicates the presence of alkaloids.

4. Hager's Test:

Filtrates were treated with Hager's reagent (saturated picric acid solution). Presence of alkaloids confirmed by the formation of yellow coloured precipitate.

DETECTION OF SAPONIN

1. Froth Test:

Extracts were diluted with distilled water to 20ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.

2. Foam Test:

0.5 gm of extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.

DETECTION OF TANNINS

Lead acetate test:

The extract was taken and 0.5 ml of 1% lead acetate solution was added and the formation of precipitate indicates the presence of tannins and phenol compounds.

DETECTION OF PHYTOSTEROLS

Small quantity of the extract was suspended in 5 ml of chloroform separately. The above obtained chloroform solution was subjected to the following tests.

1. Libermann Burchard's test:

The above prepared chloroform solution was treated with few drops of concentrated sulphuric acid. A bluish green colour solution obtained in chloroform extract shows the presence of phytosterols.

2. Salkowski test:

To 1 ml of the prepared chloroform solution, few drops of concentrated sulphuric acid were added. Formation of brown ring with chloroform extract indicates the presence of phytosterols.

DETECTION OF TERPENOIDS

1. Chloroform test:

5ml of extract was mixed with 2 ml of chloroform and 3 ml of concentrated H_2SO_4 was carefully added to form a layer. A reddish brown colouration of the inter face was formed to show positive results for the presence of terpenoids.

2. Libermann Burchard's test:

The above prepared chloroform solution was treated with few drops of concentrated sulphuric acid. A bluish green colour solution obtained in chloroform extract shows the presence of phytosterols.

DETECTION OF PHENOLS

1. **Phenols test:**

A positive reaction is the development of intense colour by the addition of ferric chloride solution to the plant extract solution.

2. **Ellagic Test:**

Plant extract is treated with few drops of 15% acetic acid and a few drops of 5% sodium nitrate solution appearance of muddy or niger brown colour indicates the presence of phenols.

Antioxidant Activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) Radical Scavening Activity

DPPH radical scavenging activity was carried out by the method of Molyneux 2004 (16). To 1 ml of 100 µm DPPH solution in Methanol, equal volume of the test sample in Methanol of different concentration was added and incubated in dark for 30 minutes. The change in coloration was observed in terms of absorbance using a spectrophotometer at 514 nm. 1 ml of Methanol instead of test sample was added to the control tube. Different concentration of ascorbic acid was used as reference compound. Percentage of inhibition was calculated from the equation:

[(Absorbance of control - Absorbance of test)/ Absorbance of control)] X 100. IC_{50} value was calculated using Graph pad prism 5.0].

Hydroxy Radical Scavenging Activity

The hydroxyl radical scavenging activity of the test sample was estimated according to the method of Halliwell *et al.*,1992.(10) The hydroxyl radical was generated by a fenton-type

reaction. The reaction mixture contained 2.0 ml of sample in varied0.1 ml concentration to which, 0.1 ml EDTA (1mM), FeCl3 (10mM) mixture, H2O2 (10mM), 0.36 ml deoxyribose(10mM), 0.33 ml phosphate buffer (50mM, pH 7.4) and 0.1 ml of ascorbic acid (1mM) was added in sequence.

The mixture was incubated at 37°C for 1 hr. To this mixture was added 1.0 ml each of TCA (10%) and TBA (0.67%) and kept in boiling water bath for 20 minutes. The colour developed was read at 532 nm. The control tube contains phosphate buffer, instead of sample. Different concentration of ascorbic acid was used as reference compound.

MTT Assay for Cell Cytotoxicity

Cell culture

MCF-7 (Human breast cancer cells) cell line were cultured in liquid medium (DMEM-(Dulbecco's Modified Eagle Medium) supplemented 10% Fetal Bovine Serum (FBS), 100 u/ml penicillin and 100 µg/ml streptomycin, and maintained under an atmosphere of 5% CO_2 at 37^{o}C.

Cell viability assay

The plant extract (*Ipomoea biloba*) was tested for *in vitro* cytotoxicity, using MCF-7 cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the cultured MCF-7 cells were harvested by trypsinization, pooled in a 15 ml tube. Then, the cells were plated at a density of 1×10^5 cells/ml cells/well (200 µL) into 96-well tissue culture plate in DMEM medium containing 10 % FBS and 1% antibiotic solution for 24-48 hour at 37°C. The wells were washed with sterile PBS and treated with various concentrations of the plant extract in a serum free DMEM medium. Each sample was replicated three times and the cells were incubated at 37°C in a humidified 5% CO_2 incubator for 24 h. After the incubation period, MTT (20 µL of 5 mg/ml) was added into each well and the cells incubated for another 2-4 h until purple precipitates were clearly visible under an inverted microscope. Finally, the medium together with MTT (220 µL) were aspirated off the wells and washed with 1X PBS (200 µl). Furthermore, to dissolve formazan crystals, DMSO-(Dimethyl sulfoxide) (100 µL) was added and the plate was shaken for 5 min. The absorbance for each well was measured at 570 nm using a micro plate reader (Thermo Fisher Scientific, USA) and the percentage cell viability and IC50 value was calculated using Graph Pad Prism 6.0 software (USA).

Statistical analysis:

The statistical analyses were carried out using Microsoft Excel 2010 and SPSS (16) soft wares. The results were expressed as means of three experiments ± standard deviation (SD).

RESULTS AND DISCUSSION

Preliminary Phytochemical Screening

The results of preliminary qualitative phytochemical analysis on leaves of Methanol solvent extract of *Ipomoea biloba* were showed in (Table 1).

Table 1: Preliminary phytochemical analysis of Methanol extract of *Ipomoea biloba*

S.NO	PHYTOCHEMICAL COMPOUNDS	PLANT EXTRACT
1.	Carbohydrate	+
2.	Protein	-
3.	Amino acids	+
4.	Flavanoids	-
5.	Alkaloids	+
6.	Saponin	+
7.	Steriods	+
8.	Terpenoids	+
9.	Phenols	+

Analysis of the plant extracts revealed the presence of phytochemicals such as phenols, tannins, flavonoids, saponins, glycosides, steroids, terpenoids, and alkaloids (4)**.** Plant derived natural products such as flavonoids, terpenoids and steroids etc. pharmacological properties including antioxidant and anticancer activity they possess biological properties such as antiapoptosis, antiaging, anticarcinogen, antiinflammation, antiatherosclerosis, cardiovascular protection and improvement of endothelial function, as well as inhibition of angiogenesis activities (18)**.** Saponins have the property of precipitating and coagulating red blood cells. Cholesterol binding properties and bitterness (7).

Different concentrations ranging from 25-200 µg/ml of the Methanol extract of leaves of *Ipomoea biloba* were tested for their antioxidant activity in different i*n-vitro* models. The percentage of inhibition was observed and found that the free radicals were scavenged by the test compounds in a concentration dependent up to the given concentration in all the models.

DPPH Radical Scavenging Activity

The activity of DPPH radical scavenging of the leaves extract was presented in Figure 1. The percentage of inhibition in DPPH in different concentration like respectively where as the percentage inhibition of ascorbic acid in concentration like 25, 50,75,100,200 µg/ml 32,45,55,68,72 respectively whereas the percentage inhibition of ascorbic acid in concentration like 25, 50,75,100, 200µg/ml were found to be 15, 20, 25, 42,54 respectively. The IC 50 values for DPPH scavenging activity for Methanol extract of leaves of *Ipomoea biloba* and ascorbic acid were 0.51 µg/ml and 0.93µg/ml respectively.

% scavenging = [Absorbance of control - Absorbance of test sample/Absorbance of control] X 100

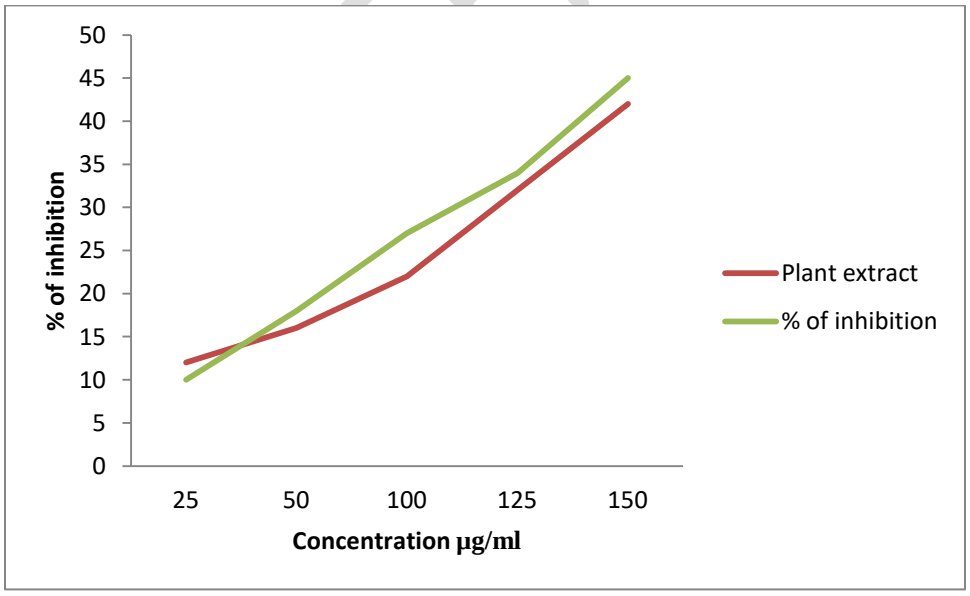


Figure 1: DPPH Radical Scavenging Activity of Methanol extract of leaves of *Ipomoea biloba*

Hydroxyl Radical Scavenging Activity

The hydroxyl radical scavenging activity of plant extract was presented in Figure 2. Hydroxyl radicals were scavenging in different concentration like 25, 50, 75, 100, 150µg/ml were observed in 12, 16, 22, 32, 42 respectively where as the percentage 10, 18, 27, 34, 45 inhibition of ascorbic acid in concentration like 25, 50, 75, 100, 150 µg/ml were found to be respectively. The IC50 values for hydroxyl radical scavenging activity Methanol extract of leaves of *Ipomoea biloba* and ascorbic acid were 0.99 µg/ml and 0.96 µg/ml respectively. Values are the average of *in-vitro* experiments and represented as mean ± standard deviation.

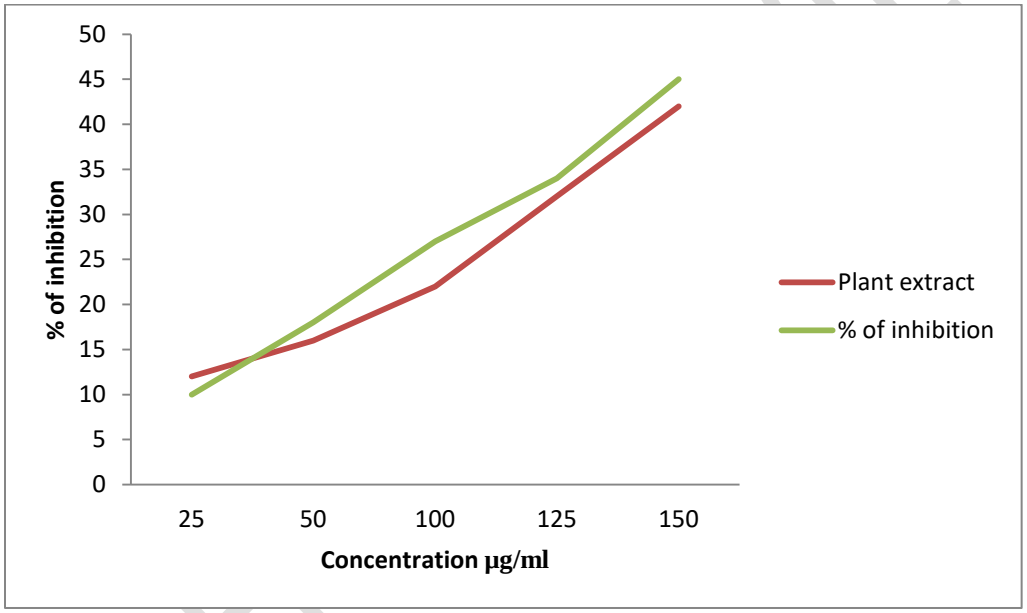


Figure 2: Hydroxyl Radical Scavenging Activity of Methanol extract of leaves of *Ipomoea biloba*

There are studies have been carried out to evaluate the antioxidant activity of *Ipomoea biloba* species using DPPH assay and reported that, particularly *Ipomoea biloba* exhibited higher antioxidant activity .Whereby the present study proof that, the leaves extract of *Ipomoea biloba* has the potential compound(s) react as antioxidant which is suitable to develop a drugs for the prevention of human disease related to free radical mechanism (20).

MTT Assay for Cell Cytotoxicity

The antiproliferative potential of the crude methanol extract of *Ipomoea biloba* leaves as well as its fractions against MCF-7 cells was assessed using MTT assay. To evaluate the impact of isolated extracts on tumor cell growth, human breast MCF7. This qualified them as invasive and, in general, resistant to conventional chemotherapeutics. Having all this in mind, it was intriguing to assess sensitivity of hormone-dependent (MCF-7) extracts of *Ipomoea biloba*, whose composition varied depending on the type of the solvent used in extraction procedure. Cells were cultivated with isolated extracts for 72 h when the number of viable cells in the culture was assessed by MTT and CV tests. As presented in Table 2 and Figure. 3. The results in table 2 showed that the methanol derived fraction had the most potent apoptotic cytotoxic activity with IC_{50}= 80.70*µg/*mL respectively.

Plant extracts displayed the different potential to decrease the number of viable cells depending on the cell type. In general, despite different origin and sensitivity to hormone stimulation of tested cell lines the strongest potential to decrease tumor cells' viability possessed Methanol extract. Breast carcinoma MCF7 displayed better response to treatment with Methanol extract. Differently, to tested cancer cell lines, Methanol extracts were not able to reduce the viability of normal cells for 50%. Since IC_{50} value was not reached upon the treatment with indicating extracts, it can be concluded that the intested range of doses they are non-toxic for normal phenotype and therefore, selective to malignant cells. Plenty of literature data confirmed that efficacy of herbal extract is defined by the mixture and interplay of its constituents. Despite the lack of scientific data about the anticancer features of ***Ipomoea biloba*** the panel of biologically active substances inside of it indicated its enormous potential in the field of anticancer therapy and needs of further evaluation.(23)

Table 2: IC_{50} values of effect *Ipomoea biloba* extracts in cytotoxicity

IC_{50} *µg/*mL^{-1}

Cellline	Assay	Methanol
MCF7	MTT	80.70 µg/ml
	CV	99.56% 10 µg/ml

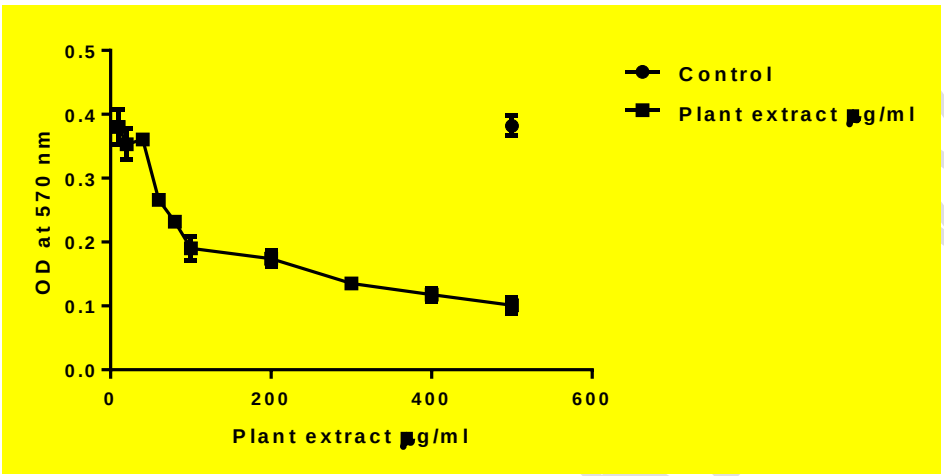
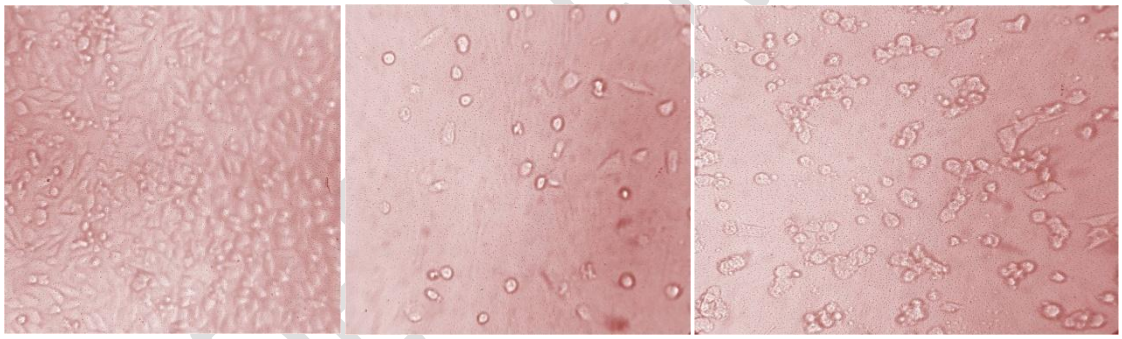
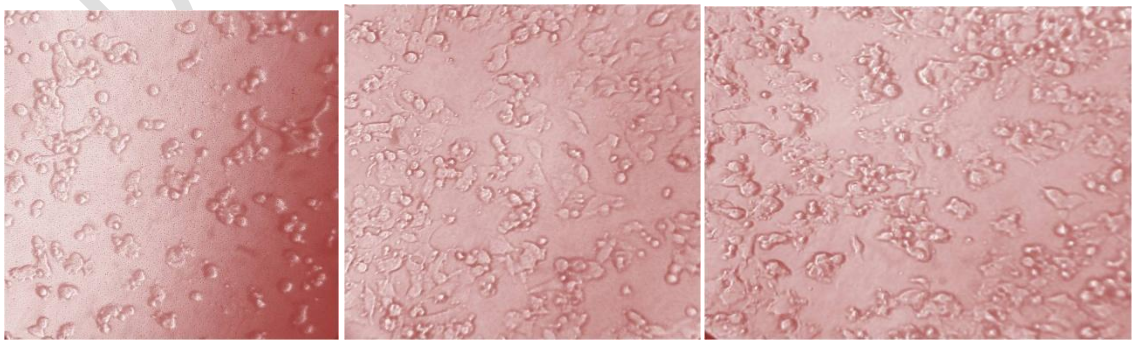


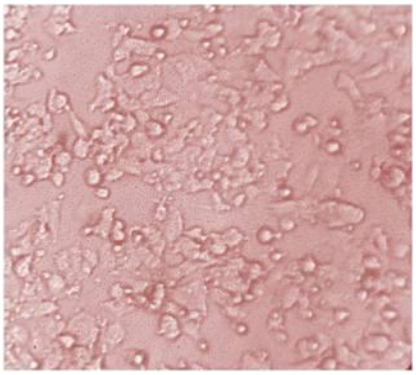
Figure 3: Effect of *Ipomoea biloba* extracts on cell lines.



Control cells **Plant extract 500 µg/ml** **Plant extract 100 µg/ml**



Plant extract 80 µg/ml **Plant extract 40 µg/ml** **Plant extract 20 µg/ml**



Plant extract 10 µg/ml

Plate 1: Morphological changes in MCF7 cells exposed to Various concentraton of Ipomoea biloba for 24 h. Images were taken using ban inverted phase contrast microscope at 205 magnification

MTT (3-4, 5 dimethylthiazol-2yl-2, 5-diphenyl tetrazolium bromide) assay, is based on the ability of a mitochondrial dehydrogenase enzyme of viable cells to cleave the tertrazolium rings of the pale yellow MTT and form a dark blue colored formazan crystals which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells (Plate 1). Solubilization of cells by the addition of detergents (DMSO) results in the liberation of crystals which are solubilized. The number of surviving cells is directly proportional to the level of formazan product created. The color can be quantified using a multi-well plate reader (Plate 1)).

MCF-7 cells are useful for ***in vitro*** breast cancer studies as a result of the cell line retaining several ideal characteristics particular to the mammary epithelium. These include the ability for MCF-7 cells to process estrogen in the form of estradiol via estrogen receptors in the cell cytoplasm. This results in the MCF-7 cell line being an estrogen receptor (ER) positive cell line. MCF-7 is also progesterone receptor positive and HER2 negative (8)..*In vitro* anticancer studies has suggested that an anticancer effect of *Ipomoea biloba* extracts is possibly due to inhibition of DNA replication in cancer cell lines. It also reported anticancer property of *Ipomoea biloba* (14).

CONCLUSION

Our results obtained in this study thus suggest the identified phytochemical compounds, antioxidant properties and anti breast cancer activity of hormone dependent cancer lines for this plant. The plant extract should be emphasized due to antioxidant and enzyme activity and plant contain bioactive constituents of substantial medicinal merit. *Ipomoea biloba* is a generally poorly studied plant, apart from its long and known traditional use world while. Plant extracts, indicated the justification of use of the plant in traditional medicine, mainly for woman related diseases due to hormone dependent anticancer activity (breast cancer), as well as having the antioxidant activity. Our results present a first report on anticancer activity of hormone dependent cancer lines, for this plant.

Hence, the above plant extract could be explored for its highest therapeutic efficacy by pharmaceutical companies in order to develop safe drugs for various ailments. Further studies are required to understand the mechanism(s) of action of these extracts on MCF7 cells

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