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ORIGINAL ARTICLE

Melastoma malabathricum Ethyl Acetate Fraction Induces Secondary Necrosis in Human Breast and Lung Cancer Cell Lines

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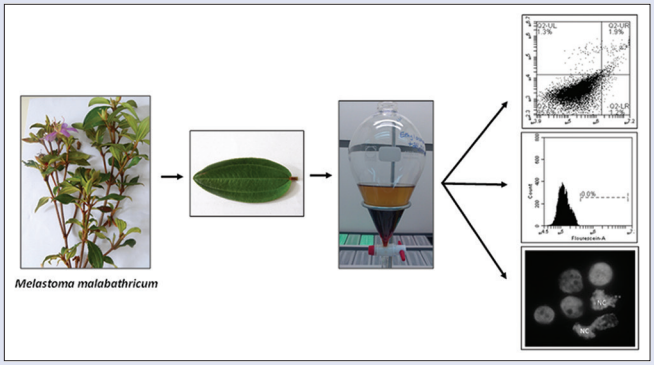
ABSTRACT

Background: *Melastoma malabathricum* (MM) is a traditional plant used in the Borneo region. The cytotoxic effects of methanol extracts from MM leaves have been reported in a number of human cancer cell lines. However, the mode of cell death by MM has not been investigated. **Objective:** We investigated the cytotoxic effects of MM in both human breast and lung cancer cell lines, MCF-7 and A549, respectively, and defined the mode of cell death. **Materials and Methods:** Cell viability was measured using the 3-(4-, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Annexin-V/propidium iodide (PI) and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining was done to determine the mode of cell death. **Results:** The MTT assay revealed that MM extract had an IC50 of >400 μg/ml on both cell lines at 24 h posttreatment. Flow cytometric and fluorescence microscopy analysis of Annexin-V/PI stained MM-treated cells revealed that the majority of the cells underwent secondary necrosis/late apoptosis. TUNEL assay showed that little to no DNA nicks were present in MM-treated cells, suggesting that cells have undergone secondary necrosis, not late apoptosis, at that time point. **Conclusion:** MCF-7 and A549 cells undergoes secondary necrosis 24 h post-treatment with MM extract. MM leaf extract could be a potential source for a novel anti-tumor agent for cancer therapy.

Key words: Apoptosis, cancer, cytotoxic, flow cytometry, *Melastoma malabathricum*, necrosis

SUMMARY

- *Melastoma malabathricum* (MM) extract was toxic on human breast and lung cancer cell lines
- Majority of MM-treated cells died by either secondary necrosis or late apoptosis at 24 h post-treatment
- Terminal deoxynucleotidyl transferase dUTP nick-end labeling assay confirmed that MM-treated cells underwent secondary necrosis, not late apoptosis.



Abbreviations used: DMSO: Dimethyl sulfoxide; MM: Melastoma malabathricum; MTT: 3-(4-, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PI: Propidium iodide; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick-end labeling.

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INTRODUCTION

Melastoma malabathricum (MM) is a shrub or small tree found commonly in the South East Asian region including countries on the Borneo Island, Malaysia, and Brunei.[1-4] The traditional uses of MM include treating diarrhea, dysentery, leucorrhea, hemorrhoids, wounds, infection during confinement, toothache, flatulence, sore legs, and thrush.[2,5] Extracts of MM have been found to exhibit effective anti-inflammatory,[6] antinociceptive, antipyretic,[7] antioxidant, antiviral (herpes simplex virus-1, poliovirus),[8] antifungal,[9] antimicrobial, and anti-obesity activities.[10] MM is also toxic in a range of cancer cell types, including breast,[8,11,12] ovarian,[13] and cervical cancer cells.[13] Although both MM flower[11,12] and leaf[11,13] extracts have revealed toxicity, MM leaf extract exhibits the most toxic activity. In all these studies, the 3-(4-,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to determine the toxicity of MM extracts. However, this is not adequate for determining the type of cell death in response to MM extract. Apoptosis and necrosis, the two major types of cell death, are characterized by different cell morphological features and pathways. For anti-cancer therapy, the successful induction of cell death of the tumor is one of the most important objectives. Here, we examine the mode of cell death by MM extract in two human cancer cell lines.

MATERIALS AND METHODS

All chemicals were purchased from Merck, Germany and were of analytical grade.

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Plant materials

Wild grown MM plants were collected from the Brunei-Muara and Tutong districts in Brunei Darussalam. The plant was identified by the Institute of Biodiversity and Environmental Research, Universiti Brunei Darussalam, Brunei Darussalam. Fresh leaves were air-dried under shade and pulverized into powder form. Ground dried Melastoma leaves (~224.1 g) were successively extracted with chloroform in Soxhlet extractor to remove chlorophyll. The marc was extracted with methanol in Soxhlet extractor. The extract was evaporated under reduced pressure with a rotary evaporator. The methanol crude extract was then fractionated with ethyl acetate and water to separate different bioactive compounds based on polarity. The obtained fractions were evaporated using a rotary evaporator. The solid formed from the ethyl acetate fractions were stored at 4°C.

Preparation of *Melastoma malabathricum* ethyl acetate fraction

Dried MM ethyl acetate fraction was dissolved in sterile dimethyl sulfoxide (DMSO) solution before filtering through a sterile 22 μm syringe filter (Millipore).

Cell culturing

MCF-7 and A549 cells were purchased from the American Type Culture Collection and cultured in Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, California, United States) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 20 U/ml penicillin, and 20 μg/ml streptomycin.

3-(4-,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

MTT assay[14] was used to measure cell viability. MCF-7 and A549 cells seeded in 96-well plates (Corning® Incorporated, Corning, New York, United States) were incubated with or without varying concentrations of MM over 24 h before performing the MTT assay. MM ethyl acetate fraction was tested at concentrations ranging from 50 ng/ml to 500 μg/ml. DMSO was used as the vehicle control.

Annexin-V/propidium iodide flow cytometry analysis

Cells were plated in 24 well plates (Corning® Incorporated) at 50,000 cells/well and incubated at 37°C for 48 h to allow the cells to adhere to the bottom of the wells. At more than 90% cell confluence, MM ethyl acetate fraction (500 μg/ml) was added to the wells. After 24 h, the cell suspension was centrifuged and the supernatant discarded. Cells were stained using the Alexa Fluor 488 Annexin-V/Dead Cell Apoptosis Kit (Invitrogen) according to the manufacturer's instructions and analyzed on a BD Accuri® C6 Flow Cytometer (BD Biosciences, San Jose, California, United States).

Nuclei staining and microscopy

Cells were grown on glass coverslips before treatment for 24 h and fixed in ice-cold methanol (−20°C) for 1 min at room temperature. Cells were washed once in PBS before staining with 0.4 μg/ml of DAPI in PBS for 10 min in the dark. Cells were washed again in PBS before mounting with Dako-Cytomation fluorescent mounting medium (Dako North America, Inc, Carpinteria, California, United States) under a coverslip. Cells were analyzed using a fluorescence microscope (Nikon inverted microscope Eclipse 90 L) and viewed under ×100 magnification.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay

Cells were stained using the *In Situ* Cell Death Detection Kit, Fluorescein (Roche Holding AG, Basel, Switzerland) according to the manufacturer's instructions and analyzed on a BD Accuri® C6 Flow Cytometer.[15]

RESULTS

Melastoma malabathricum extract is toxic on MCF-7 and A549 cells

Previous studies showed that the methanol extract of MM leaf exhibited the most toxic effect on different cancer cell lines,[11,13] suggesting that these extracts contain an active anti-proliferative agent. MTT assay of the methanol extract on MCF-7 and A549 cells confirmed a more than 50% loss of cell viability in both cell lines treated with 500 μg/ml of MM compared to a lower concentration (50 μg/ml) [Figure 1]. This shows that MM leaf extracts kill MCF-7 and A549 cells at high concentrations with an IC50 more than 400 μg/ml.

Melastoma malabathricum extract-mediated cell death is consistent with secondary necrosis

We next assessed the mode of cell death of MM extracts using Annexin-V/propidium iodide (PI). Cells undergoing apoptosis generally stain positive for Annexin-V as phosphatidylserine is externalized to the outer cell membrane during apoptosis.[16] Non-apoptotic cell death, such as necrosis, allows PI to enter the cells as the cell membrane becomes permeable due to the loss of plasma membrane integrity. Flow cytometric analysis revealed that the majority of MM extract-treated cells were Annexin-V (+)/PI (+), consistent with secondary necrosis, although this difference was more marked in MCF-7 cells than A549 cells [Figure 1b and c]. Microscopic analysis confirmed that nuclei morphology of MM extract-treated cells was consistent with necrotic features [Figure 2a]. This suggests that MM extracts drive MCF-7 and A549 cells predominantly to a secondary necrotic type of cell death.

Annexin-V (+)/PI (+) staining also suggests that cells could be undergoing late apoptosis. To distinguish between these possibilities, we performed a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay, which detects extensive DNA fragmentation, a hallmark of apoptosis.[15] There was no difference in the number of TUNEL-positive cells between MM extract and DMSO alone-treated MCF-7 and A549 cells, suggesting that the cells did not undergo late apoptosis [Figure 2]. Together these data suggest that MM extract kills MCF-7 and A549 cells by secondary necrosis.

DISCUSSION

The main objective of this study was an in-depth analysis of the mechanism of action of the induction of cancer cell death by MM extracts. Here, we report that the ethyl acetate fraction from methanol MM extracts are toxic on MCF-7 and A549 cells (IC50 >400 μg/ml) over 24 h treatment and that the cell death mediated by MM occurs predominantly by secondary necrosis.

MM leaves have been shown to exert a range of biological effects including cell killing, which makes MM a potential source for an anti-cancer drug. Furthermore, the toxic effect of MM leaves is well known, with the methanol extract showing the most potent anti-proliferative effect. An early study reported an IC50 of more than 400 mg/ml of methanol leaf MM extracts on MCF-7 cells.[8] Another study reported an IC50 value of 88 μg/ml for methanol leaf MM extracts on MCF-7 cells.[13] Both studies did not report exposure time. Recent

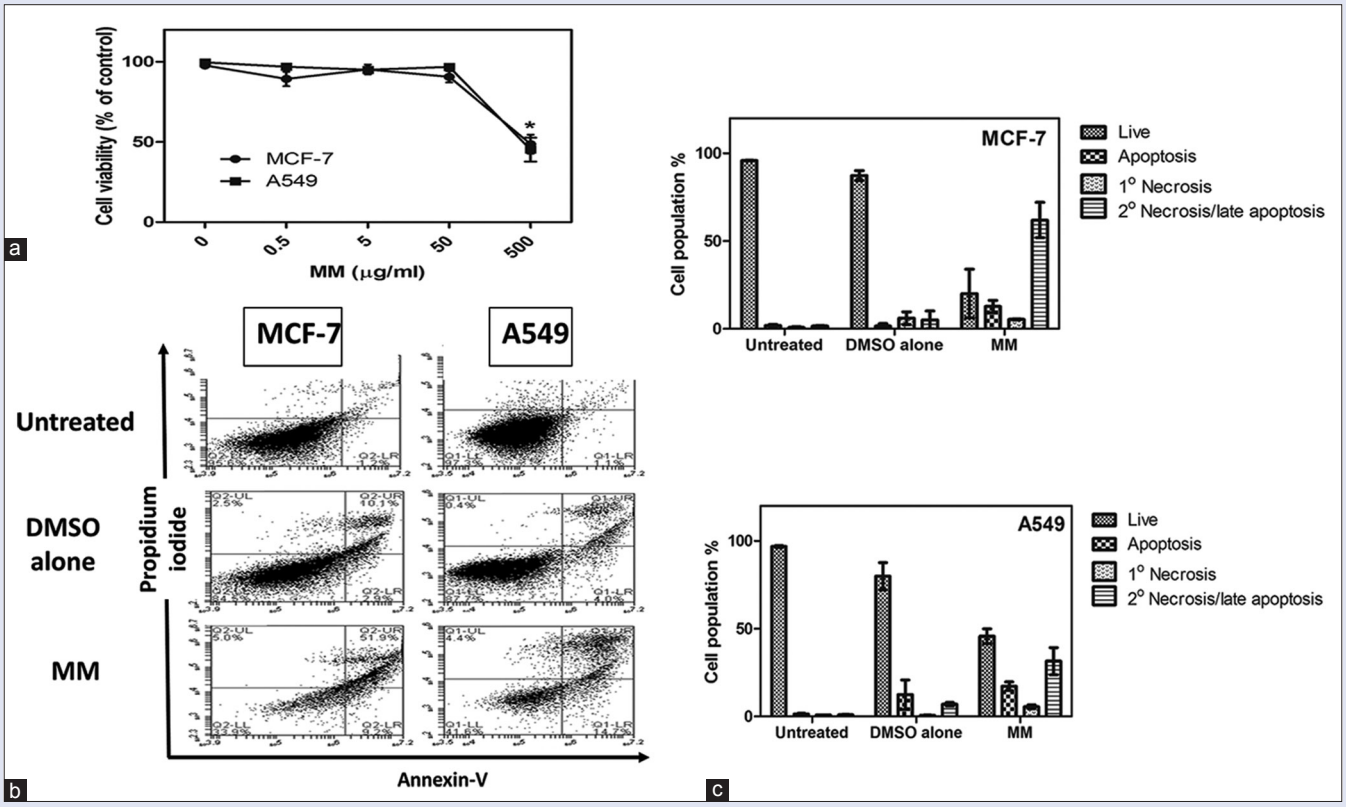

Figure 1: *Melastoma malabathricum*-induced cell death in MCF-7 and A549 cells occurs predominantly by secondary necrosis/late apoptosis. (a) MCF-7 and A549 cells were incubated with varying concentrations of *Melastoma malabathricum* over 24 h before measuring cell viability by 3-(4-, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Percentage cell viability was determined by comparing the effect of *Melastoma malabathricum* to the vehicle control (dimethyl sulfoxide). Data represents the mean ± standard error mean of four independent experiments. *$P < 0.005$, by Student's *t*-test. (b) MCF-7 and A549 cells were incubated with 500 μg/ml *Melastoma malabathricum*, dimethyl sulfoxide or no treatment for 24 h before staining with Annexin-V and PI and analysis by flow cytometry. Results are typical of two independent experiments. (c) Examples of (b) are shown, with graphs showing the mean ± range of two independent experiments

work by Roslen *et al.*[11] showed potent anti-proliferative activity of methanol leaf MM extracts (IC50 = 7.14 μg/ml) on MCF-7 cells after 72 h treatment. Flower extracts have been shown to have anti-proliferative activity on MCF-7 cells in one study[17] but not in another.[11] Thus, the concentration of the methanol MM leaf extract required to kill cells varies across different studies. Inhibition of human breast cancer cells proliferation was reported using the isolated bioactive compound, Kaempferol-3-O-(2",6"-di-O-p-*trans*-coumaroyl)-β-glucoside, from MM methanol leaf extract.[17] We hypothesize that the ethyl acetate fraction from the methanol extract may contain this compound and is responsible for the cytotoxic effects. Hence, we fractionated bioactive compounds from the methanol extracts using ethyl acetate.

Cell death of a cancerous tumor is one of the favorable outcomes in anti-cancer therapy. There are two major types of cell death, which can be morphologically distinguished from each other.[18,19] Apoptosis is a programmed cell death characterized by plasma membrane blebbing and nuclei condensation that eventually leads to the formation of "apoptotic bodies," which carry "eat-me" signals on the surface and are cleared by major scavengers such as macrophages. Classical morphologic characteristics of necrosis are cell swelling, loss of nuclear and cytoplasmic integrity, rupture of cellular membranes and release of intracellular contents into extracellular milieu. Secondary necrosis occurs when cells initially undergo apoptosis and after a certain time show signs of necrosis.

Despite initial studies linking MM to cell toxicity, the mode of cell death induced by MM has not been previously investigated. We are the first to report that the MM ethyl acetate fraction induces secondary necrotic death in cancer cells 24 h post-treatment. It is possible that cells could be dying by apoptosis at earlier times post-treatment and eventually switch to secondary necrosis over time. Unlike apoptosis, necrosis is considered immunologically harmful at all times due to the sudden release of pro-inflammatory mediators.[20] However, this notion does not withstand current experimental verification in which some apoptosis-inducing treatments caused immune-dependent tumor regression, whereas others did not.[21,22] The release of soluble factors that accompany later stages of cell death, like secondary necrosis, is advantageous in anti-cancer therapy as this type of cell death allows the efficient activation of the immune system and thereby the clearance of tumor cells from the tumor microenvironment. For instance, the early exposure of calreticulin, a Ca^{2+}-binding chaperone protein that becomes exposed on the outer leaflet of the cells during the early phase of cell death (e.g., apoptosis)[21,23,24] and followed by high-mobility group box 1-release during later stages of cell death (e.g., secondary necrosis)[25] facilitates efficient dendritic cell activation and maturation.[26] The presentation of tumor antigens by mature dendritic cells finally leads to CD4+ and CD8+ T-cell activation for T-cell mediated killing of tumor cells.

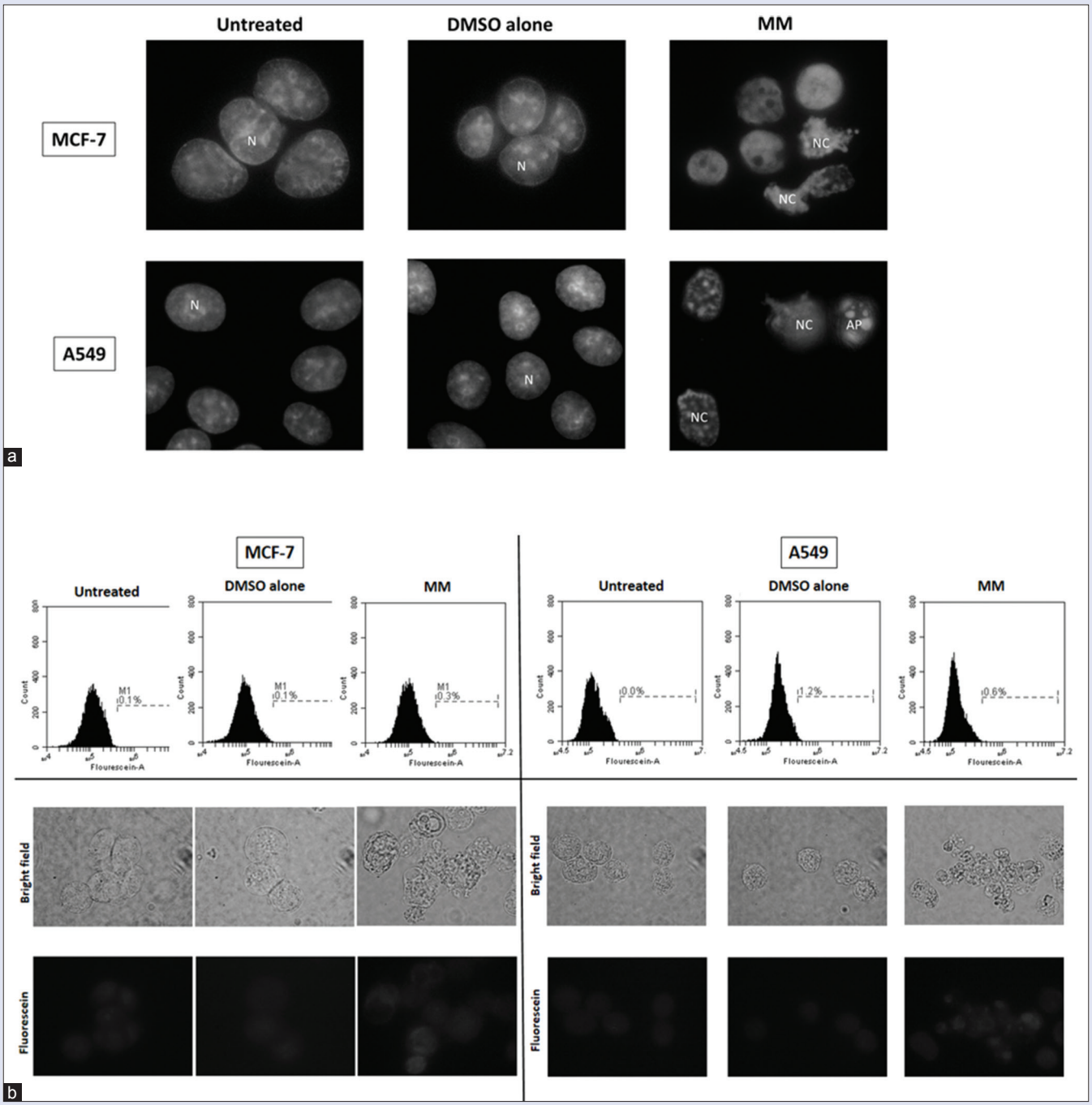

Figure 2: *Melastoma malabathricum*-mediated cell death at 24 h was not apoptotic. MCF-7 and A549 cells were fixed with 100% cold methanol at 24 h post-treatment (untreated, dimethyl sulfoxide or 500 μg/ml of *Melastoma malabathricum*). (a) Cell nuclei were stained with DAPI and observed under ×100 magnification using a fluorescence microscope. N, normal nuclei; NC, necrotic nuclei; AP, apoptotic nuclei. (b) Cells were terminal deoxynucleotidyl transferase dUTP nick-end labeling stained before measuring terminal deoxynucleotidyl transferase dUTP nick-end labeling -positive events by flow cytometry. Histogram plots show change in fluorescein intensity (terminal deoxynucleotidyl transferase dUTP nick-end labeling-positive cells) over time post-*Melastoma malabathricum* treatment. Data are representative of three independent experiments

CONCLUSION

Extracts from MM leaves show promise as potential anti-cancer agents. We will pursue isolation and characterization of the specific compound responsible for the biological activity and investigate its detailed molecular mechanism.

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Conflicts of interest

There are no conflicts of interest.

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