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Phytochemical Composition, *in-vitro* Antioxidant and Cytotoxic Effects (MCF-7) of *Ipomoea biloba*

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Ipomoea biloba is a plant, which belongs to Convolvulanceae family and also has more medicinal values. It is an aquatic creep 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 current investigation, the Methanol extract of *Ipomoea biloba* leaves was experimented to evaluate the phytochemical properties, *in vitro* antioxidant activity and *in vitro* 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 comes froth as estradiol via estrogen receptor in the cytoplasm of the cell. 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 it and against with standard ascorbic acid. The results of this analysis highlight the interest of *Ipomoea biloba* extract for the isolation of anticancer molecules.

Keywords: Phytochemicals; antioxidants; Ipomoea biloba; cytotoxicity; anticancer.

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1. 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 [1]. In the medicinal practice of West Indies, a leaf brew is used as remedy for asthma, rheumatism and also drunk daily during 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 [3]. These chemicals are often referred to as “secondary metabolites [4]. Most of the phytochemicals compounds from plant extract such as phenolics and flavoniods have been resulted to have positive impact on health and cancer prevention [5]. High level of phenolic and flavonoids presences in plant extract will prevent the oxidative stress, which causes age-related diseases [6]. 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 [7]. 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 [8]. Antioxidants showed a substantial role in safeguarding the body against reactive oxygen damage [9].

Cancer is a disease caused when cells divide uncontrollably and spread into surrounding tissues and grow out of control. Cancer is a genetic disease—that is, cancer is caused by certain changes to genes, located within cell structure called chromosomes that control the way our cells function, especially how they grow and divide. Cancer develops when mutations take place in genes [10]. According to the survey of the International Agency for Research on Cancer of the World Health Organization which has published in 2014, estimated that approximately 14 million new cases and forecasted to increase around 19.3 million in 2025 [11]. The most common is breast cancer. Particularly in US women 1.3 million are affected with breast cancer. While some are being undergoing treatment. In the survey of 2015, around 40,290 women are forecasted to die from breast cancer [12]. Medicinal compounds dervied from plant extract is used to treat many diseases like Cancer, migraine, ulcer, Hormonal imbalances, diabetes, inflammation etc., [13]. Treating the diseases with plant extract for medicinal purpose, which is very safe to the boby as there is no or minimal side effects involved [14].

2. MATERIALS AND METHODS

2.1 Plant Collection

The fresh leaves of *Ipomoea biloba* were collected in and around Salem District, Tamilnadu, India was identified and confirmed. In Tamil it was known as “Hadapan kodi”.

2.2 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.

2.3 Quantitative Estimation of Phytochemical Analysis

The preliminary phytochemical screenings were carried out to identify the useful constituents present in the plant extract by standard method. For preliminary phytochemical analysis, the freshly prepared Methanol extracts of *Ipomoea biloba* leaves were tested for the phytoconstituents by using standard phyto-chemical procedures [15].

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

Chart 1. List of phytochemicals

Phytochemicals	Test procedure	Observation
Carbohydrates	Filtrate + Naphthol + Sulphuric acid	Violet colour
	Filtrate + Conc. Nitric acid	Yellow colour
Proteins and Amino acids	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_2SO_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

2.4 Antioxidant Activity

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

The method of Molyneux [16] was used to test DPPH radical scavenging activity [16]. Equal volume of the test sample in Methanol of varied concentrations was added to 1 ml of 100 m DPPH solution in Methanol and incubated in the dark for 30 minutes. A spectrophotometer set to 514 nm was used to measure the colour shift in terms of absorption. Instead of test sample, 1 ml of Methanol was put 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].

2.4.2 Hydroxy radical scavenging activity

The hydroxyl radical scavenging activity of the test sample was estimated according to the method of Halliwell et al., 1992 [17]. 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), $FeCl_3$ (10mM) mixture, H_2O_2 (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.

2.5 MTT Assay for Cell Cytotoxicity

2.5.1 Cell culture

The MCF-7 (Human Breast Cancer Cells) cell line was grown in liquid media (DMEM-Modified Dulbecco's Eagle Medium) supplemented with 10% Fetal Bovine Serum (FBS), 100 u/ml penicillin, and 100 g/ml streptomycin, and kept at 37°C in a 5% CO_2 atmosphere.

2.5.2 Cell viability assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheny-ltetrazolium bromide (MTT) assay was used to test the plant extract (*Ipomoea biloba*) for in vitro cytotoxicity in MCF-7 cells. Trypsinization was used to collect the cultivated MCF-7 cells, which were then pooled in a 15 ml tube. The cells were then seeded at a density of 1105 cells/ml cells/well (200 L) into a 96-well tissue culture plate in DMEM medium containing 10% FBS and 1% antibiotic solution for 24-48 hours at 37°C in DMEM medium containing 10% FBS and 1% antibiotic solution. In a serum-free DMEM medium, the wells were rinsed with sterile PBS and treated with various doses of the plant extract. Each sample was tripled, and the cells were cultured for 24 hours at 37°C in a humidified 5 percent CO_2 incubator. 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 IC_{50} value was calculated using Graph Pad Prism 6.0 software (USA).

2.6 Statistical Analysis

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

3. RESULTS AND DISCUSSION

3.1 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	+

The Methanol extract of *Ipomoea biloba* plant shows the presence of phytochemicals such as phenols, Carbohydrate, Protein, Amino acids, tannins, flavonoids, saponins, glycosides, steroids, terpenoids, and alkaloids [18]. Extract contains natural compunds such as flavonoids, Phenols and steroids etc. Medicinal properties including antioxidant and anticancer activity and also contains biological properties such as antiinflammation, antiatherosclerosis, antiaging, anticarcinogen, cardiovascular protection and improvement of endothelial function and also inhibition of angiogenesis activities [19]. Saponins have various properties which involved in precipitating and coagulating red blood cells and also Cholesterol binding properties [20].

3.2 Antioxidant Activity

Concentrations ranging from 25-200 µg/ml of the Methanol extract of leaves of *Ipomoea biloba* were experimented for their antioxidant activity in various methods of in-*vitro* models. The percentage of inhibition was found that the free radicals were scavenged by the test compounds in a concentration dependent up to the given concentration in all the models.

3.3 DPPH Radical Scavenging Activity

The activity of DPPH radical scavenging of the leaves extract was presented in Fig. 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

3.4 Hydroxyl Radical Scavenging Activity

The hydroxyl radical scavenging activity of plant extract was presented in Fig. 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.

Analysis of various studies have been examined to evaluate the antioxidant activity of *Ipomoea biloba* species using DPPH assay and Hydroxyl Radical Scavenging Activity stated that, particularly *Ipomoea biloba* exhibited high concentration of antioxidant activity and also the current activity evidence that, the leaves extract

of *Ipomoea biloba* has the essential compound(s) react as antioxidant which the prevents the diseases related to free radical mechanism which is worth for the preparation of drugs [21].

3.5 MTT Assay for Cell Cytotoxicity

The MTT assay was used to assess the anti-proliferative potential of the crude methanol extract of Ipomoea biloba leaves, as well as its fractions, against MCF-7 cells. Human breast MCF7 cells were used to assess the effect of separated extracts on tumour cell proliferation. They were classified as invasive and, in general, resistant to standard chemotherapeutics as a result of this. With this in mind, evaluating the sensitivity of hormone-dependent (MCF-7) extracts of Ipomoea biloba, whose composition changed depending on the kind of solvent used in the extraction technique, was intriguing. MTT and CV assays were used to determine the number of viable cells in the culture after 72 hours of cultivation using isolated extracts. As presented in Table 2 and Fig. 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.

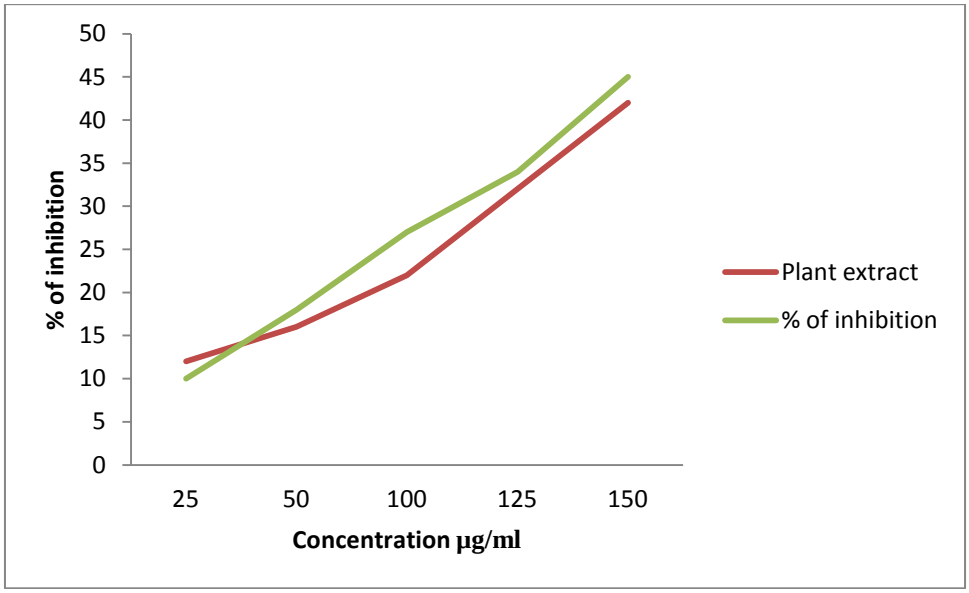


Fig. 1. DPPH radical scavenging activity of methanol extract of leaves of *Ipomoea biloba*

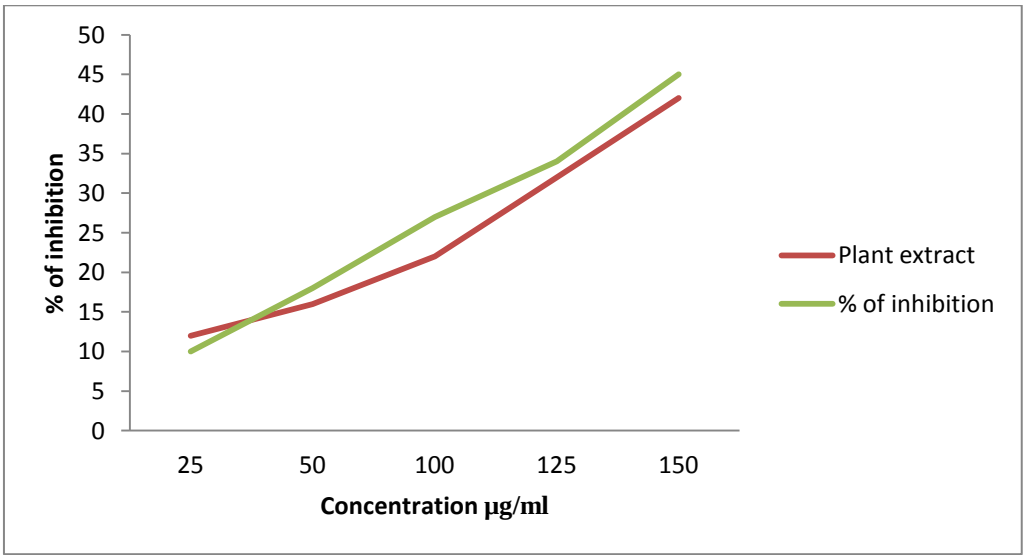


Fig. 2. Hydroxyl radical scavenging activity of methanol extract of leaves of *Ipomoea biloba*

Depending on the cell type, plant extracts had varying degrees of ability to reduce the number of viable cells. Despite the diverse origins and susceptibility to hormone stimulation of the cell lines studied, Methanol extract had the greatest capability to reduce tumour cell viability. Methanol extract therapy improved the responsiveness of MCF7 breast carcinoma. Methanol extracts, on the other hand, were unable to diminish the viability of normal cells by 50% in the tested cancer cell lines. Because no IC50 value was obtained after treatment with the indicated extracts, it may be concluded that the doses tested are non-toxic to normal phenotypic cells and hence selective to malignant cells. The efficacy of herbal extracts is characterised by the mixing and interplay of their elements, according to a large body of literature. Despite the absence of scientific data on Ipomoea biloba's anticancer properties, a panel of biologically active compounds found inside it suggested that it has significant potential in the field of anticancer therapy and warrants more investigation [22].

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)).

Table 2. IC_{50} values of effect *Ipomoea biloba* extracts in cytotoxicity IC_{50} *µg*/mL^{-1}

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

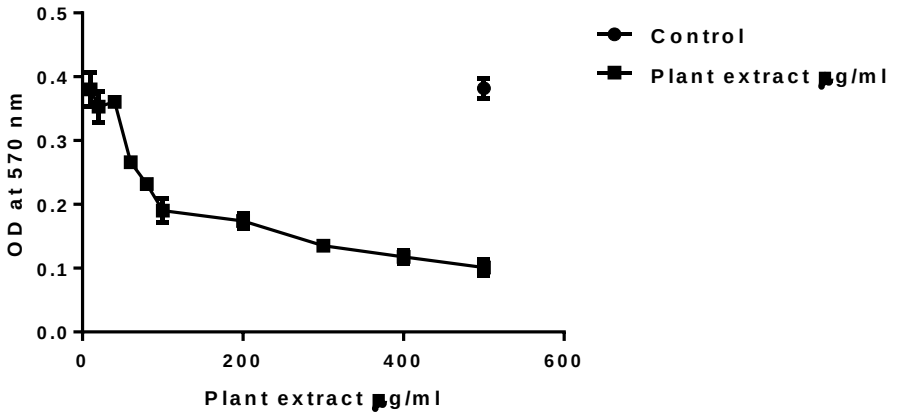
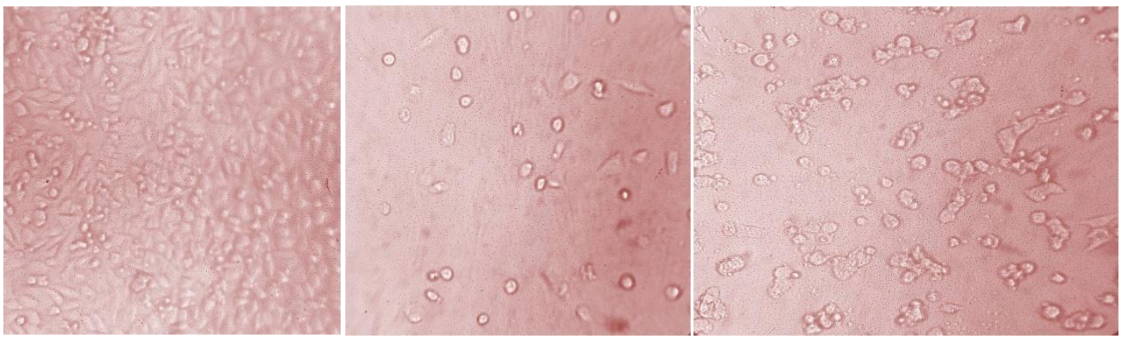
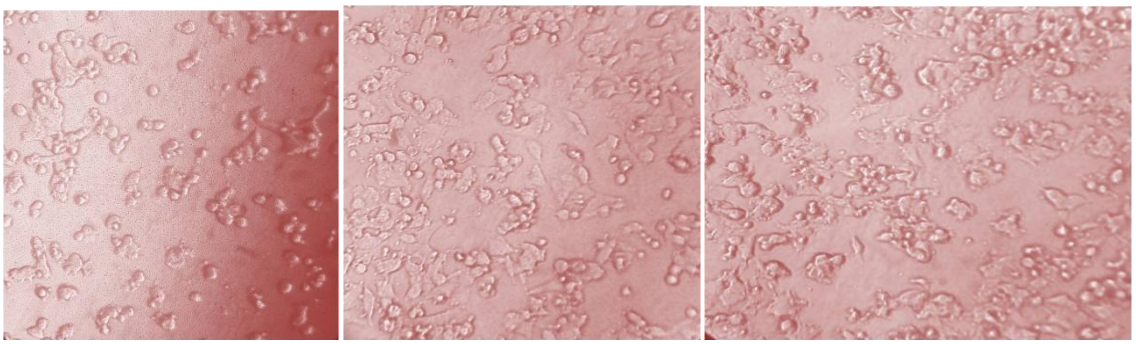


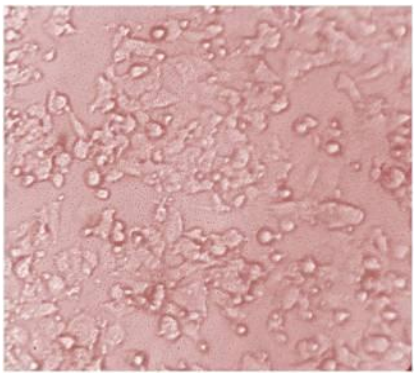
Fig. 3. Effect of *Ipomoea biloba* extracts on cell lines



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

MCF-7 cells preserve certain perfect properties specific to the mammary epithelium, they are suitable for in vitro breast cancer investigations. MCF-7 cells have the ability to metabolise oestrogen in the form of estradiol through oestrogen receptors in the cytoplasm. As a result of this, the MCF-7 cell line is oestrogen receptor (ER) positive. MCF-7 is also positive for progesterone receptors and negative for HER2 [23]. *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* [24].

4. CONCLUSION

From the results of this study, it is concluded that 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. Apart from its lengthy and well-known traditional use around the world, *Ipomoea biloba* is a plant that has received little attention. Plant extracts demonstrated the plant's validity for use in traditional medicine, particularly for women's disorders, due to hormone-dependent anticancer action (breast cancer) and antioxidant activity. Our findings are the first for this plant to show anticancer action in hormone-dependent cancer cells.

Pharmaceutical companies could thus investigate the aforementioned plant extract for its best therapeutic efficacy in order to manufacture safe medications for a variety of diseases. Further studies are required to understand the mechanism(s) of action of these extracts on MCF7 cells. Most anticancer drugs have been discovered through random screening of plant materials. This shows that the present research carried out paves a pathway for further pharmacological studies and isolation and novel components from the leaves of *Ipomoea biloba.*

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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