MECHANISTIC BASIS OF CYTOTOXIC ACTION OF *GARCINIA CELEBICA* ETHEREAL OILS IN CULTURED BREAST CELLS

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Abstract

Garcinia celebica (*G. celebica*), an aromatic plant which is domestically planted for fruits and ornamental purposes, is exceptionally rich in important phytochemicals that may contribute to its medicinal properties. The mechanistic basis of cytotoxic action of *G. celebica* essential oil (EO-GC) in MCF-7, MCF-7/TAMR-1, and MCF-10A cell cultures was explored in this study. The study findings showed that the EO-GC potently inhibited the proliferation of the MCF-7 and MCF-7/TAMR-1 cells by 50% at 37.5 µg/mL and 18.8 µg/mL concentrations, respectively. In contrast, a higher concentration (77.5 µg/mL) of the essential oil was needed to inhibit the MCF-10A cells. These findings demonstrated that the EO-GC possesses selective cytotoxic effects by two-fold and four-fold towards the MCF-7 and MCF-7/TAMR-1 cells, respectively, than the MCF-10A cells. Flow cytometric analysis has proven that the EO-GC can effectively induce apoptotic cell death in the cultured MCF-7 and MCF-7/TAMR-1 cells, while having minimal effect on the MCF-10A cells. Therefore, these preliminary findings may add novel scientific data related to the potential therapeutic efficacy of the ethereal oil derived from *G. celebica* leaves.

Keywords: *Garcinia celebica* L., Ethereal Oil, Cytotoxicity, Apoptosis Breast Cells

Introduction

Garcinia celebica Linnaeus (*G. celebica* L.) is taxonomically clustered under the Clusiacae family (1, 2). To date, there are more than 400 Garcinia species that had been identified thus far. The tree is commonly distributed in the lowveld and mountain ranges of Africa and Peninsular Malaysia (3). Within the Garcinia genus, *G. celebica* L. was first proclaimed and published in 1754 by Carl Linnaeus, the Father of Taxonomy (4).

G. celebica was reported to be geographically distributed in Peninsular Malaysia, the upper part of Borneo, Thailand, Papua New Guinea, India as well as the island of Andaman and Nicobar (1). Ecologically, the *G. celebica* tree can be found natively in the lowland forest near the sea as well as in the inland forest up to 1200 m of altitude (5). As this tree is regularly found in coastal areas, it has been named as the "Seashore Mangosteen". In Malaysia, specifically, it is commonly pronounced as "*Beruas*" or "*Manggis Hutan*" (4). The *G. celebica* tree is small to medium in height in which it can grow up to 30 m tall (6). Meanwhile, the fruits bear some resemblance to mangosteen; They are round, rose red in colour when ripen, and taste slightly sour (4).

In Peninsular Malaysia, *G. celebica* trees are planted domestically for fruits and ornamental purposes. This, in turn, may enhance the understandings about their nutritional values and thus commercialisation aspects in promoting the socio-economic development among the villagers. In some villages in this country, the leaf and root extracts of *G. celebica* are boiled in water as it is

believed by the communities that the decoction may relieve pain in women after childbirth (1). In addition, the plant parts of the tree, such as roots and leaves, are also used in folk medicine to treat itchiness (4). As for India, the rootstock of *G. celebica* is generally used to cultivate the fruits (7).

Catechin, friedelin, and garcihombronane D are some examples of bioactive principles identified from *G. celebica* leaves (2, 5). The leaves were reported to exhibit antiplasmodial properties. Furthermore, as reported by a number of studies, numerous parts of *G. celebica* L. extracts (such as from leaves, barks and twigs) exhibited interesting biological activities, such as antioxidant (8, 9), anti-bacterial (10), anti-trypanosomal (11), antidiabetic (12), antiplatelet aggregation (13), anticholinesterase (14), hepatoprotective (15), and anticancer (16). In our previous study (17), we reported about the main phytochemicals found in *G. celebica* essential oil (EO-GC). Those metabolites are classified under the terpenoids and sesquiterpenes functional groups.

Several studies reported that the *Garcinia* species possess anti-cancer properties against the breast cancer. The anti-cancer effects of this tree species could be attributed to its underlying inhibitory mechanisms, such as anti-proliferative, anti-metastasic, and pro-apoptosis. Furthermore, the experimental findings from a study also demonstrated that the *Garcinia* species are able to synergise the therapeutic activity of some anti-cancer drugs (18). For instance, in our another recent study (16), the findings showed that the *Garcinia atroviridis* ethereal oil was cytotoxic to cultured MCF-7 cancer cells. Furthermore, the *Garcinia* oil was also found to enhance the cytotoxic activity of tamoxifen (16). However, the success of tamoxifen treatment is commonly hampered by the occurrence of drug resistance. This problem may result in clinical severity and mortality (19). Hence, the present study aimed to investigate the mechanistic basis of the cytotoxic action of the EO-GC in cultured human breast cells.

Materials and Methods

Plant material

In this study, *G. celebica* leaves were obtained from Penang Botanical Garden, Penang. The plant material was assessed and authenticated by a botanist (Mr. Baharudin Sulaiman) from the School of Biological Sciences, Universiti Sains Malaysia (USM), Penang, Malaysia. A voucher specimen of *G. celebica* (USM 11748) had been deposited in the herbarium of the university.

Isolation of EO-GC oil

The extraction of ethereal oil from *G. celebica* leaves was carried out by the following method, as described in our previous study (17). Briefly, the collected leaves were firstly washed with tap water, and the debris was removed. About 130 grams of *G. celebica* of leaves were crushed into small pieces and subjected to hydrodistillation using Clevenger-type apparatus for five hours with distilled *n*-pentane as the collecting solvent. The resulting oil (EO-GC) was concentrated under a gentle flow of nitrogen gas at the room temperature. Then, the EO-GC was preserved in a sealed vial at 4 ℃ until it was ready for analysis. The extraction was carried out in triplicate.

Cell cultures and treatments

MCF-7 and MCF-10A cells were cultured according to the American Type Culture Collection (ATCC) recommendations, as described in previous studies by Tan *et al.* (16) and Yaacob and Ismail (20). Following Merck Manufacturer's guideline, MCF-7/TAMR-1 cells were cultured in DMEM/F-12 medium without phenol red, buffered with 10% phosphate buffer saline (FBS), and added with 10 µg/ml human insulin, 1% PenStrep, and 1 µM of 4-hydroxytamoxifen every alternate passage.

The EO-GC was dissolved in dimethyl sulphoxide (DMSO) at 10 mg/mL stock concentrations. Tamoxifen (Nacalai Tesque, Japan) was dissolved in ethanol at 10 mM stock concentrations. Then, the freshly diluted EO-GC and tamoxifen solutions were applied for the cell treatments.

Analysis of cytotoxicity

The cytotoxic effects of the EO-GC against cultured breast cells were measured quantitatively using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay (Merck Millipore, Germany). The cells were seeded, treated, and analysed by following the manufacturer's protocols, as described previously by Tan *et al*. (17). Dose-response curves and inhibitory concentration at 50% (IC_{50}) value were plotted and calculated based on the formula described in the study (17). MTT assay was repeated thrice independently whereby all samples, controls, and blanks were prepared in triplicates.

Selectivity index (SI)

The degree of selectivity of which the EO-GC displays for the cancer cells was expressed by its selectivity index (SI) value, which was calculated using the following formula:

$SI = IC_{50}$ normal cells / IC_{50} cancer cells

Formula 1 was adapted from the study by Badisa *et al*.

(21) with minor modifications.

Analysis of mode of cell death

Prior to the analysis, the cells were seeded at an optimum density in a T-25 cm^2 culture flask. Following an overnight incubation, the cells were treated with the EO-GC and controls. After 24 hours, the treated cells were harvested, washed twice with PBS, and pelleted by centrifugation at 1000 rpm for five minutes. Based on the BD Biosciences (USA) guidelines, the cell pellets were processed and stained with annexin-FITC and PI dye. Each sample was analysed using the FACS Calibur instrument and CellQuest Pro software (Becton Dickinson, USA), as described in a previous study by Yaacob *et al*. (22).

Statistical analysis

The statistical significance of the differences between treated and untreated cells from the three independent experiments was assessed using an independent Student's t-test. Significant differences were considered as $P < 0.05$ (*). The IBM SPSS Statistics software was used for the statistical analysis whereby all data were presented as a mean ± standard deviation (SD).

Results

Cytotoxic effect

The results showed that the inhibitory effects of the EO-GC against cultured MCF-7 and MCF-7/TAMR-1 cells were significantly affected by its concentration and treatment time. Specifically, the EO-GC decreased the proliferation of the MCF-7 and MCF-7/TAMR-1 cells by more than 50% at 50 µg/mL and 25 µg/mL concentrations, respectively, after 24 hours of treatment (Figures 1 A and B).

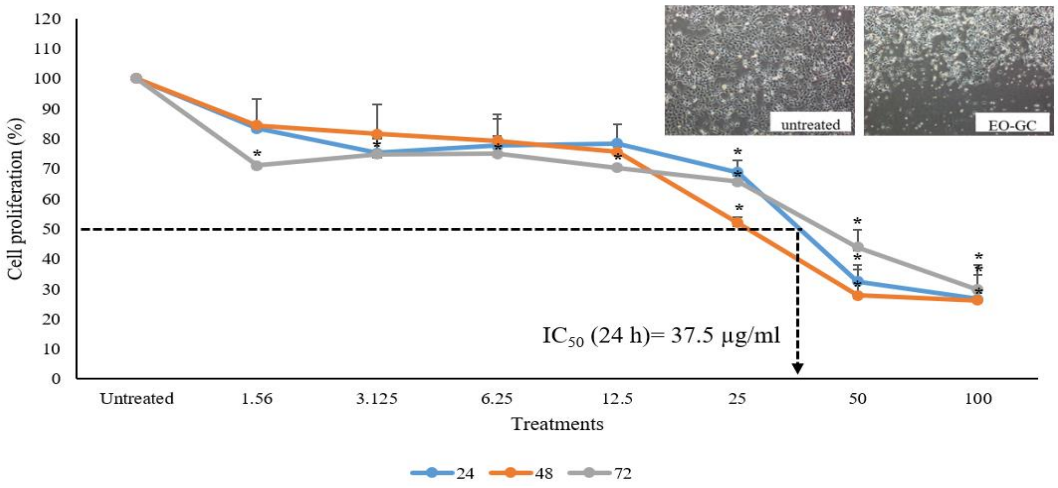

Figure 1: (A). Effect of EO-GC in MCF-7 cell culture. MCF-7 cells were treated with EO-GC at 1.56 to 100 µg/mL concentrations for 24-72 hours. *p < 0.05 is significantly different to untreated cells

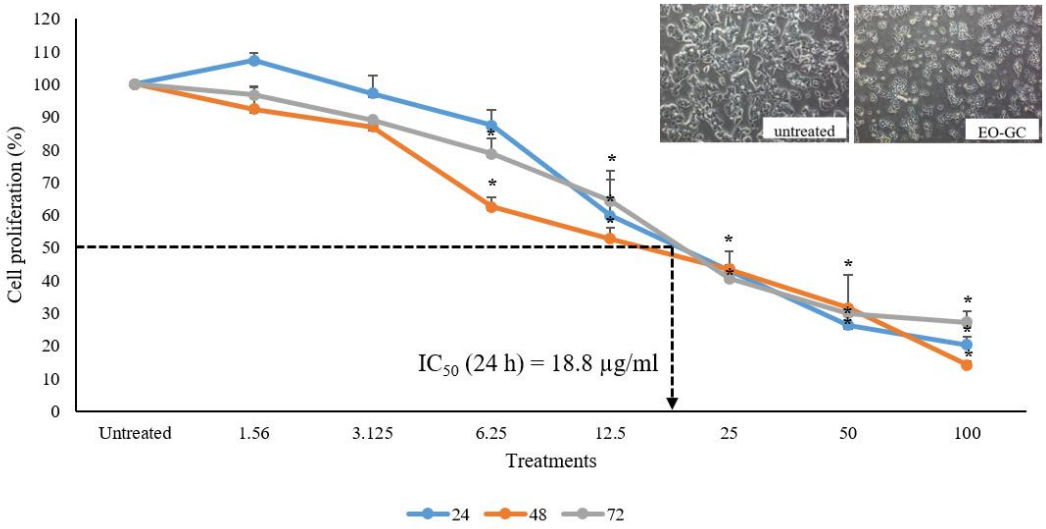

Figure 1: (B). Effect of EO-GC in MCF-7/TAMR-1 cell culture. MCF-7/TAMR-1 cells were treated with EO-GC at 1.56 to 100 µg/mL concentrations for 24-72 hours. *p < 0.05 is significantly different to untreated cells

The concentrations that inhibited 50% of the cells (IC_{50}) obtained following the EO-GC treatment on the MCF-7, MCF-7/TAMR-1, and MCF-10A cells were indicated in Figures 1 (A), (B) and (C), respectively. After 24 hours of exposure, IC_{50} values of the EO-GC determined in the MCF-7, MCF-7/TAMR-1, and MCF-10A cells were recorded at 37.5 µg/mL, 18.8 µg/mL and 77.5 µg/mL, respectively.

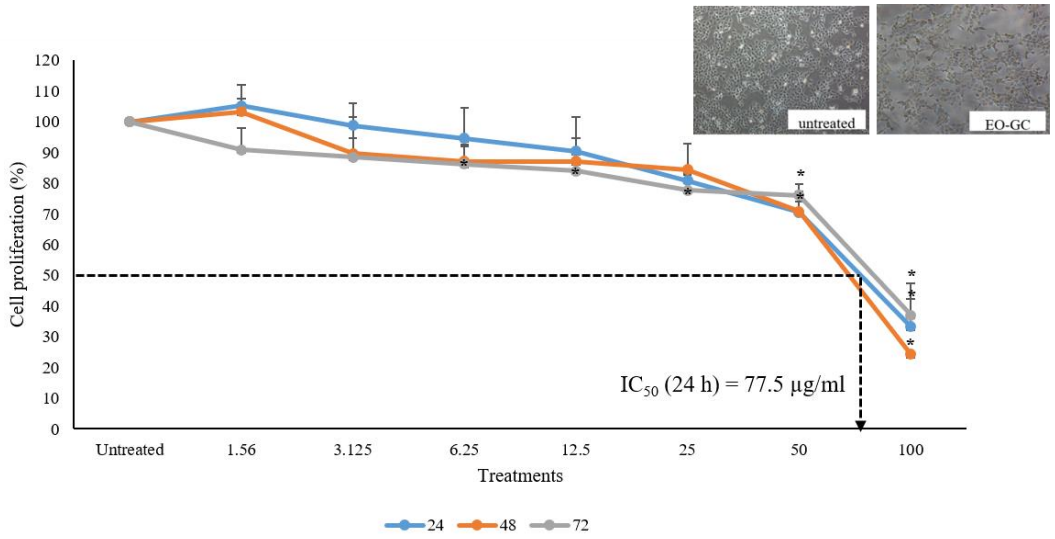

Figure 1: (C). Effect of EO-GC in MCF-10A cell culture. MCF-10A cells were treated with EO-GC at 1.56 to 100 µg/mL concentrations for 24-72 hours. *p < 0.05 is significantly different to untreated cells

The SI values in this study were calculated by comparing the IC_{50} values of the EO-GC in MCF-10A normal cells against those treated in the MCF-7 or MCF-7/TAMR-1 cancer cell lines. A higher SI value indicates a higher selectivity of the compounds or extracts toward the cancer cells (23). Based on the study results in Table 1, the SI values of the EO-GC exhibited two-fold and four-fold cytotoxic selectivities in the MCF-7 and MCF-7/TAMR-1 cells, respectively, compared to MCF-10A.

Table 1: The selectivity index (SI) values of EO-GC were calculated based on Formula 1 (IC_{50} normal cells / IC_{50} cancer cells)

Cell lines	MCF-10A	MCF-7		MCF-7/TAMR-1	
EO-GC	IC_{50} (µg/mL)	IC_{50} (µg/mL)	SI	IC_{50} (µg/mL)	SI
24 h	77.5 ± 4.8	37.5 ± 1.7	2.1	18.8 ± 3.4	4.1
48 h	73.3 ± 2.5	32.3 ± 7.6	2.3	16.0 ± 2.9	4.6
72 h	87.7 ± 5.6	48.0 ± 8.2	1.8	21.0 ± 1.7	4.2

Mechanism of apoptosis

Apoptosis, which is a type of mechanism of cell death, occurs in a programmed biochemical event. It is important in maintaining the cellular equilibrium between growth and death (24). In general, apoptosis biochemical events involve a cascade of cellular self-destruction that is regulated by the pro-apoptotic molecules. Cytotoxic agents can induce apoptosis in cultured cell lines (25). This characteristic provides promising and potential cytotoxic agents which are applicable in pharmaceutical and agricultural sciences.

In order to determine whether apoptosis was the key factor influencing the EO-GC-induced growth inhibitory effects in the cultured cells, qualitative and quantitative assessments of the apoptosis and necrosis as described in the methodology section was carried out after 24 hours of treatment. The EO-GC-treated cells were shrink and rounded as observed under inverted light microscopy (Figure 1 (A), (B), and (C); refer *inserts*). The cells were

analysed further by flow cytometry, and then clustered into four stages, namely viable, early apoptosis, late apoptosis and necrosis, as shown in Figure 2 (A).

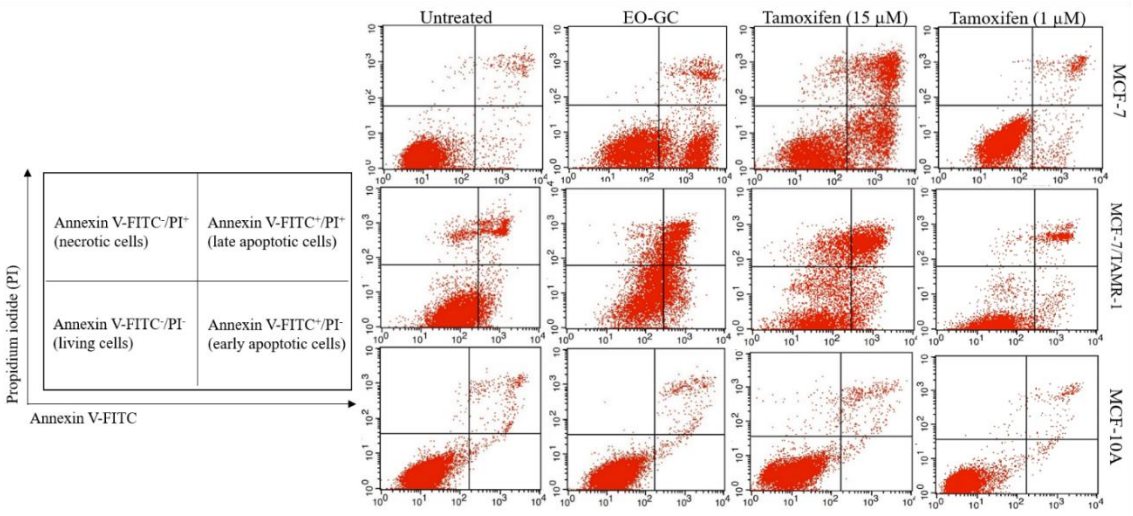

Figure 2 (A). Flow cytometric analysis of EO-GC. Each cell line was treated with culture medium alone (untreated), EO-GC (IC_{50} value) and tamoxifen (positive control)

As the findings show, although the 24 hours of EO-GC treatment had induced apoptosis for almost 50% in both the MCF-7 and MCF-7/TAMR-1 cells, the treatment did not lead to significant apoptosis effect in the MCF-10A cells (Figure 2 (B)). This effect was comparable to the apoptosis induced by 15 µM of tamoxifen (positive control). In the present study, The MCF-7/TAMR-1 cell line was alternately maintained with 1 µM of tamoxifen, following the manufacturer's recommendation. Therefore, 1 µM of tamoxifen was also included as a control for the apoptosis assay whereby the treatment showed negligible apoptotic effects in all cell lines tested in this study. Taken together, it can be postulated that the inhibitory effects of the EO-GC occurred through the mechanism of apoptotic cell death.

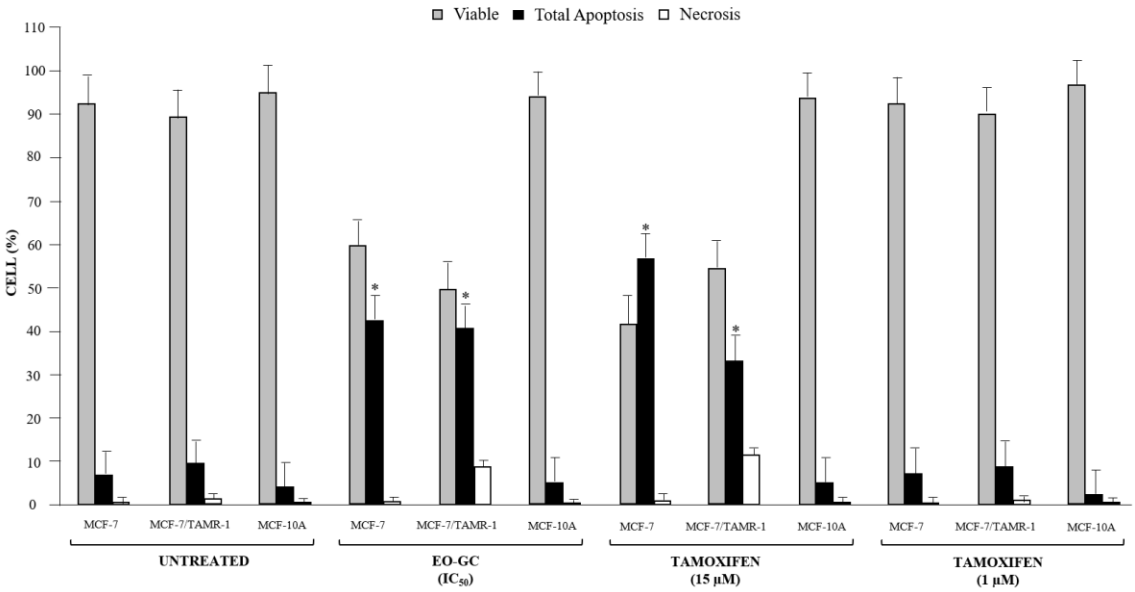

Figure 2 (B). Comparison of viable, apoptosis and necrosis in cultured human breast cells. The percentage of viable, total apoptotic (early and late) and necrotic cells. *$p < 0.05$ is significantly different to untreated cells

Discussion

Generally, the major composition of ethereal oils is from secondary metabolites which are commonly concentrated in the regions of leaves and bark of aromatic plants. Selected plants under the *Clusiaceae* family have been reported to contain these volatile organic compounds (16, 17, 26). These include *Garcinia* species which have been scientifically documented as potent cytotoxic agents (27, 28). In our previous study, it was identified that the EO-GC contains sesquiterpenes that are classified under the terpenoids group (17). In particular, the oil contains 22 compounds which are dominated by α-copaene, germacrene D, and β-caryophyllene. The aforementioned study described the chemical compositions of the EO-GC obtained by the capillary gas chromatography and gas chromatography-mass spectrometry (17). In this paper, the mechanism basis underlying the EO-GC as potential cytotoxic agent in cultured MCF-7, MCF-7/TAMR-1 and MCF-10A cells is discussed in detailed.

The results of the MTT assay have shown that the EO-GC inhibited the proliferation of the breast cancer cells with IC_{50} values lower than the concentration needed to kill normal cells, i.e. MCF-10A. The present findings also provide conclusive evidence on the significant cytotoxic effects of the EO-GC on breast cancer cell lines compared to the normal cell line, as indicated by the SI values which are greater than 2.0. A similar finding on selective cytotoxic effects was also discussed by Amiel *et al*. (29) and Salleh *et al*. (30). For instance, Amiel *et al.*'s study reported that β-caryophyllene, which is one of the bioactive compounds commonly found in ethereal oils, exhibited more selective cytotoxic effects against cancer cell lines in comparison to normal cells (29). In similar vein, AVO-l, which is the oil from *Artemisia vulgaris*, was found to exhibit more selective growth inhibitory effects against various cancer cell lines than in normal human skin fibroblast BJ and kidney epithelial cells (30).

Next, apoptosis was quantified using the flow cytometry method to investigate whether the decreased cell number by the EO-GC was due to the induction of apoptosis or necrosis. The findings indicate that the essential oil had induced the morphological changes of the MCF-7 and MCF-7/TAMR-1 cells, which corresponded to the typical apoptotic features. The apoptosis effect was also evidenced by the increase in the percentage of positive annexin-V-FITC cells. These findings are in accordance with the reported cell death effects of oil isolated from *Artemisia capillaris* (31). The apoptosis experiment using the flow cytometric analysis in this present study has also provided the evidence on the insignificant effect of the EO-GC on normal cell line, i.e. MCF-10A. A similar result was observed by Manjamalai *et al*. (32) who reported the significant apoptosis effect of oil extracted from *Tridax procumbens* in lung cancer-induced mice, compared to the normal control group.

Given these observations, the EO-GC was proven to be cytototoxic and pro-apoptotic agents against the cultured MCF-7 and MCF-7/TAMR-1 cells. Therefore, these findings contribute to the body of literature by adding novel evidence-based data related to the biological and therapeutic properties of *G. celebica*. Further detailed investigation on the EO-GC is necessary to unravel the pro-apoptotic downstream mechanisms of actions responsible for the anticancer properties as discussed in this study.

Conclusion

This study concludes that the EO-GC exhibited potent cytotoxic properties against the cultured MCF-7 and MCF-7/TAMR-1 breast cancer cells whereby the effects were dependent on the concentration and treatment duration. Furthermore, the selective cytotoxicity by the EO-GC was mediated by apoptosis. These preliminary findings may be helpful for further understanding on the efficacy of ethereal oil derived from G. celebica as a potential source of pharmaceuticals.

Acknowledgements

The corresponding author gratefully acknowledges the financial support from the Ministry of Higher Education (MOHE), Malaysia under the Fundamental Research Grant Scheme (FRGS; Reference code: FRGS/1/2021/STG01/USM/02/11, Account no. 203.CIPPT.6711976). Musthahimah Muhamad was sponsored under the Universiti Sains Malaysia Fellowship Scheme.

Conflict of Interest

No potential conflict of interest was reported by the authors.

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