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Effects of *Calophyllum inophyllum* fruit extract on the proliferation and morphological characteristics of human breast cancer cells MCF-7Shanmugapriya¹, Yeng Chen², Jagat Rakesh Kanwar³, Sreenivasan Sasidharan^{1*}¹Institute for Research in Molecular Medicine, Universiti Sains Malaysia, USM 11800, Pulau Pinang, Malaysia²Dental Research & Training Unit, and Oral Cancer Research and Coordinating Centre, Faculty of Dentistry, University of Malaya, Kuala Lumpur 50603, Malaysia³Nanomedicine-Laboratory of Immunology and Molecular Biomedical Research (LIMBR), School of Medicine, Faculty of Health, Institute for Frontier Materials, Deakin University, Waurn Ponds, VIC 3217, Australia

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ABSTRACT

Objective: To evaluate the antiproliferative activity of *Calophyllum inophyllum* (*C. inophyllum*) fruit extract against human breast cancer cells MCF-7.**Methods:** The cytotoxic effect of *C. inophyllum* fruit extract against MCF-7 cancer cells was evaluated through MTT and CyQuant assays for 24 h and the morphological investigation of treated MCF-7 cells was observed under optical microscope using Giemsa staining.**Results:** The cytotoxic effect of *C. inophyllum* fruit extract against MCF-7 cancer cells was evaluated through MTT and CyQuant assays simultaneously for 24 h after treatment, which demonstrated the inhibition of cell viability with the IC₅₀ values of 19.63 µg/mL and 27.54 µg/mL, respectively. The preliminary time-based morphological investigation of MCF-7 cells treated with the IC₅₀ value (23.59 µg/mL) of *C. inophyllum* fruit extract was observed under an optical microscopy via Giemsa staining, which exhibited prominent histological characteristics of apoptosis.**Conclusions:** This study clearly proved that the proliferation of human breast cancer cell MCF-7 was inhibited by *C. inophyllum* fruit extract resulted from the induction of apoptosis in MCF-7 cells.

1. Introduction

Medicinal plants have been used for the treatment of various diseases since ancient times which is now greatly gaining attentions due to their rich contents of phytochemicals playing an essential role in treating diseases especially cancers[1,2]. Hence, it is highly recommended to evaluate the effectiveness and cytotoxicity of the plant extracts before developing them into anticancer agents in order to quantitatively identify the toxicity of the plant extracts to the cancer cells[3,4]. This can be accomplished through the *in vitro* cytotoxicity screenings where several cell-based assays can

be employed to study the toxic effects of the plant extracts against cells.

The association between fruit and medicinal plant consumption and cancer threat has been reported in hundreds of scientific studies. The findings of these studies have been hypothesized that the wide variety of nutrients and phytochemicals in fruits and medicinal plants can prevent carcinogenesis during the initiation, promotion, and progression stages. Therefore, the current study was undertaken with the objective to study the cytotoxicity effect and to observe the morphological characteristics of MCF-7 breast cancer cells treated with *Calophyllum inophyllum* (*C. inophyllum*) fruit extract which is a local medicinal plant. *C. inophyllum* is a medium to a large evergreen tree presenting elliptical leaves, fragrant white flowers and large round nuts. The tree is widely distributed along the coasts of the Indian and Pacific Oceans, especially in Melanesia and Polynesia. It is locally called “Tamanu” in Polynesia, “Tamanou de bord de mer” in New Caledonia, “Dilo” in Fiji, “Nyamplung” in Indonesia and latterly is traditionally used notably in the treatment

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of suppurating wounds. This important medicinal plant is also used in various forms to treat various diseases. Moreover, the decoction of the leaves is used to relieve dermatosis, urticaria and eczema, the juice of the bark is purgative, the first cold pressed seed oil is used in wound healing and to relieve neuropathy associated with leprosy[5-7]. Oil from the plant is suggested for all kinds of burns, post-surgical and suppurating wounds, dermatoses, acne, psoriasis, herpes, rheumatism and gonorrhoea[6]. The present study was to evaluate the antiproliferative activity of *C. inophyllum* fruit extract against human breast cancer cells MCF-7.

2. Materials and methods

2.1. Plant sample collection

The fruits of *C. inophyllum* were collected from various areas in Universiti Sains Malaysia, Penang, in October 2015, and authenticated at the Herbarium of the School of Biological Sciences, Universiti Sains Malaysia, Pulau Pinang, Malaysia, where a sample was deposited (voucher specimen: USM/HERBARIUM/11604). The fruits were washed thoroughly with tap water and then washed again with distilled water. The fruits were then cut into small pieces and dried under shade for 3 weeks at room temperature (28 °C). The dried fruits were grounded into powder using a grinder and stored in clean labelled airtight bottles.

2.2. Preparation of *C. inophyllum* fruit extract

The powdered dried fruits sample was sequentially extracted with methanol by adding approximately 100 g of the dried sample into 500 mL of methanol. The extraction was carried out at room temperature (28 °C) by soaking for 7 days with intermittent stirring. The extracts were filtered through clean muslin cloth and the extraction process was repeated a second time by adding another 400 mL of methanol to the sample residue. The filtrate from each extraction was combined and concentrated under vacuum on a rotary evaporator (Buchi, Switzerland) at 40–50 °C in order to evaporate the excess methanol solvent and until a dark brown methanol extract was produced. The concentrated extract was poured into Petri dishes and brought to dryness at 60 °C in the oven until a paste-like mass was obtained. Then, the paste-form extract was sealed in the petri plates and stored at room temperature (28 °C).

2.3. Cell culture

2.3.1. Media preparation

The purchased Dulbecco's modified Eagle's medium (DMEM) was resuspended assiduously before dispensing out an accurate volume of 44.5 mL into the sterile 50 mL conical centrifuge tubes and stored at 4 °C which was warmed to 37 °C in a water bath (Grant, Cambridge, UK) each time before use. Complete media

were prepared by supplementing it with 0.5 mL of 100 IU/mL penicillin and 100 µg/mL streptomycin and 5 mL of 10% sterile heat inactivated fetal bovine serum. Complete media were preferred to be prepared freshly for each 44.5 mL DMEM media. The formulated complete media were also stored at 4 °C and pre-warmed to 37 °C in water bath each time before use.

2.3.2. Cell line

MCF-7 which is the breast adenocarcinoma cells was procured from the Tissue Culture Laboratory of Institute for Research in Molecular Medicine. The cells were cultured in complete media. These cell lines were cultivated as adherent monolayer cultures in cell culture flasks (Corning) in a humidified atmosphere of 5% CO₂ incubator (Thermo Scientific, Canada) at 37 °C.

2.4. MTT assay

Cytotoxicity of *C. inophyllum* fruit extract against MCF-7 breast cancer cells was determined with the employment of MTT assay as reported by Kim *et al.*[8]. The MCF-7 cells were harvested from the culture flask which was then quantified by using a hemocytometer. Approximately, 3000–5000 cells in 100 µL medium were seeded per well in the 96-well plate and allowed to incubate overnight at 37 °C with 5% (v/v) CO₂ for the attachment of the cells. Subsequently, the cells were treated with different concentration (100 µg/mL to 0.781 µg/mL) of *C. inophyllum* fruit extract prepared through serial dilution in DMEM ranging from 100 µg/mL to 0.781 µg/mL. The assay for each concentration of the extract was performed in triplicates with additional wells of untreated cells to serve as the negative control. The plate was incubated at 37 °C with 5% (v/v) CO₂ for 24 h. The following protocol was conducted in dark since MTT is light sensitive. Meticulously, 20 µL of MTT reagent was added to each well followed by further incubation for 4 h. After 4 h of incubation period, the media were removed from each well and 100 µL of DMSO was added to each well to dissolve the water-insoluble purple formazan crystals. The plate was kept on a plate stirrer for 30 s to completely dissolve the crystals. The absorbance values were measured at a wavelength of 540 nm with the incorporation of a microplate reader (Molecular Devices Inc., USA). The IC₅₀ value of *C. inophyllum* fruit extract against MCF-7 cells was determined using GraphPad Prism version 6.00 (GraphPad, San Diego, CA, USA) and used in the subsequent experiments. The results were expressed as percentage of treated cell number relative to the untreated control cells.

2.5. CyQuant cell proliferation assay

Cytotoxicity of the fruit extract against MCF-7 breast cancer cells was also determined by another means of assay known as CyQuant cell proliferation assay where the viable cells were calculated through the presence of cellular nucleic acid. This assay was

conducted with an incorporation of CyQuant® Cell Proliferation Assay Kit (Invitrogen, USA).

The MCF-7 cells were harvested from the culture flask which was conducted with the same steps used in MTT assay above. The following protocol was adopted from the Molecular Probes, Inc. (USA). After the incubation period, the cells were washed carefully with phosphate buffered saline (PBS) and freezed by storing the microplate at -70°C for up to 4 weeks. Before quantifying the samples, the plate was thawed at room temperature. Then, 200 μL of CyQuant GR dye/cell lysis buffer was added to each well and mixed gently. The samples were then incubated for 2–5 min at room temperature protected from light. The fluorescence readings of the samples were measured using a fluorescence microplate reader with filters appropriate for $\sim 480\text{ nm}$ excitation and $\sim 520\text{ nm}$ emission. The IC_{50} values of *C. inophyllum* fruit extract against MCF-7 cells were determined using GraphPad Prism version 6.00 (GraphPad, San Diego, CA, USA) and used in the subsequent experiments. The percentage of cell viability was calculated by using the formula below with the fluorescence readings obtained from the CyQuant assay.

$$\text{Cell viability (\%)} = \frac{[\text{Sample} - \text{Blank}]}{[\text{Control} - \text{Blank}]} \times 100\%$$

The results were expressed as percentage of treated cell number relative to the untreated control cells.

2.6. Morphological detection of apoptosis in MCF-7 breast cancer cells

Preliminary study of the morphological detection for apoptosis is equally imperative to confirm the action of the mechanism of *C. inophyllum* fruit extract in MCF-7 breast cancer cells which was accomplished by using optical microscopy upon Giemsa staining. The brief protocol of the staining begins with cell seeding. The cells were then treated with an average IC_{50} value ($23.59\ \mu\text{g}/\text{mL}$) derived from the data of both the cytotoxicity assays in this study. Each treated samples were incubated for different time frames starting from 6 h, followed by 12 h, 24 h and 48 h, respectively. An untreated sample was prepared to serve as a negative control of the experiment. Upon their respective treatment period, the medium was discarded and washed twice with PBS. The cells were then fixed by adding 1 mL of McDowell trump's fixative followed by an overnight storage at 4°C . The fixed cells were gently rinsed with PBS to remove the excess fixation agent. After that, the cells were stained with Giemsa and incubated for 30 min. Once the staining period was over, the cells were washed twice with double distilled H_2O and allowed to air-dry completely before observing the samples under an inverted light microscope (Olympus, Japan) and photographed.

2.7. Statistical analysis

Quantitative data obtained from the cytotoxicity assays were expressed as mean \pm SD from the least three independent

experiments ($n = 6$ for each experiment). The IC_{50} values were obtained from a linear regression model based on sigmoidal dose response curve and computed using Graphpad Prism version 6.00 (GraphPad, San Diego, CA, USA).

3. Results

3.1. MTT assay

The cytotoxic effect of *C. inophyllum* fruit extract against MCF-7 breast cancer cells was determined with the incorporation of colorimetric MTT assay at a range of 0–100 $\mu\text{g}/\text{mL}$ after 24 h of treatment period. The cell viability can be qualitatively assessed by the color intensity of the purple formazan dye. The intensity of the purple formazan dye increased when the concentration of the drug decreased (Figure 1). This indicated that the increase of the metabolically active cells was inversely proportional to the drug concentration. The absorbance values obtained from the microplate reader for respective concentration of the drug were summarized and a graph of cytotoxicity (%) versus the concentration of *C. inophyllum* fruit extract was plotted (Figure 2). The IC_{50} value which was the concentration of the drug resulting in 50% cytotoxicity was determined from Figure 2. The *C. inophyllum* fruit extract showed effective cytotoxic effects with an IC_{50} value of $23.59\ \mu\text{g}/\text{mL}$ in MCF-7 breast cancer cells.

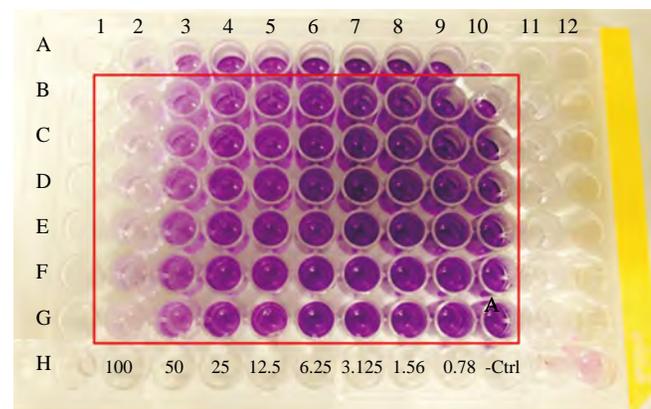


Figure 1. The 96-well micro plate used in MTT assay.

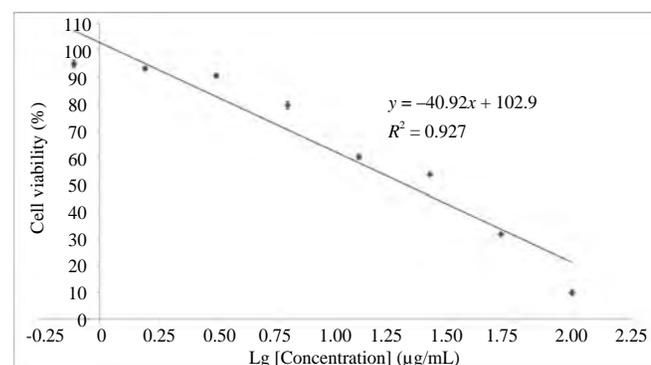


Figure 2. Cell viability of MCF-7 cells treated with *C. inophyllum* fruit extract by using MTT assay.

3.2. CyQuant proliferation assay

The cytotoxic effect of *C. inophyllum* fruit extract on MCF-7 breast cancer cells was further investigated by CyQuant® proliferation assay in which the cell viability was assessed based on a different aspect from MTT assay. The cells were treated with serially decreasing concentrations of *C. inophyllum* fruit extract from 0–100 µg/mL for 24 h and a cytotoxicity study was investigated by means of DNA content in cells. The results are graphically represented in Figure 3. The DNA content in cells was observed to be conversely proportional to the concentration of the extract, which indicated that the treatment for growth inhibition responded in a dose-dependent manner. The IC₅₀ of the crude extract was obtained from the graph. The *C. inophyllum* fruit extract showed effective cytotoxic effects with the IC₅₀ value of 27.54 µg/mL in MCF-7 breast cancer cells.

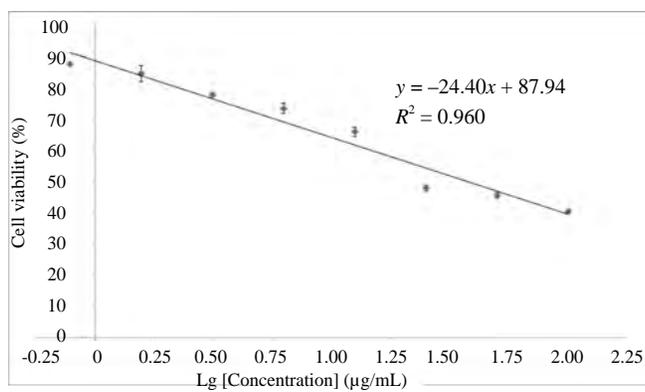


Figure 3. Cell viability of MCF-7 cells treated with *C. inophyllum* fruit extract by using CyQuant assay.

3.3. Morphological detection of apoptosis using Giemsa staining

Morphological alteration in MCF-7 cells was observed under a light microscopy to construe the preliminary mechanism of action of *C. inophyllum* fruit extract treatment in comparison with untreated cells. The morphological characteristics of untreated and *C. inophyllum* fruit extract treated MCF-7 cells upon Giemsa staining were observed and photographed. The typical morphological feature of untreated MCF-7 cells was observed with its common morphology of luminal epithelial by noticeable formation of domes (Figure 4). However, Giemsa staining revealed that *C. inophyllum* fruit extract treatment at the concentration of 23.59 µg/mL induced apoptotic cell death in MCF-7 cell lines. The photomicrograph obtained at 6 h of treatment period displayed enlargement of MCF-7 cells (Figure 5) as compared to the untreated cells. When the treatment period extended to 12 h, chromatin condensation was able to observe besides the enlargement of cells (Figure 6). At 24 h exposure of cells to the *C. inophyllum* fruit extract revealed remarkable morphological alterations such as the cell blebbing, appearance of apoptotic bodies, cell clumping, enucleation, and cytoplasmic extrusion mass with a shortened lamellopodia (Figure 7). Finally, the 48-hour-treated MCF-7 cells depicted an exceptional decrease in number of cells with all of the

apoptotic features (Figure 8). The alteration in cell morphology appears to gradually excruciate with a significant decrease in number of cells when the treatment period extends.

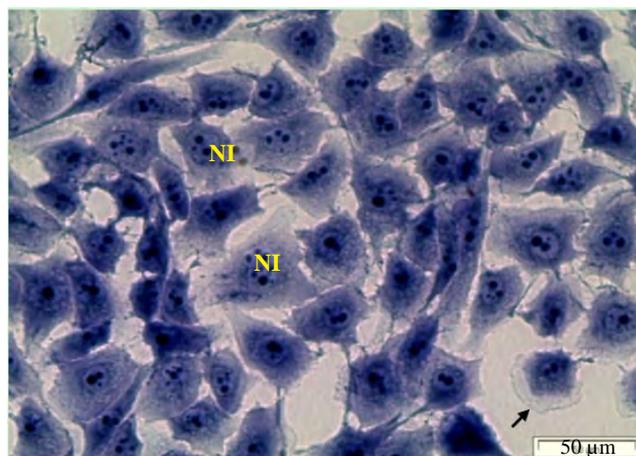


Figure 4. Morphology of untreated MCF-7 cells as a negative control, viewed under an inverted light microscope upon Giemsa staining (20× magnifications).

Black arrow: The lamellopodium; NI: Nucleoli.

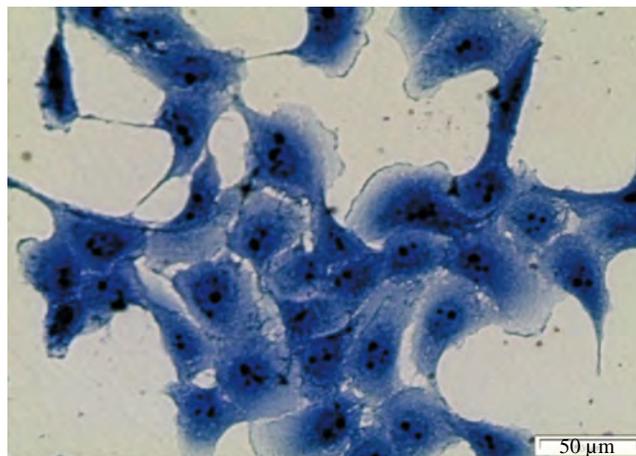


Figure 5. Morphological changes of MCF-7 cells treated with IC₅₀ concentration (23.59 µg/mL) of *C. inophyllum* fruit extract for 6 h assessed by Giemsa staining and viewed under an inverted light microscope (20× magnifications).

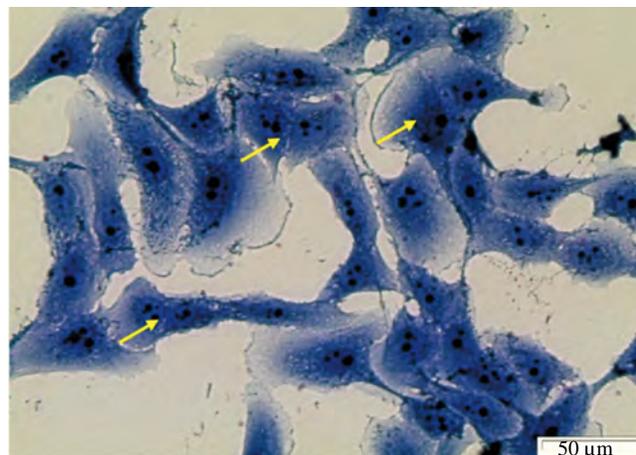


Figure 6. Morphological changes of MCF-7 cells treated with IC₅₀ concentration (23.59 µg/mL) of *C. inophyllum* fruit extract for 12 h assessed by Giemsa staining and viewed under an inverted light microscope (20× magnifications).

Yellow arrow heads indicate nuclear condensation.

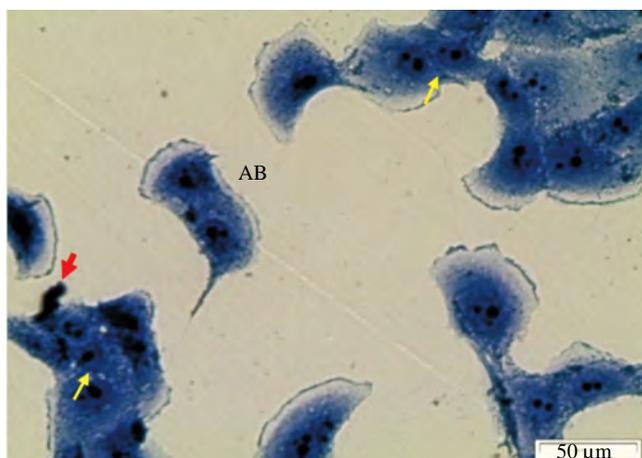


Figure 7. Morphological changes of MCF-7 cells treated with IC_{50} concentration (23.59 $\mu\text{g/mL}$) of *C. inophyllum* fruit extract for 24 h assessed by Giemsa staining and viewed under an inverted light microscope (20 \times magnifications).

Yellow arrow heads: Nuclear condensation; Red arrow head: Enucleation; AB: The presence of apoptotic body.

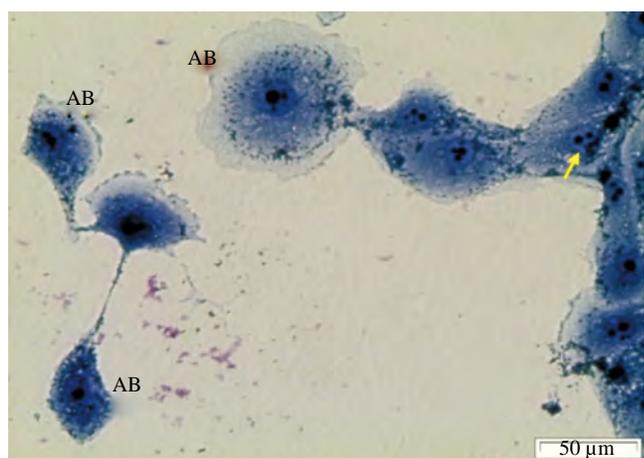


Figure 8. Morphological changes of MCF-7 cells treated with IC_{50} concentration (23.59 $\mu\text{g/mL}$) *C. inophyllum* fruit extract for 48 h assessed by Giemsa staining and viewed under an inverted light microscope (20 \times magnifications).

Yellow arrow heads: Nuclear condensation; AB: The presence of apoptotic body.

4. Discussion

4.1. In vitro cytotoxicity activity

Plants have been the major source of medication since ancient times and are serving as the basic foundation in modern medicine nowadays. Plants with a long history of traditional medication have drawn increasing attention and being studied further to investigate its efficiency as chemotherapeutic drugs especially for the treatment of cancer. Although natural products have been utilized traditionally, methodical screening or scrutiny of its cytotoxicity is highly decisive in the drug discovery field particularly for cancer treatment and chemoprevention[9]. Several cytotoxicity tests are available not only for the hazard evaluation of a particular plant extract but also to disclose the molecular mechanisms of action of the extract[10].

In this study, two different assays have been employed to evaluate the cytotoxicity of the *C. inophyllum* fruit extract with an index of cell viability from different aspects, namely the MTT assay and the CyQuant assay which appraises through the metabolic activity and the amount of DNA presenting from the viable cells, respectively. According to McCauley *et al.*[11], MTT assay is considered to be one of the sensitive cytotoxicity assay in despite of its momentous statistical results obtained for the treated cells in contrast to the controls as well as its sensitivity in encountering early apoptosis. Besides MTT assay, CyQuant cell proliferation assay has been developed and characterized to be an eminently sensitive nucleic acid stain-based assay which is contemplated to be a more rapid and convenient way to quantify viable cells[12]. Moreover, DNA and RNA levels are highly associated with the cell proliferation which eventually provides a concrete reason for accepting cellular nucleic acid content as a legitimate indicator in quantifying viable cells[13].

The results obtained from both MTT and CyQuant assays confirmed that the *C. inophyllum* fruit extract has significant cytotoxic activity against MCF-7 breast cancer cells at an exposure time of 24 h. This was characterized by IC_{50} of the MCF-7 breast cancer cell line. The *C. inophyllum* fruit extract exhibited cytotoxicity with the IC_{50} values of 19.63 $\mu\text{g/mL}$ and 27.54 $\mu\text{g/mL}$ through MTT and CyQuant assays, respectively. According to the plant screening program of the American National Cancer Institute (NCI), the criterion of cytotoxicity activity for the crude extracts is the one with an $IC_{50} < 30 \mu\text{g/mL}$ in the precedent tests[14,15]. Therefore, based on the results obtained, it can be concluded that the *C. inophyllum* fruit extract has a promising anticancer potential with a wide safety margins, since both the cytotoxicity assays applied showed an IC_{50} value less than 30 $\mu\text{g/mL}$ that falls within the NCI criteria.

The phytochemical literature investigation of this *Calophyllum* species has revealed that it accommodates secondary metabolites such as xanthenes[16-19], coumarins[17,20-23], flavonoids[24-26] and triterphenoids[27,28]. For example, prenylated xanthenes from *C. inophyllum* called caloxanthone O was reported to show cytotoxicity against human gastric cancer cell line (SGC-7901) with an IC_{50} value of 22.4 $\mu\text{g/mL}$ [29]. In addition, caloxanthone N and gerontoxanthone C from twigs of *C. inophyllum* were reported to exhibit cytotoxicity against chronic myelogenous leukemia cell line (K562) with IC_{50} values of 7.2 and 6.3 $\mu\text{g/mL}$, respectively[30]. Furthermore, calocoumarin A from *C. inophyllum* showed significant inhibitory effect on epstein-barr virus early antigen activation induced by 12-O-tetradecanoylphorbol-13-acetate in Raji cells and further displayed its inhibitory effect on mouse skin tumor promotion in an *in vivo* two-stage carcinogenesis stage, concluding it to be a promising anticancer agent[31]. Several coumarins have also been reported to display strong cytotoxic activity against KB cells[21]. Triterpenoids from *C. inophyllum*, namely friedelin, canophyllic acid, 3-oxo-friedelan-28-oic acid and oleanolic acid, have been accounted for antiproliferative effect in human leukemia HL-60 cells[28].

4.2. Morphological study of optical microscopy

Although it was evident that *C. inophyllum* fruit extract exerted cytotoxic properties against MCF-7 breast cancer cells via cytotoxic assays performed in this study, it is decisive to identify the mechanism behind the cell death, which is due to a natural event of programmed cell death rather than an unplanned cell death of necrosis. The detection of apoptosis was initially made based on morphological changes in cells which are thus far remains as a preliminary evaluation of apoptosis before proceeding to molecular study of cell death[32]. Observance under light microscopy upon cell staining is reported to appraise the manifestation of apoptosis commensurately meticulously[33]. Fixing the cells prior to staining is highly imperative in order to preserve the morphology and to prevent smearing while performing staining procedure[34]. Giemsa dye is widely used to stain the cells as it is economical and requires a short period of staining which is less than an hour[35].

Dettmeyer[36] has previously stipulated an overview of the significant facets of photographical structures observed via Giemsa staining, whereby the nuclei are presented to be purple-red, nucleoli to be blue, and cytoplasm to be light blue-grey. The photographs obtained in this study via Giemsa staining are homogenous with the previous proclaimed study. Furthermore, the morphological alterations observed in MCF-7 cells upon exposure to *C. inophyllum* fruit extract in contrast to the untreated cells apparently indicate the induction of apoptosis. The morphological changes noticed at different treatment time periods significantly present the continual process of cell death, showing an apparent reduction in cell number since the completely detached dead cells were removed in the washing step.

The microscopy investigation of *C. inophyllum* fruit extract treated MCF-7 cells via Giemsa staining revealed an enlargement of cells, condensation of chromatin, cell blebbing, enucleation, diminished lamellopodia and the formation of apoptotic bodies which is consistent with the histological characteristics of apoptosis. Contrastingly, the untreated cells were observed to withhold their typical morphology with significantly levelled veil-like projection called a lamellipodia, prominent nucleus and several nucleoli as described earlier by Majumdar *et al.*[37]. The micrographic outcomes are perpetual with plethora morphological studies conducted previously using Giemsa staining to define apoptosis which have also reported cell enlargement[38], chromatin condensation[39-42], membrane blebbing[42] leading to the formation of apoptotic bodies[37,39,43] and enucleation[44]. Preliminary study on morphological changes in MCF-7 cells upon *C. inophyllum* fruit extract treatment through Giemsa staining confirmed the induction of apoptosis. Hence, it can be considered that *C. inophyllum* fruit extract is a propitious anticancer candidate though further molecular mechanistic study is highly required.

In conclusion, the cytotoxicity activity of *C. inophyllum* fruit extract was confirmed through MTT assay and CyQuant assay with IC₅₀ values of 19.63 µg/mL and 27.54 µg/mL, respectively. The viability of cells tends to decrease upon the increasing

concentrations of the crude extract. It can be proposed that the crude extract has an immense safety boundary to be utilized as a chemotherapeutic agent since both the evaluated IC₅₀ values fall within the NCI criteria of cytotoxicity of crude extract. The average of both the IC₅₀ values was calculated (23.59 µg/mL) and adopted as a general IC₅₀ value of the *C. inophyllum* fruit extract throughout the research. Furthermore, the mechanism of cell death is assessed preliminarily through the morphological analysis under light microscope using Giemsa staining which urged the induction of apoptosis upon *C. inophyllum* fruit extract treatment in MCF-7 cells.

Conflict of interest statement

We declare that we have no conflict of interest.

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