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ORIGINAL ARTICLE

Effect of Ethanolic Extract of *Excoecaria agallocha* Leaves on the Cytotoxic Activity and Cell Cycle Arrest of Human Breast Cancer Cell Lines – MCF-7

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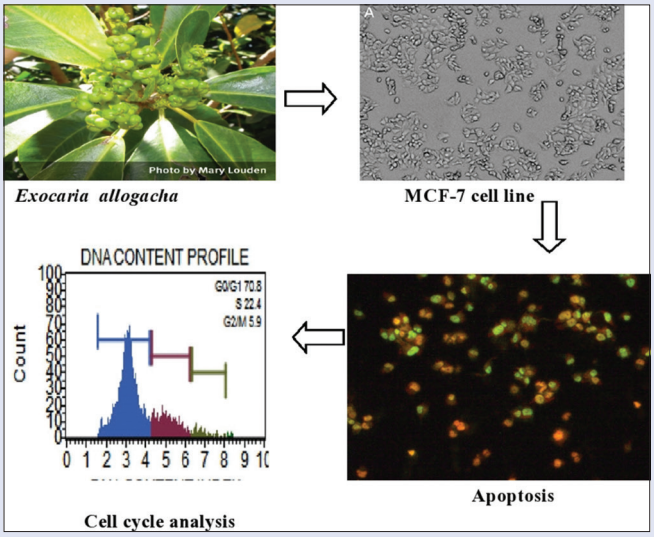
ABSTRACT

Background: At present, Mangrove plants have gained importance in drug discovery due to the presence of many phytomolecules of medicinal importance. *Excoecaria agallocha* is widely distributed medicinal mangrove which has been traditionally used to treat sores, ulcers, and leprosy. **Objective:** The aim of the present study is to analyze the cytotoxic potential of *Exoecaria agallocha* leaves in terms of its antiproliferative activity, apoptosis induction, and cell cycle arrest in the breast cancer MCF-7 cell lines. **Materials and Methods:** The ant proliferative nature of the EEEA extract was determined by direct microscopic observation as well as 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay by employing the different concentrations of the plant extract, and the IC_{50} values were calculated. Apoptosis-inducing potential was determined by double staining with acridine orange/ethidium bromide staining method and further confirmed by annexin V staining. Flow cytometry was performed with IC_{50} concentration of EEEA drug treated MCF-7 cells to determine arresting stage in the cell cycle progression. **Results:** There was a significant cytotoxic activity exhibited by the ethanolic extract of *E. agallocha* leaves (EEEA) in the direct microscopic as well as MTT assay with IC_{50} value of 56.5 µg/ml. Well prominent fluorescent microscopic images of morphological changes in the double staining method indicate the apoptotic potential of the extract which was further confirmed by annexin V staining. Suppression of cell cycle progression at $subG_1$ and G_1/G_2 phases were also evidenced in the flow cytometry analysis. **Conclusion:** From the above results, it may be concluded that the ethanolic leaf extract of *E. agallocha* (EEEA)-induced apoptosis mediated cytotoxic and antiproliferative activity in the breast cancer MCF-7 cell lines which can be developed into a new drug for treating breast cancer.

Key words: Breast cancer, cell cycle, cytotoxicity, *Excoecaria agallocha*, flow cytometry, MCF-7

SUMMARY

- In this study, it may be concluded that the ethanolic extract of *Excoecaria agallocha* through the presence of several phytochemicals suppressed the $subG_1$ and G_1/G_2 phases of cell cycle progression in MCF-7 cell lines (human breast cancer cell lines) by apoptosis mechanism thereby exert its antiproliferative activity. This extract can be developed as a therapeutic candidate for the treatment of breast cancer after, undertaking proper clinical and preclinical evaluation.



Abbreviations Used: PBS: Phosphate-buffered saline; FBS: Fetal bovine serum; DMEM: Dulbecco's modified eagle medium; MTT: 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-tetrazolium bromide; EB: Ethidium bromide; AO: Acridine orange.

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INTRODUCTION

Worldwide, breast cancer is ranking fifth among different cancer types for the cause of death and the second most common type of non-skin cancer accounting approximately 10.4% among women.[1] The major pathology involved in this disease is loss of cell cycle control leading to the uncontrolled proliferation and dedifferentiation of cells.[2] Most breast cancers begin either in the cells that line the ducts (ductal cancers) or in the cells that line the lobules (lobular cancers), while a small number start in the other tissues. As far as breast cancer is concerned, chemotherapy, radiotherapy, and surgery were followed as treatment protocol. However, each type of treatment has got several side effects including death, which has motivated the researchers to search of a new plant-based anticancer drug which could cure cancer with minimal or no side effects.[3] Herbal medicine forms a big part of many traditional medicine systems. The knowledge of these traditional medicine systems

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has provided key information for the discovery of anticancer agents from plants. Traditional medicine further forms a very important source of affordable and readily accessible health care system for most people in developing countries.[4]

Excoecaria agallocha L (Euphorbiaceae) is an ancient mangrove small tree found abundant in Pichavaram mangrove forest, Indian coastal regions.[5] *E. agallocha* L. belongs to Euphorbiaceae family is commonly called as "blinding tree" since the milky sap present in the tree can cause temporary blindness if it enters the eyes and Thillai, Kampetti in Tamil. The sap present in the plant causes skin blisters and irritation. Clinical trials conducted by Peter and Sivasothi[6] have reported that the plant possess anticancer, antiviral, antibacterial, and anti-HIV properties. In India, the breast and cervical cancer are predominantly identified in women.[7] Hence, the objective of the present study is to investigate antiproliferative and apoptosis-inducing activities of the *E. agallocha* leaves on human breast carcinoma cell line (MCF-7).

MATERIALS AND METHODS

Chemicals

Trypsin was procured from Gibco, USA while fetal bovine serum (FBS), Dulbecco's modified eagles medium (DMEM), 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide (MTT), Penicillin-Streptomycin, and ethidium bromide (EB) were purchased from Himedia Laboratories, Mumbai, India. Rest of the chemicals employed in the study was analytical grade.

Collection and preparation of ethanolic extract *Excoecaria agallocha* (EEEA)

The leaves of the mangrove plant *E. agallocha* were obtained from mangrove forest at Pichavara, Southeast coast of Tamil Nadu, India. The plant specimen was identified and confirmed by the Herbaria of Centre of Advanced Study in Marine Biology, Annamalai University, Tamil Nadu, India. The leaves were cleaned in the running tap water and shade-dried. The dried material was further subjected to a fine coarse powder using a blender, and ethanolic extract of *E. agallocha* (EEEA) was prepared using 90% ethanol in room temperature by cold maceration process. The excess ethanol is removed using rota flash evaporator after filtering the extract, and the sample yield was calculated. The yield was found to be 2.12% w/w which was stored in 4°C until further use.

Maintenance of MCF-7 (human breast carcinoma) cell lines

The MCF-7 (Human breast carcinoma) cell line was initially obtained from National Centre for Cell Sciences, Pune, India and initially grown in DMEM and then passaged in DMEM cell culture medium by providing the supplements such as L-glutamine, sodium bicarbonate, 10% FBS with antibiotic solution containing streptomycin (100 µg/ml), and penicillin (100 U/ml) under appropriate cell culture conditions. A 2-day-old confluent monolayer cells were trypsinized and suspended in 10% growth medium. A 100 µl cell suspension (5×10^4 cells/well) was seeded in 96 well tissue culture plates and incubated to study the anti-proliferative activity in humidified 5% CO_2 incubator for 24 h.

MCF-7 cell lines treatment for the cytotoxic assay

The 24 h incubated 96 well seeded MCF-7 (Human breast carcinoma) cell lines in growth medium were treated with different concentrations of ethanolic extract of *E. agallocha* (EEEA) dissolved in DMSO and incubated continuously for 72 h to analyze the anti-proliferative potential of the extract. MCF-7 cells without any treatment served as negative control and Doxorubicin served as a positive control in the present study.

Direct microscopic observation for cytotoxicity

At the end of 72 h, the treated tissue culture plate was observed in an inverted phase contrast Microscope (Olympus CKX41 with Optika Pro5 CCD camera), and the appropriate changes in the cell morphology like shrinking or rounding of cells as well as granulation and vacuolization in the cytoplasm were noted as they are the cytotoxic indicators as the result of EEEA treatment.

3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-tetrazolium bromide assay

The MCF-7 cell viability in the presence of EEEA extract was studied using MTT assay by the method of Horiuchi *et al.*[8] Briefly to the aliquots of EEEA extract treated and 24 h incubated MCF-7 cell lines, 30 µl of MTT solution (15 mg of MTT reconstituted in 3 ml phosphate-buffered saline [PBS] and filter sterilized) was added and incubated for another 4 h at 37°C in a humidified 5% CO_2 incubator. After the incubation period, the supernatant was removed and 100 µl of MTT solubilization solution (DMSO) was added and the wells were mixed gently by pipetting up and down to solubilize the formazan crystals. The absorbance values were measured using microplate reader at a wavelength of 570 nm. The percentage viability was calculated as follows:

Cell viability = optical density of samples/optical density of control × 100

Treatment of cells for apoptosis and cell cycle analysis

MCF-7 breast cancer cell lines at the concentration of 10^5 cells per ml were seeded in a tissue culture and incubated at 37°C in 5% CO_2 incubator for 24 h. The EECA extract at the concentration of 25 µg/mL, 50 µg/mL and 100 µg/mL were added and once again incubated for 24 h under same condition. Apoptosis and cell cycle analysis were performed using these treated cells.

Double staining by acridine orange, ethidium bromide for the apoptosis determination

The administration of EECA extract causes the death of cancer cells, and according to Zhang *et al.*'s[9] method, the mode of cell death was determined by acridine orange (AO)/EB staining. The drug treated and 24 h incubated cells were washed in cold PBS, subsequently stained with a mixture of AO (100 µg/ml) and EtBr (100 µg/ml) at room temperature for 10 min, washed again twice with PBS and examined under the fluorescence microscope (blue filter-Olympus CKX41 with Optika Pro5 camera). The visualized cells were categorized into 4 types (normal cells having green nucleus, early apoptotic cells with condensed or fragmented chromatin with bright green, late apoptotic with orange-stained nuclei, and uniformly orange-stained cell nuclei indicating the necrotic cells). The control cells and positive control also received the same treatment.

Apoptosis by annexin V-FITC/PI assay

The annexin *V-FITC/PI* assay was performed to quantify the apoptosis due to the drug treatment. Initially, the EEEA and doxorubicin treated cells were trypsinized and centrifuged for 5 min at 1200 rpm. Supernatant was discarded, and cell pellets were resuspended in PBS after washing with the same, centrifuged once again to produce a concentration of 1×10^6 cells per ml. By following the manufacturer's instructions of annexin *5-FITC* apoptosis detection kit, stained apoptotic cells were analyzed in the flow cytometer.

Cell cycle analysis by flow cytometry

The drug-treated cells were washed in PBS and 70% cold ethanol and stored at 4°C for 12 h. After centrifugation at 3000 rpm for 5 min, the supernatant was discarded and the pellet was suspended in 250 µl PBS and centrifuged once again. The pellet was dissolved in 250 µl of MUSE cell cycle reagent and again centrifuged. After removing the supernatant, the resultant solution was incubated for 30 min at dark condition to perform the cell cycle analysis. The percentage of cells gated in G_0/G_1, S and G_2/M phases of the cell cycle along with DNA content were determined.

Statistical analysis

All the experimental procedures were performed in triplicate to analyses the test results. Statistical analysis was performed using latest SPSS version (IBM software, India).

RESULTS

Measurement of cell viability

In the present investigation, cytotoxicity is noticed in the MCF-7 cells treated with ethanolic extract of *E. agallocha* (EEEA). There was morphological changes like rounding or shrinking of cells with granulation and vacuolization in the cytoplasm were observed [Figure 1]. Increased cytotoxic activity is noticed in the cells treated with 25 mg/ml and 50 mg/ml of EEEA extracts.

3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-tetrazolium bromide assay

The MTT assay is usually performed to find the toxicity profile of any extract because it determines the viability of cells after treatment. The drug treatment considerably reduced the MCF-7 cells viability indicating the cytotoxic potential of the plant extract [Figure 2].

A concentration-dependent cytotoxic activity is observed in the present study, and at higher concentration of 50 and 100 µg/ml, the cell viability showed a deep decrease which was comparable with the doxorubicin the positive drug used in the present study. IC_{50} values were calculated, and it was found to be 56.5 µg/ml. This concentration was used in cell cycle analysis by flow cytometry analysis.

Apoptosis assessment by double staining

To differentiate between apoptosis and necrosis of the MCF-7 cells due to EEEA treatment, the DNA binding dyes AO and EB were used to stain the nucleus. Figure 3 shows the increased cell death in the MCF-7 cells due to plant extract treatment when comparable to control untreated cells. The appearance of uniform cells with green color-stained nucleus in the control group indicates the live cells with their membrane intact.

The cells treated with 56.5 µg/ml and 113 µg/ml of the EEEA extract showed bright green colored nuclei with chromatin condensation and bright orange-colored nuclear areas with condensed chromatin indicating the presence of early apoptotic cells and late apoptotic cells. At the same time, necrotic cells with intact membrane and bright orange-colored uniform nucleus are also visualized. The cells which were treated with 113 µg/ml of EEEA extract showed an increased late apoptosis and necrosis which was comparable to the positive drug doxorubicin.

Apoptosis by annexin V-FITC/PI assay

Any anticancer drug is expected to induce apoptosis, and in the present study, the annexin *V-FITC/PI* assay was performed to find the apoptotic potential exhibited by EEEA extract. It was observed that the plant extract significantly induced apoptosis of MCF-7 cells at both the concentration, and at 113 µg/ml, the apoptosis exhibited by the EECA extract was higher than the positive control doxorubicin used in this study [Table 1].

Cell cycle analysis by flow cytometry

The flow cytometry measures the cell cycle progression through apoptotic induction as a result of EEEA leaf extract treatment [Figure 4]. The IC_{50} value of 56.5 µg/ml plant extract treated MCF and 7 cells were gated through the flow cytometry analysis which determines the number of cells present in the different stages of cell cycle.

In the untreated group, 70.8% cells were found in G_0/G_1 phase, against the EEEA-treated group which has only 34.5% in that phase. In the

Table 1: Apoptosis-AnnexinV-FITC/PI assay of PA-1 cell lines of control, EEEA and doxorubicin

MCF-7 Cell Treatment	% Apoptosis
Control (Untreated)	3.65 ± 0.95
EEEA Treated (56.5µg/ml)	42.45 ± 3.29a**
EEEA Treated (113.0 µg/ml)	79.60 ± 1.43b**
Doxorubicin Treated (10µg/ml)	70.15 ± 3.49c**

Each value represents the mean ± SEM (n = 3).Comparison between [a]Control versus EEEA 56.5 µg/ml treated, [b]Control versus EEEA 113.0 µg/ml treated, [c]Control versus Doxrubicin. *P<0.05, **P<0.01, ***P<0.001, NS: Not significant; SEM: Standard error of mean; EEEA: Ethanolic extract of *Excoecaria agallocha* leaves

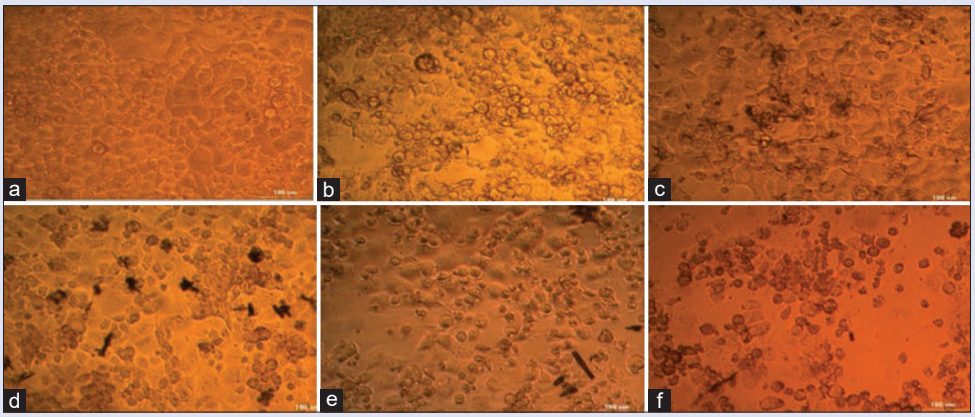

Figure 1: Morphological changes of MCF-7 cells after 72 h of treatment (a) untreated (control) treated with (b) 6.25 mg/ml (c) 12.50 mg/ml (d) 25 mg/ml (e) 50 mg/ml of *Excoecaria agallocha* leaf extract. (f) Doxorubicin-treated cells viewed under an inverted light microscope (×200)

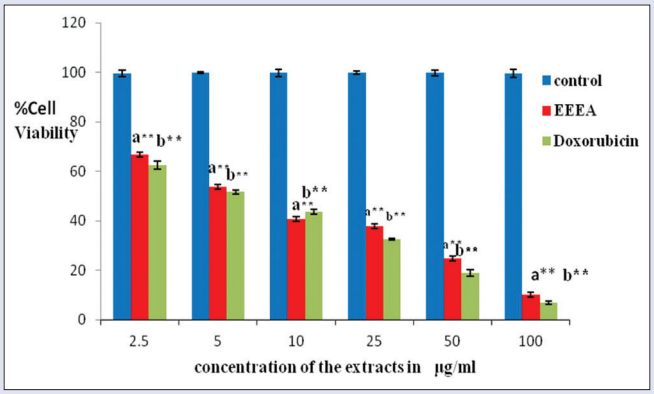

Figure 2: Cell viability of MCF-7 cell lines (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-tetrazolium bromide assay) of control, EEEA, and doxorubicin. Each value represents the mean ± standard error of mean (n = 3). Comparison between a: Control versus EEEA and b: Control versus doxorubicin. *P < 0.05, **P < 0.01, ***P < 0.001, NS: Not significant

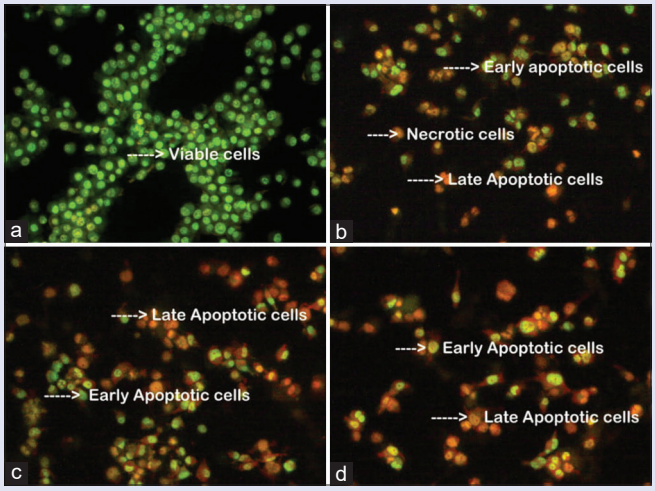

Figure 3: Apoptosis assessment in MCF-7 cells by double staining acridine orange/ethidium bromide staining of MCF-7 cells treated with ethanolic extract *Excoecaria agallocha* for 72 h and viewed under the fluorescence microscope (×200). (a) Control untreated viable cells with normal green nucleus (b) 56.5 µg/ml extract treated cells showing early and late apoptotic cells and necrotic cells (c) 113 µg/ml extract treated cells showing late apoptotic cells and necrotic cells (d) doxorubicin-treated cells showing the late apoptotic cells and necrotic cells

"S" phase, there is no much difference found in the untreated and drug-treated groups which showed the presence of 22.4% and 27.6% cells, respectively. Significant increase in the cell population was found in the G_2/M phase of the treated cells when comparable to untreated cells. These results indicate although there was increase in the gated cells in "S" phase and G_2/M phase of treated cells, the EEEA extract could hinder initial multiplication process cells in the G_0/G_1 phase indicates the antiproliferative potential of our extract.

DISCUSSION

In Mangrove vegetation, the plant which normally grows in the marine coastal environments intertidal zones and estuarine margins require wet and muddy soil for their growth. Most of the plants have the ability to tolerate and grow in very high salt concentration and they possess medicinal and commercial importance. *E. agallocha* is commonly known as milky mangrove which possesses milky latex which causes skin blistering and irritation. In recent times, mangrove plants have gained scientific interest as alternative medicine in cancer treatment. Already Patil *et al.*[10] have reported through the chromatographic finger printing, the presence of cardiac glycosides, and saponin which has showed potential cytotoxic activity on cancer cell lines such as Miapaca-2, BxPC-3, PANC-1, and Capan-1 cells. With the above facts in mind and owing to the presence of less work on the cytotoxic activity of ethanolic extract of *E. agallocha* on MCF-7 cancer cell lines, we made an attempt to study cytotoxic activity of *E. agallocha* ethanolic leaf extract in MCF-7 cell lines which is used to study the breast cancer.

In the present investigation, the EEEA extract-treated MCF-7 cells exhibited rounding or shrinking of cells, granulation, and vacuolization in the cytoplasm, etc., confirming the phenotypic apoptosis of cells due to drug treatment. At the same time, the MTT assay (4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide) which is an accepted colorimetric method for screening the cytotoxic activity of crude extracts, isolated compounds, and synthetic drugs also shows the antiproliferative potential of the EEEA extract in terms of decreased viability of the treated cells which further confirms the cytotoxic nature of the drug. Peter and Sivasothi[6] and Subhan *et al.*[11] in terms of modern clinical trials have reported the anti-HIV, anticancer, antibacterial, and antiviral potential of this plant.

To be an anticancer drug, the plant extract is expected to induce apoptosis and arrest cell proliferation.[12] In the present study, AO/EB staining methods were conducted to analyze the antiproliferative activity of the EEEA extract on MCF-7 cells in terms of apoptosis and necrosis. Significant increase in the death of cells is evidenced in the AO/EB staining either as apoptosis or necrosis in the EEEA-treated cells. The apoptotic anticancer drug is considered as beneficial agent, when it not only induces programmed cell death but also scavenges the unregulated or abnormal cells unlike necrosis an inflammatory response which invariably destroys the normal cells the surrounding microenvironment.[13] Annexin/PI flow cytometric analysis is performed which evaluates the potential of the any extract as a apoptosis biomarker through phosphatidylserine the flipping[14] since inherent property of cancer cells is to resist apoptosis to continue their cell proliferation without any control.[15] The EEEA extract induced considerable apoptosis in the present investigation as evidenced by shrinkage of cells, chromatin condensation in the MCF-7 cancer cells. Several researchers have reported the presence of diterpenoid, triterpenoid derivatives, alkaloids, and diterpenes were found to possess antitumor activity.[16-22]

In the cell cycle analysis, the ethanolic extract of *E. agallocha* extract significantly decreased the gating of cells in the sub-G_1 and also G_0/G_1 phase when compared to the untreated cells indicating the inhibition of cell cycle progression through decreased DNA content. Among the several tumor suppressor proteins, p53 protein is considered as a potent transcription factor involved in the induction of apoptosis and regulator of cell cycle arrest. Another protein – the p21, a cyclin-dependent kinase inhibitor gets activated either in the presence or absence of the p53.[23] Together p-53 and p21 pathway gets activated in the DNA damaged cells and arrests the G_1 and G_2 phases of cell cycle leading to the termination of cell progression.[24] Rifai *et al.*[25] and Abidi,[26] have isolated six flavonoid glycosides from the leaves of *E. agallocha* and reported their cytotoxic potential against the human pancreatic (PANC1) and prostate (DU145) and Hep-2 cancer cells by Batsa and Periyasamy.[27] According to Zou *et al.*,[19] the anticancer potential of the *E. agallocha* extract may be due to the presence triterpenoids (β-amyrin acetate, epilupeol, epitaraxerol, 3 β-[(2E,4E)-5-oxodeca-2,4-dienoyloxy] olean-12-ene, taraxerol, and taraxerone). The EEEA owing to the presence of several phytochemicals could arrest the cell progression and exert the anticancer activity.

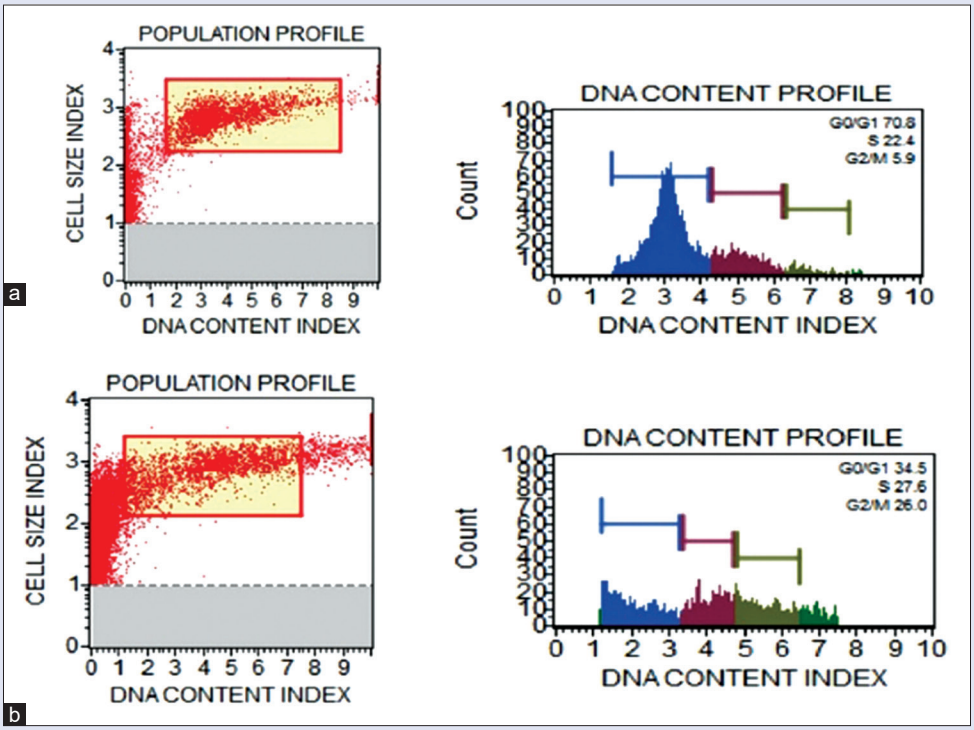

Figure 4: Cell cycle analysis MCF-7 cells by flow cytometry. (a) Gated cells of untreated group (b) gated cells of EEEA-treated group

CONCLUSION

It may be concluded that the ethanolic extract of *E. agallocha* through the presence of several phytochemicals suppressed the subG$_1$ and G$_1$/G$_2$ phases of cell cycle progression in MCF-7 cell lines (human breast cancer cell lines) by apoptosis mechanism thereby exerting its antiproliferative activity. This extract can be developed as a therapeutic candidate for the treatment of breast cancer after undertaking proper clinical and preclinical evaluation.

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Conflicts of interest

There are no conflicts of interest.

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