Future Journal of Pharmaceutical Sciences

RESEARCH

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Exploring *Clinacanthus nutans* leaf different solvent extracts on antiproliferative effects induced metastasis through apoptosis and cell cycle against MCF-7 human breast cancer cell lines

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Abstract

Background: Medicinal herbs in Malaysia like *Clinacanthus nutans* (CN) traditionally are used in the treatment of various diseases and cancers. The present research was conducted to determine the effects of *C. nutans* leaf different solvent extracts on the human breast cancer cell lines (MCF-7). The antiproliferative growth and survival effects of dichloromethane CN leaf extracts (CNDCM), as well as the short- and long-term effects through metastasis, apoptosis and cell cycle effects, were observed. The chemical profiles were done to evaluate the properties of the CNDCM.

Results: The evaluation of GC–MS identified 16 major phytochemical compounds present in this extract with biological activities. Antiproliferative assay used is the SRB assay, which showed the CNDCM induced strong antiproliferative property compared with the other extracts, so its IC_{50} dose was selected for further testing with value 108 µg/mL at 72 h after exposure on MCF-7 and MCF-10A cell lines. The clonogenic survival effects of CNDCM in various concentrations (31.25, 62.5, 125, 250 and 500 µg/mL) inhibited the ability of MCF-7 cells to form colonies, and the metastasis result was indicated in an image of wound healing assay. Moreover, the CNDCM extract significantly induced apoptosis in all the cell cycle phases. Finally, the experiments with various extract concentrations on normal human breast cell lines showed no antiproliferative effects for all the extracts tested.

Conclusion: Among all the extracts of CN, the CNDCM extracts demonstrated the highest antiproliferative activity and survival against the MCF-7 cell lines tested.

Keywords: *Clinacanthus nutans*, Dichloromethane extract, MCF-7 cell lines, Clonogenic cell survival assay, Apoptosis

Background

Cancer is one of the mortal diseases affecting the human population nowadays. This disease has become the second highest mortality causing disease in humans after cardiovascular disease. In 2020, the World Health Organization (WHO) was estimated about 24.6 million people from all over the globe were death caused by cancer [1]. Usually, cancer can be treated with chemotherapy, surgery, immunotherapy, radiation or monoclonal antibody therapies [2]. Among the various forms of cancer, breast cancer is considered the most serious and is causing health problems for women worldwide. Breast cancer leads to 15% of all deaths occurring

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in women. The cancers and types of adenocarcinomas malignant tumour with a potential of tumour cell growth are widely dependent on the immune responses, cell survival and growth in the target tissues [3]. Surgery and chemotherapy based on paclitaxel have been the standard therapies for cancer in the past two decades [4]. Currently, the treatment and therapy of breast cancer are performed with drugs, radiation therapy and surgery. However, many cases of these treatments failed due to drug resistance and toxicity; therefore, a more effective and safe anticancer treatment of breast cancer is required [1]. The development of Nobel and natural nutraceuticals for cancer remedies as the alternative treatment or therapy, with minimal side effects and cost-effective for the chemoprevention of cancer, is urgently needed [5]. The herbal extracts with anticancer properties are acting via diverse mechanisms, including the inhibition of proliferation by apoptosis, modulation of diverse cell signalling pathways, suppression of adhesion, migration of cancer cells, inhibition of topoisomerase I and cell cycle arrest [6].

The medicinal plant is one of the nutraceutical alternatives that have received considerable attention because of its presumed safety and potential nutritional and therapeutic effects [7]. Medicinal plants derivatives have been used in various cancer treatments and have gained focus for being effective with value-added sources of anticancer agents [1]. The efficiency depends on the stage of cancer, and the ethnobotanical study literature indicated that in the early days of humankind, herbs were used to maintain health and for the treatments of disease [8]. However, the side effects of medicinal herbs are still unknown, but through the supports from scientific researches, these herbs were mostly proven to be safe for consumption. Currently, there is an increasing trend in utilizing medicinal herbs to prevent health problems, especially cancers. Meanwhile, the studies of medicinal plants, like *Clinacanthus nutans*, are increasing due to their potentials as a medicinal herbal treatment for cancer.

Many plants in the Acanthaceae family have anticancer potential. *C. nutans*, also known as 'Sabah snake grass' or 'belalai gajah', is a species from the Acanthaceae family that has become a popular need in Malaysia [9]. The habitat area of this plant is sandy and peat soils. The characterization of CN is full of green leaves. CN is a traditional herbal remedy used in Malaysia, Singapore and Thailand to treat cancer [10]. Previous studies conducted to determine the phytochemical analysis of CN observed the presence of beta sitosterol, chlorophyll, vitexin, isovitexin, shaftoside, clinamide and others [11, 12]. The pharmacological effects and potentials of CN include antibacterial, antivirus, antioxidant, antiproliferative, antiinflammatory [9]) and antitherapies [13].

In recent years, CN leaf and stem extracts as alternative remedies for cancer have been popularly consumed by the Asian populations. Therefore, many cancer researches involving CN extracts have been currently done. These studies tested the effects of CN on various cancer cell lines, and the extracts were scientifically proven as a potential alternative cancer treatment and prevention for some selected cancer types [14]. CN contains natural antioxidant and anticancer compounds, as reported in the study by Arullappan et al. [15]. The treatments of CN extracts extracted from the chloroform leaves against several cell lines (K562, HepG2, IMR32, NCL-H23, SNU-1, HeLa, LS-174T, Raji and IMR32) showed significant antiproliferative activities to these cells lines [16]. For the CN methanol extracts studies, the extracts with the highest total flavonoid contents exhibited the cytotoxic effects on HEPG2 cell line [17]. The antioxidant and anticancer effects of CN extracts on human cervical cancer cell line (HeLa) were studied and successfully showed anticancer activities conferred by the CN extracts [10, 18]. A study on breast cancer cell line (MCF-7) showed that the CN extracts were exhibiting antioxidant and cytotoxic activities [19]. A recent study on the cytotoxicity potentials of CN as a medicinal herb indicated its prospects and future development in cancer research. Thus, the objective of this study was to determine the antiproliferative property of CN extracts on the human breast cancer cell line (MCF-7) and normal human breast cell line (MCF-10A). Further study was also performed to confirm the potentials of antiproliferative property through the clonogenicity, wound healing, apoptosis and cell cycle analyses of MCF-7, coupled with gas chromatography–mass spectra (GC–MS) analysis.

Methods

Plant collection and botanical identification

The plants of *Clinacanthus nutans* samples were collected and harvested from a farm located at Pongsu Seribu (HERBagus), 13200 Kepala Batas, Seberang Perai Utara, Pulau Pinang, Malaysia. The complete specimen, including roots, flowers, leaves and all parts of plant, was collected. These complete samplings were then sent to Unit Herbarium, School of Biology, Universiti Sains Malaysia (USM), for voucher specimen preparation of the herbarium. The last step involved herbarium and plant identification by an expert taxonomist from USM. The complete herbarium was deposited with a reference number (number 111536) and the voucher specimen.

Plant preparation of solvent extraction using partition coefficient

Freshly cleaned CN leaves were blended into small pieces, soaked in the methanol as solvents mixture,

then filtered and continuously soaked for three times to ensure exhaustive extraction. The soaking was making 72 h in total for the three-time soaking and each soaking involved 24 h (Fig. 1). The yield in the form of crude extracts was kept at − 20 °C until used. The filtrate was concentrated in a vacuum rotary evaporator at 40 °C until about 1/10 of the original volume was left. Then, the leftover filtrate was freeze-dried to obtain the 80% methanol crude extract. The crude was used for successive solvent and partitioning extractions with different solvents of increasing polarity, starting with hexane, dichloromethane, chloroform, butanol and aqueous. The extracts of partitioning were concentrated to dryness by using a rotary evaporator. The dried powdered extracts were then stored in screw-capped glass bottles and kept in a refrigerator at 4 °C until further use.

Water and hot water extraction

Water extraction involved two types of extractions, namely water and hot water extractions which differ by the heat used. The water extraction did not use any heat, while the hot water extractions used boiled water heated to the temperature of 60 °C. This process started with the CN leaves, and stems were separated and then washed three times with distilled water to remove microorganisms. About 500 g of the fresh leaves was blended into small pieces and soaked in distilled water for 24 h at room temperature on a shaker. The water extract was then centrifuged and filtered. This process was repeated twice. After that, the filtrate was freeze-dried to obtain the crude extract. The final yield of extract was stored in a bottle at 4 °C until used. All the steps were performed according to the protocol published by Shebaby et al. [20].

Approximately 500 g of cleaned fresh CN leaves was blended and soaked in distilled water. The mixture was incubated in a water bath at 60 °C for 24 h. The extract was then centrifuged and filtered by using Whatman No.1 filter paper. After filtration, the extract was freeze-dried and frozen at 4 °C until further used (Fig. 2).

Chemical profile of CNDCM extract (gas chromatography–mass spectrometry (GC–MS) analysis)

GC–MS analysis was performed based on the methods described by Abdul Rahim [21]. The samples were analysed by using an Agilent 7890A Gas Chromatograph

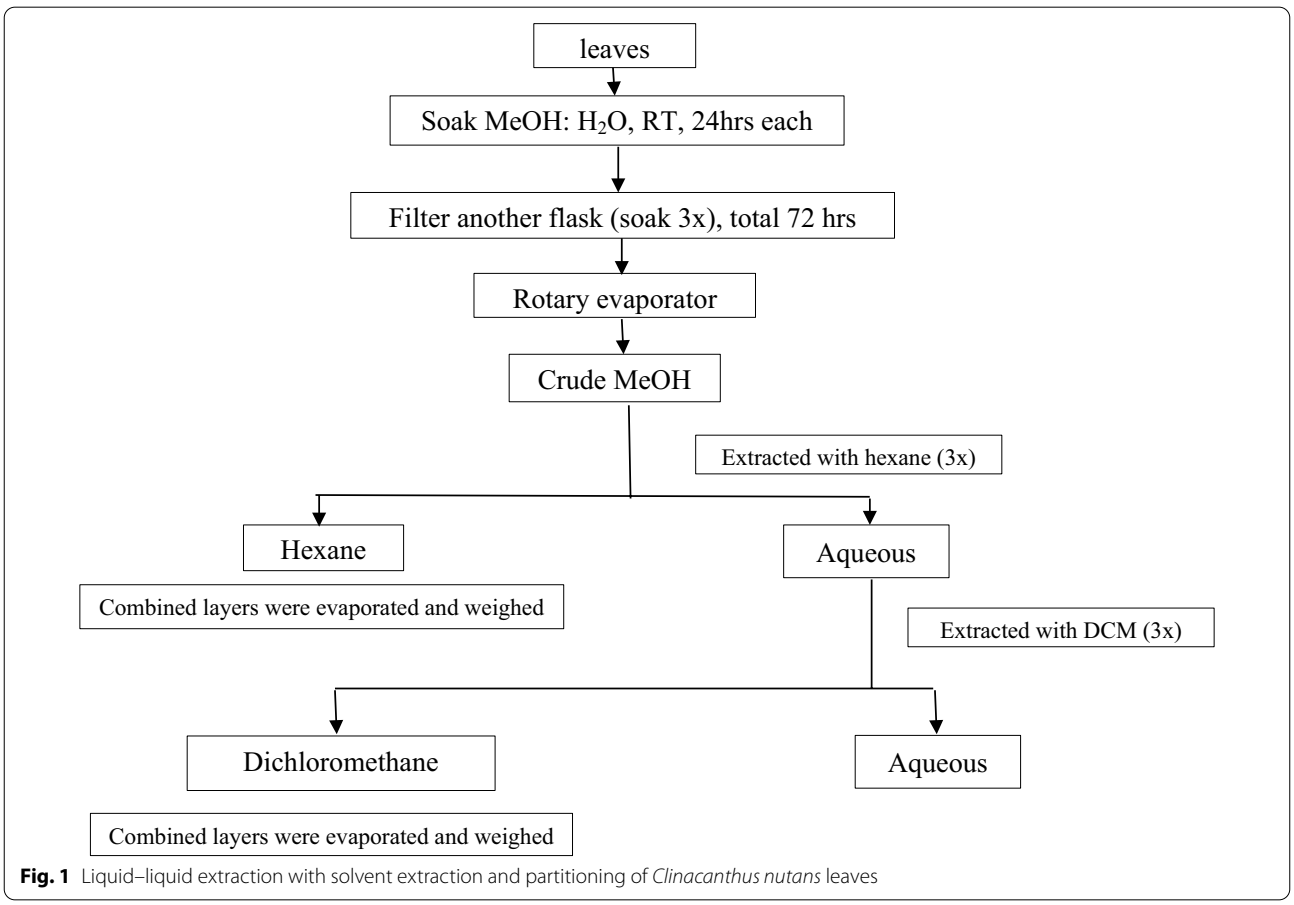

Fig. 1 Liquid–liquid extraction with solvent extraction and partitioning of *Clinacanthus nutans* leaves

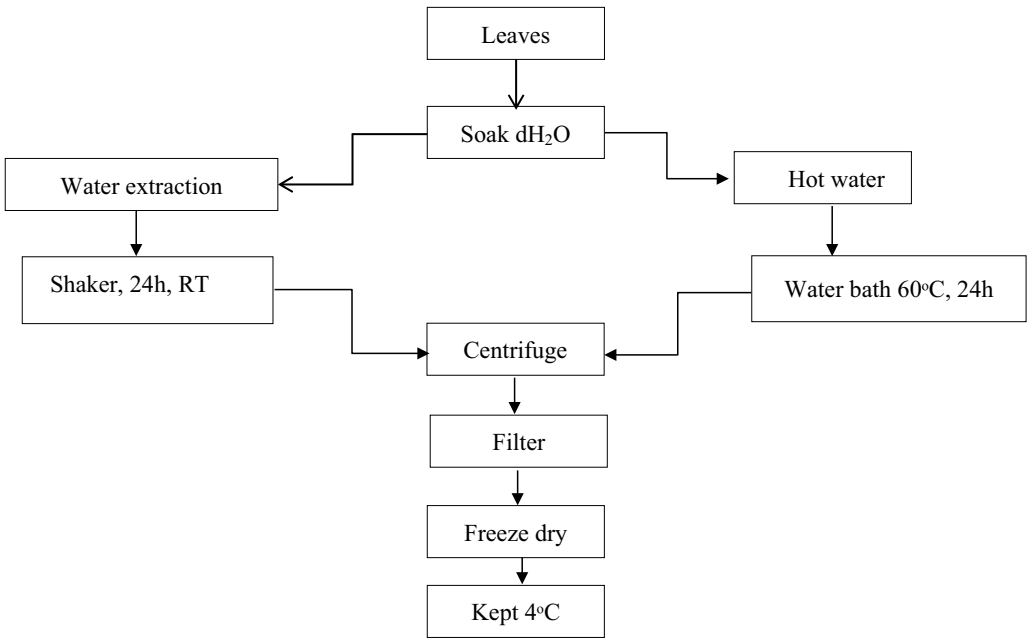

Fig. 2 Water and hot water extractions

(Agilent Technologies, Wilmington, DE, USA). Briefly, a 1 mg aliquot of an CNDCM extract was weighed and added into a GC vial. The powder was then mixed with 1 mL of methanol. Next, the 10-µL mixture was injected into the GC–MS system via splitless mode. The GC–MS system was connected to an MS/MS triple quad detector. The HP-5MS GC column with an inner column and a film thickness diameter of 0.25 mm × 30 m × 0.25 µm was used for the GC–MS analysis,. The initial oven temperature was set at 100 °C for 2 min before it was increased gradually to the final temperature of 280 °C within 17 min at a rate of 10 °C/min. Helium was used as the carrier gas with a rate of 1 mL/min. All samples were analysed in three replicates. The spectra of the chromatogram peaks were compared against the National Institute of Standards and Technology (NIST) database library to identify the retention time (RT) index of common primary and secondary metabolites.

Cell culture and human breast cancer cell line

In this study, human breast epithelial adenocarcinoma cells (MCF-7) and normal human breast cell lines (MCF-10A) were utilized with the code numbers ATCC®HTB-22™ and ATCC® CRL-10317TM, respectively. These cell lines were obtained from American Type Culture Collection (Manassas, VA, USA) and were adherent cells retrieved from breast epithelial mammary glands. Thus, they are originated from humans. MCF-7 breast cancer cells overexpress oestrogen receptors, whereas the MCF-10A cells are non-tumorigenic and non-metastatic epithelial breast cells. These cell lines were maintained in a specific complete growth medium under an atmosphere of 5% CO2 and a temperature of 37 °C in a humidified incubator. The cells were allowed to passage and grow every 2 to 3 days.

The complete growth medium for MCF-7 cells was formulated with ATCC-formulated Eagle's minimum essential medium (EMEM, catalog no. 30-2003). Two supplements, which were 0.01 mg/mL of human recombinant insulin and 10% foetal bovine serum, were added to this medium to create a complete growth medium. On the other hand, the complete growth medium for MCF-10 cells was added with base medium (MEBM) and Lonza with kit MEGM (CC-3150, Lonza) that contains bovine pituitary extract, human recombinant epidermal growth factor, insulin, hydrocortisone, gentamicin–amphotericin B mix and 100 ng/mL of cholera toxin. Upon preparation, the two different complete growth mediums were kept at 4 °C until use.

Cell proliferation assay

The cell proliferation activities of MCF-7 and MCF-10A cell lines were determined by using sulforhodamine B (SRB) assay. This assay is an efficient, inexpensive and highly cost-effective technique [22]. SRB assay is a type of in vitro antiproliferative activity assays that can be extrapolated to measure cell proliferation activity in cell-based studies [23]. The SRB assay was performed

according to the method explained by Sekehan in 1990 [24].

The cells were seeded in a 96-well plate containing 100 µL of completed growth media with a plating density of around 5×10^3 cells/well. These cells were then incubated at conditions of 37 °C, 5% CO_2, 95% air and 100% relative humidity for 24 h before the addition of CN extracts. The cells treated with the extracts were incubated for between 24 and 72 h. During the incubation period, the SRB assay was performed progressively. The treated cells were fixed with 50% cold trichloroacetic acid and then incubated for 30 min at room temperature. Next, the plates were washed with distilled water five times and dried, before the cells were stained with 100 µL of 0.4% SRB in 1% acetic acid for 30 min at room temperature. The cells were repeatedly rinsed four times with 1% acetic acid. Upon drying, 100 µL of 10 mM Tris buffer was added and the plates were shaken for 5 min. Finally, the absorbance at a wavelength of 540 nm was read by using a microplate reader (BMG LABTECH, Germany). The cell proliferation activity was measured as the percentage of absorbance by comparing it to the absorbance value recorded for the control sample. The following formula was used to calculate the cell proliferation: (absorbance of sample/absorbance of control) × 100%. The in vitro study required the calculation of IC_{50} value to determine the concentration of an inhibitor where the response is reduced half. It caused the inhibition of 50% of the cell proliferation at inhibition concentration. The IC_{50} value was interpolated from the dose–response graph curves percentage.

Trypan blue exclusion assay and clonogenic assay

MCF-7 cell lines treated and untreated with CNDCM extracts were harvested by trypsinization and centrifugation. The effects on the exposures of extracts were determined by staining the cells with trypan blue solution (0.4%). The cells were collected and counted with a haemocytometer under a microscope.

The harvested cells were then plated in a well plate, reseeded at 100 cells per well and were allowed to adhere overnight. The cells were maintained for dose extracts in a 2-mL medium and incubated for around 6 to 7 days at 37 °C with 5% CO_2. The culture medium in each of the well was removed gently by aspiration with the help of a Pasteur pipette with a fine angled tip. The cells colonies were washed with cold PBS. The wells were stained with methylene blue (1% in 50% methanol), and the colonies that contained 50 or more cells were scored as survivors. The number of surviving cells colonies was calculated and expressed as a percentage of appropriate vehicle-treated controls.

Wound healing assay

The proliferation or migration capabilities of MCF-7 cells were assessed by using a scratch wound healing assay that was slightly modified from Liang et al. [25] and Fronza et al. [26]. The MCF-7 cells were seeded at 2×10^6 cells per well and cultured for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2 in a medium containing 10% FBS to produce a nearly confluent cell monolayer. The monolayer cells were grown to confluence in a 6 well plate. The cells were subsequently treated with CNDCM at the IC_{50} concentrations of 108 µg/mL. A linear wound was introduced by using a sterile yellow pipette tip (200 mL) to produce an approximately 1-mm-wide scratch. Any cellular debris was removed by washing the wells with PBS. Pictures of the wounded areas were captured with an inverted microscope periodically until 72 h of post-treatment.

Apoptosis assay

The harvested cells at a density of 5×10^5 cells/mL were washed with 1 mL of 1X binding buffer and centrifuged at 300×*g* for 5 min. The supernatants were completely discarded, and the pellets were then resuspended with 100 µL of 1 × binding buffer. Then, the cells were stained with 10 µL of annexin V-FITC and allowed to incubate in a dark room for 15 min. Next, a 1 × binding buffer was added into each tube and centrifuged at 300×*g* for 10 min. Next, the supernatants were discarded. After that, 500 µL of 1 × binding buffer was added and the cell pellets were resuspended by pipetting. The last step of apoptosis staining was performed by adding 5µL of PI working solution, and the cells were immediately analysed by flow cytometry FACSCalibur. The fluorescence emission was measured at between 488 and 600 nm excitation wavelengths. The data analysis of FACSCalibur was performed by using the quadrant statistics for apoptotic and necrotic cells population available in the BD CELLQuest Pro software. The electronic compensation of the instrument was carried out to exclude the overlapping emission spectra of the two dyes. The fluorescence intensity was detected at the FL1-A (*X*-axis) and FL2-A (*Y*-axis) channels.

Cell cycle analysis

MCF-7 cells were seeded and cultured in a six-well plate at a density of 5×10^5 cells/mL per well. The cells were incubated overnight in a CO_2 incubator. The cells were then treated with CNDCM extract, followed the SRB assay analysis at IC_{50} dose. The cells treatment for duration times of 24, 48 and 72 h was carried out. The positive control used 4,-hydroxytamoxifen (4-TAM-OH), while the negative control was without any CN extract

treatment. After the treatment, the cells were harvested and fixed by using ice-cold 70% ethanol. The cells were then stained with FxCycle™ PI/RNase staining solution (Life Technologies Corporation, USA) by following the manufacturer's protocols. The cell cycle distribution was determined using a FACS Calibur flow cytometer (BD Biosciences, USA) with 405 nm excitation wavelength. Finally, the DNA content and the percentage of the cells at different cell cycle phases (i.e. G1, S and G2/M) were determined through ModFit LT™ software (version 3.2, Verity Software House). The data obtained were analysed in triplicate.

Statistical analysis

The results of statistical analysis were obtained by plotting the percentage of cell survival against the respective extracts concentrations used in the assay. The comparison of untreated and treated values was made by using the Student *t* test. The level of significance (*P* value) was set as less than 0.05. The values from three independent experiments were recorded for data collection.

Results

Gas chromatography–mass spectrometry analysis of active CNDCM fraction

The GC–MS analysis was performed on the dichloromethane extracts fractions obtained from the leaves of CN. Eighteen known major compounds were identified, as listed in Table 1. The compounds were identified by referring the similarity index of at least 80% in comparison with the corresponding compounds available in the NIST library. The highest relative peak area of 14.21% was shown for gamma sitosterol, beta stigmasterol and beta sitosterol. Out of the identified compounds, 18 compounds constituting hexadecanoic acid, octadecanoic acid, beta sitosterol, stigmasterol, etc., had existing anticancer evidences.

Antiproliferative effects of C. nutans extracts against MCF-7 cells

Human breast cancer cell lines (MCF-7) were treated with increasing concentrations of each extract at 24-, 48- and 72-h intervals. The various concentrations of 31.25, 62.5, 125, 250 and 500 µg/mL were used. The half-maximal inhibitory concentration (IC_{50}) values are given in Table 2, while the antiproliferative effects of the extracts are illustrated in Figs. 3 and 4. Among the six organic solvents extracted, only those from the dichloromethane (Fig. 3B) fraction had shown significant antiproliferative effects ($31.25 < IC_{50} < 500$ µg/mL) on the MCF-7 cell lines. The extracts with an IC_{50} value of greater than 500 µg/mL, as well as other extracts (from the hexane, aqueous, methanol, hot water and water fractions), did not possess much inhibitory effects on the growth of breast cancer cells. About 70% of cell viability was observed for the other extracts, except for the CNDCM extracts that had very minimal antiproliferative effect on the MCF-7 cell lines. Thus, DCM fraction extracts with the lowest value of IC_{50} (108 µg/mL) with 72 h of treatment period were selected for further analysis on the MCF-7.

DCM extract of CN showed antiproliferative against human breast cancer cells with an effect on normal cells

Human normal breast cell lines (MCF-10A) were incubated with various extracts concentrations for 72 h. The findings indicated that all the extracts did not show any inhibitory and antiproliferative effects, as shown in Fig. 4. In addition, the maximal inhibitory effects (IC_{50}) values were more than 500 µg/mL.

The colony-forming ability of treated cells (clonogenicity assay)

The clonogenic assay was performed to determine the long-term consequences of CNDCM fraction extracts treatment on the survivability of the cells, while the clonogenic growth assay was carried out to determine the replicate ability of breast cancer MCF-7 cells. Figure 5 indicates the inhibition significance of colonies forming in the breast cancer cell lines, by showing that this situation occurs in a dose-dependent manner at the various concentrations (31.25, 62.5, 125, 250 and 500 µg/mL) of CNDCM fractions extracts. The colony-forming ability of breast cancer cell lines was statistically decreased in a dose-dependent manner after 72 h of treatment.

Wound healing properties evaluated with the scratch assay

Scratch assay techniques in wound healing assay are normally used to determine cells proliferation and migration, two processes that have a vital role in the wound healing action. The optimum incubation of 72 h was confirmed for the MCF-7 cells treated with CNDCM fraction extracts. The scratch assay was performed, and the results achieved indicated that the CNDCM fractions extract inhibits the in vitro migration of the highly metastatic MCF-7 cells, as reported in Fig. 6. The treated MCF-7 cells were not able to fill the gap between the cells, while the untreated cells filled the gap surface within 72 h. These results confirmed that the CNDCM suppresses the migration of MCF-7 breast cancer cells.

Assessment of apoptosis analysis by flow cytometry

The MCF-7 cells were treated with CNDCM (108 µg/mL) for 72 h. The resulted cells apoptosis was compared with the apoptosis effects of the control (untreated cell) and the positive control (the cells treated with

Table 1 Phytochemical constituents compounds identified and biological activities of dichloromethane *Clinacanthus nutans* leaf extracts by gas chromatography–mass spectrometry (GC–MS)

No	Identified/similar compounds	Retention time (min)	Peak area (%)	Molecular weight (g mol^{-1})	Biological activities	Molecular formula
1	Pentadecanoic acid, 14-methyl-methyl ester	19.940	3.40	270.4	No activity reported	$C_{17}H_{34}O_2$
2	Hexadecanoic acid, methyl ester	19.940	3.40	270.45	Antioxidant, flavour, hypocholesterolemic, nematicide, antiandrogenic, cancer preventive	$C_{17}H_{34}O_2$
3	Diphenyl sulfone	20.260	0.36	218.27	Antimicrobial, Antibacterial and antifungal	$(C_6H_5)_2SO_2$
4	9,12-Octadecadienoic acid, methyl ester	24.849	0.96	294.4721	Antiinflammatory, hypocholesterolemic, cancer preventive, nematicide, hepatoprotective, insectifuge, antihistaminic, antieczemic, antiacne, antiandrogenic, antiarthritic, anticoronary	$C_{19}H_{34}O_2$
5	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	24.849	0.96	294.5	Antiinflammatory, hypocholesterolemic, cancer preventive, nematicide, hepatoprotective, insectifuge, antihistaminic, antieczemic, antiacne, antiandrogenic, antiarthritic, anticoronary	$C_{19}H_{34}O_2$
6	9,15-Octadecadienoic acid, methyl ester,(Z,Z)-	24.849	0.96	294.5	Hypercholesterolemic, antiarthritic, hepatoprotective, antiandrogenic, antihelmintic, antihistaminic. Antieczemic	$C_{19}H_{34}O_2$
7	9,12,15-Octadecatrienoic acid, methyl ester	25.119	3.58	292.4562	Antiinflammatory, hypocholesterolemic and antiarthritic	$C_{19}H_{32}O_2$
8	9,12,15-Octadecatrien-1-ol,(Z,Z,Z)-	25.119	3.58	264.4	Antimicrobial	$C_{18}H_{32}O$
9	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)	25.119	3.58	292.4562	Antiinflammatory, hypocholesterolemic and antiarthritic cancer preventive, hepatoprotective, antioxidant and hypocholesterolemic	$C_{19}H_{32}O_2$
10	Octadecanoic acid, methyl ester	26.097	1.25	298.5	Antiinflammatory, hypocholesterolemic and antiarthritic	$C_{19}H_{38}O_2$
11	.gamma.-Sitosterol	39.188	14.21	414.718	Antidiabetic	$C_{29}H_{52}O_2$
12	Stigmasterol, 22,23-dihydro-	39.188	14.21	412.7	Antihypercholesterolemic activity, antiinflammatory, anticancer	$C_{29}H_{48}O$
13	.beta.-Sitosterol	39.188	14.21	414.718	Antihypercholesterolemia, anticancer	$C_{29}H_{50}O$
14	.beta.-Amyrin	42.275	7.36	426.7	Antinociceptive and antiinflammatory	$C_{30}H_{50}O$
15	.alpha.-Amyrin	42.275	7.36	426.7	Antinociceptive and antiinflammatory	$C_{30}H_{50}O$
16	9-Octadecenamide, (Z)-	45.683	1.79	281.48	Antiinsomnia	$C_{18}H_{35}NO$

4-OH-TAM), as shown in Fig. 7. The effects on control group showed that the cells were highly viable, with about 90.64% being measured, as compared to the IC_{50} (34.67%) and the positive control group (41.87%). The CNDCM fractions extract resulted in necrosis (2.55%), late apoptosis (11.73%) and early apoptosis (51.05%). In addition, the analysis of CNDCM fractions extracts indicated that the percentage of cell distribution was higher in the early apoptosis stage.

Evaluation of cell cycle analysis by flow cytometry

The IC_{50} concentrations of CNDCM fractions were used for the effects of extracts evaluation based on the cell cycle analysis of MCF-7 cells. The results of the effect on the cell cycle are shown in Fig. 8. The cells were incubated with the extracts for 72 h with IC_{50} dosage concentration CNDCM. The results showed that the percentage of cell cycle arrest was decreased in all the G0/G1, S and G2/M phases of cells treated with the CNDCM fractions extracts.

Table 2 Effects of *Clinacanthus nutans* water and fractions extracts against human breast cancer cells (MCF-7) and human normal breast cell line (MCF-10A)

Extracts	Inhibition concentration (IC_{50}µg/ml)	
	MCF-7 cells	MCF-10A cells
Hexane	312.5	> 500
Dichloromethane	108	> 500
Methanol	> 500	> 500
Aqueous	> 500	> 500
Hot water	> 500	> 500
Water	> 500	> 500

The half-maximal inhibition concentration (IC_{50}) of antiproliferative activity at various concentrations of 72-h treatment. All values are presented as mean ± SD of three replicates

Discussion

Clinacanthus nutans in the Acanthaceae family is one of the medicinal plants with a high value in Southern Asian countries, such as Thailand, Malaysia and Indonesia [27]. In Malaysia currently, many cancer patients have testified and claimed that they recovered from cancer after drinking a decoction of *Clinacanthus nutans* leaves extracts [28]. This traditional medicinal herb is highly debated for its effectiveness as a treatment to cure and prevent cancer. Women with breast cancer were reported to have up to 64% of traditional and complementary medicinal treatments, but only a few of these therapies have been tested scientifically [29]. Traditionally, people usually consume the CN leaves as in the juice or hot tea forms; thus, in this study, the preparation of CN extracts involved water and solvent extractions as a comparison. All the extractions were tested against the human breast cancer cell lines (MCF-7) because this cell line is the most sensitive and is mainly affected by its hormone dependent characteristics with the ability to express oestrogen receptors [30]. Thus, this early stage of study to discover potential of effects CN leaves on the cancer cells.

The researches on the anticancer effects of *Clinacanthus nutans* leaves have been increasing from year to year since 2014. In 2017, Ng [6] and her colleagues reported the antiproliferative effects of boiled water extractions and different polarities extraction of CN leaves and also showed that the bioactive compounds from the extracts reflected in the antiproliferative assay might contribute to anticancer effects. The sulforhodamine B (SRB) antiproliferative assay was used in this experiment to measure cell proliferation in an *in vitro* study to indicate the effects on the human breast cancer cell lines (MCF-7) and normal human breast cell lines (MCF-10A), as shown in Figs. 3 and 4, respectively . SRB is a protein stain that is used for in vitro chemosensitivity testing and is thus more sensitive than the MTT assay, as it demonstrated better linearity with the cell number and with higher reproducibility used for cytotoxic screening evaluation [31]. Both of the cells were exposed to water and organic solvent extractions with different polarities at 24, 48 and 72 h. The solvent organic extractions used in this experiment depend on the relative polarities of solvents, such as hexane (0.009), dichloromethane (0.309) and methanol (0.762).

Among the extractions tested on MCF-7, the dichloromethane CN extracts indicated highly significant antiproliferative activity at 72 h, as compared to the other extracts (hexane, aqueous, methanol, hot water and water). The graph showed that the DCM extraction (Fig. 1B) inhibited the cells proliferation in a dose-dependent manner. The half-maximal inhibition concentration (IC_{50}) of DCM was shown to be 108 µg/mL and hexane was 312.5 µg/mL. These values were interpolated from the percentage viability cells curves from the SRB experiment (as given in Table 2). Meanwhile, the methanol, aqueous, water and hot water extracts did not show any inhibition of proliferation. As the CNDCM extracts showed the highest significant antiproliferative effects on the MCF-7 cells, the extracts were chosen for the further experiments involving GC–MS analysis, growth, survival, apoptosis and cell cycle analyses. It was evident that the plant extracts, after 72 h of treatment, exhibited antiproliferative activities to the cells in a dose-dependent manner. Moreover, all the extracts were efficient, based on the lowest IC_{50} value (as listed in Table 2). These findings suggested that the antiproliferative effects activity might be exhibited by the CNDCM extract of the plant. However, it is not considered as an active anticancer agent, according to the standard set by the National Cancer Institute, because the IC_{50} value was measured to be > 20 µg/mL. Previous study on other nonpolar and semipolar extracts of CN such as chloroform, ethyl acetate, dichloromethane, hexane and petroleum ether demonstrated a higher antiproliferative activity than polar extracts (aqueous and methanol) [10].

Currently, gas chromatography–mass spectrometry (GC–MS) was established as an effective phytochemical technology that analyses the polar solvents extracts and volatile oils with bioactive compounds [2, 32]. The analyses of gas chromatography and mass spectrometry tests on the CNDCM leaves extracts are given in Table 1. The analyses resulted in 16 major chromatogram peaks of the compounds, and these were identified based on more than 80% similarity matching index with the compounds listed in the NIST library. At 39.19 min, the highest peak with 14.21% of peak area was obtained, and this identified three different compounds, such as gamma sitosterol, stigmasterol,

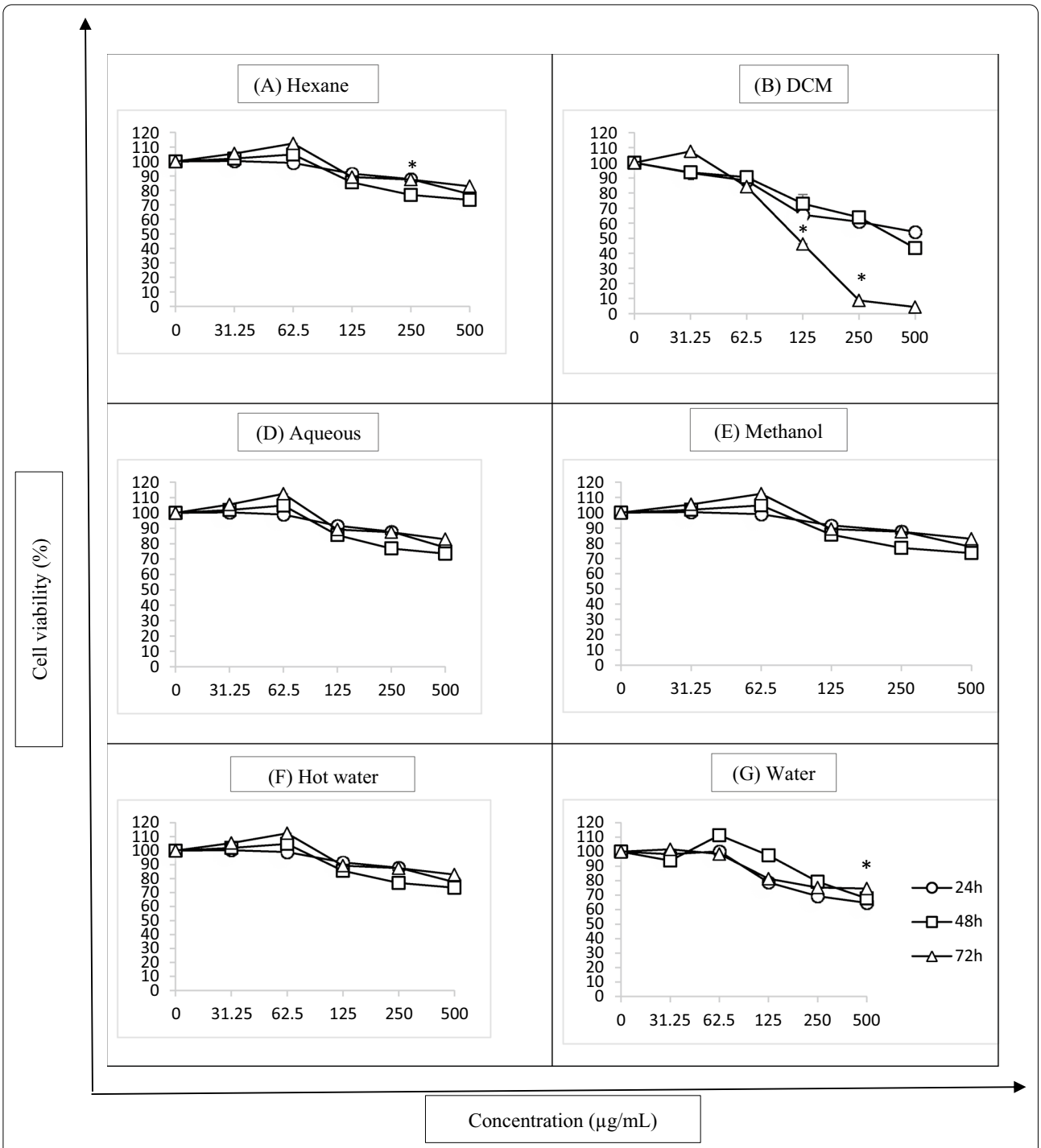

Fig. 3 Antiproliferative effects of *Clinacanthus nutans* fractions extracts in MCF-7 cell lines. The cancer cells were incubated and treated with increasing concentrations of different solvent extracts (hexane, DCM, aqueous, methanol, hot water and water). Various concentrations were used from 31.25 to 500 µg/mL for 24, 48 and 72 h. The antiproliferative effect was measured by SRB assay. Data shown are the mean values ± SD for three independent experiments, and statistical analysis was determined using Student's *T* test with $^{*}P < 0.05$ significantly different to control untreated cells

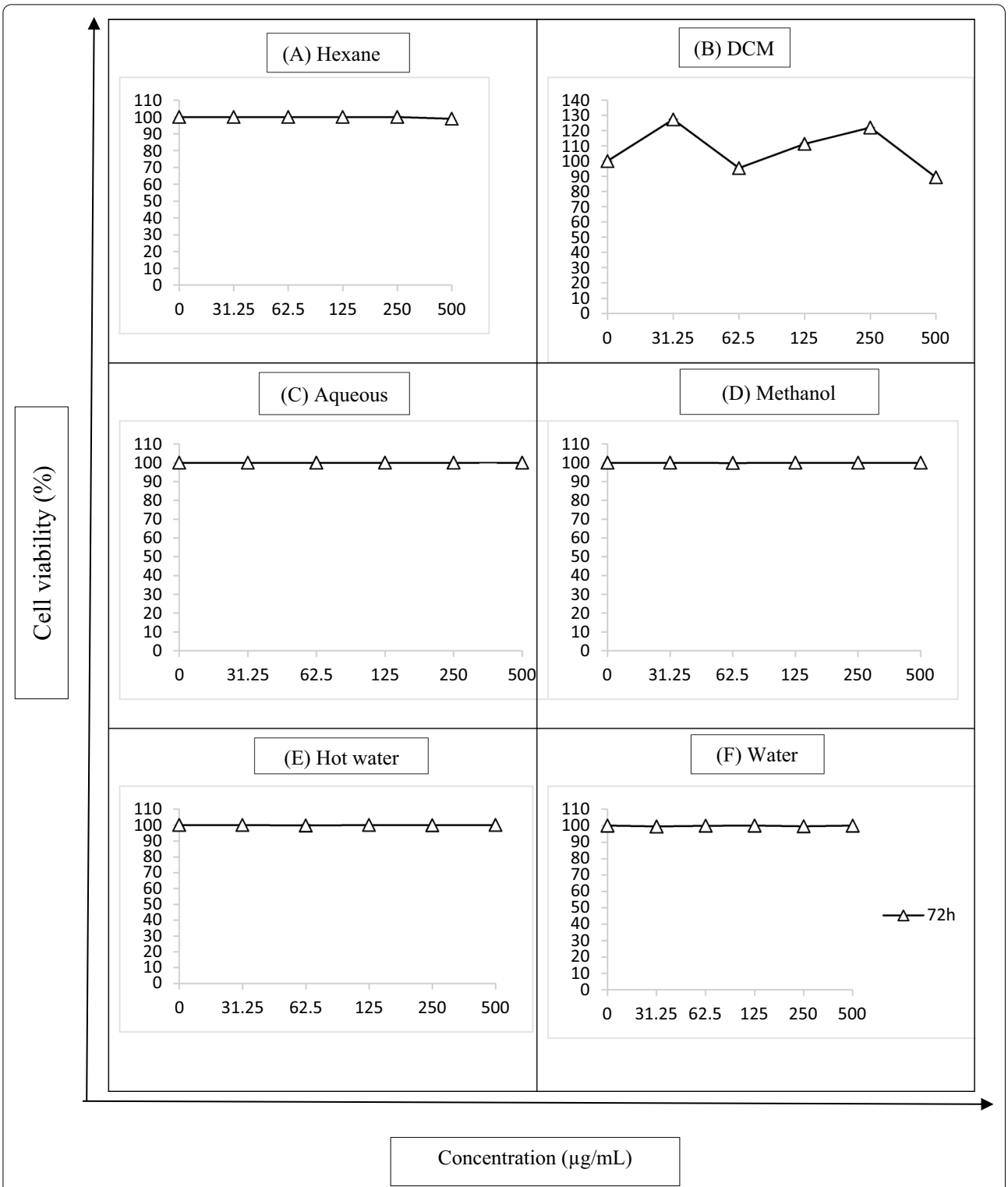

Fig. 4 Antiproliferative effects of *Clinacanthus nutans* fractions extracts in MCF-10A cell lines. The cancer cells were incubated and treated with increasing concentrations of different solvent extracts (hexane, DCM, aqueous, methanol, hot water and water). Various concentrations were used from 31.25 to 500 µg/mL for 24, 48 and 72 h. The antiproliferative effect was measured by SRB assay. Data shown are the mean values ± SD for three independent experiments, and statistical analysis was determined using Student's *T* test with $*P < 0.05$ significantly different to control untreated cells

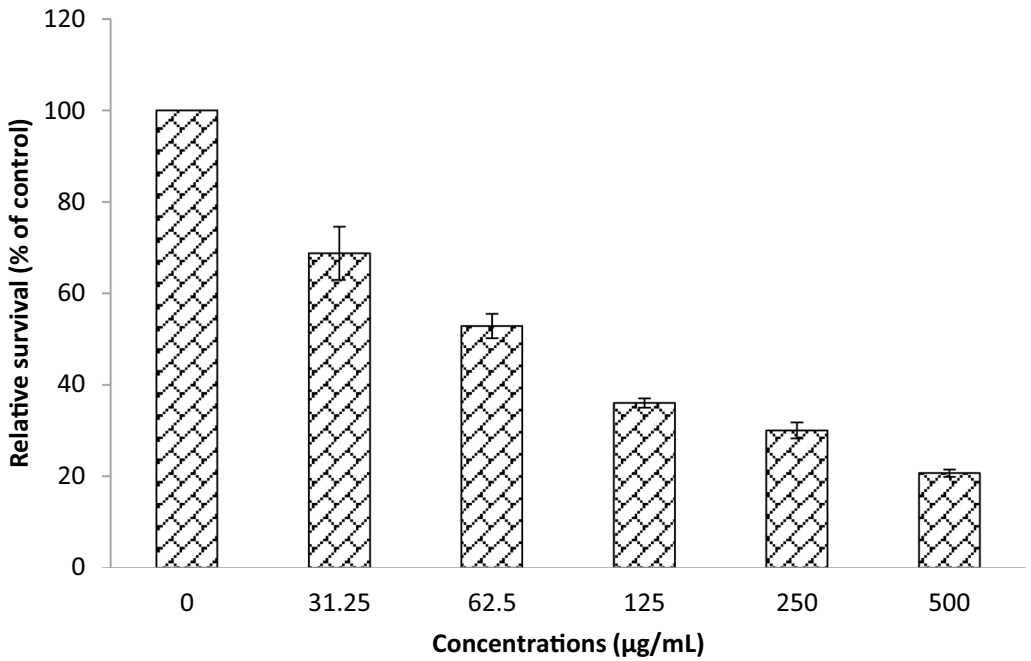

Fig. 5 Effects of CNDCM fractions extracts on the clonogenic activity of human breast cancer cells. The relative survival cells of clonogenicity assay result when treated with various concentrations of CNCDM after 72 h. All data expressed as mean ± SD compared with corresponding controls

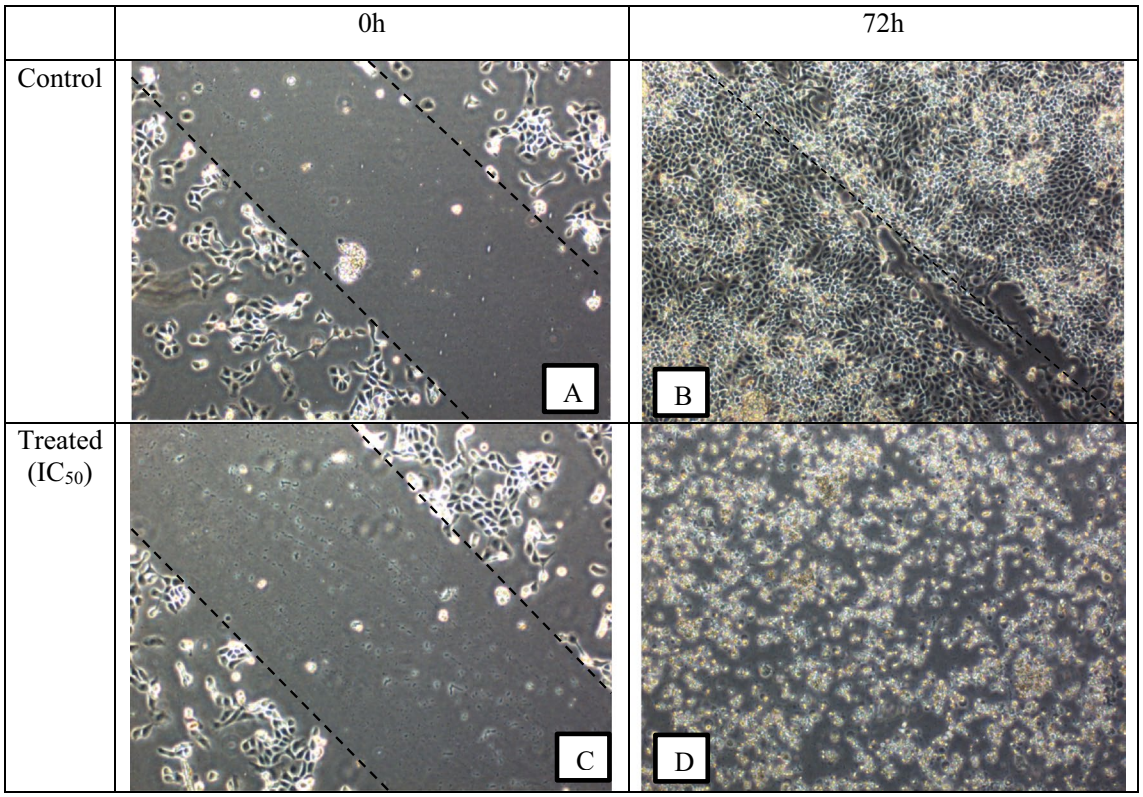

Fig. 6 Representative wound healing assay image over 72-h period detected by the inverted microscope. **A**, **B** Cellular migration of control cells MCF-7. **C** and **D** Cellular migration of cells treated with IC_{50} dose of CNDCM fractions extracts. From these images, it is clear that cellular migration is slowed and not healed, while the presence of CNDCM inhibits proliferation. The control images show a completely 'healed' scratch after 72 h

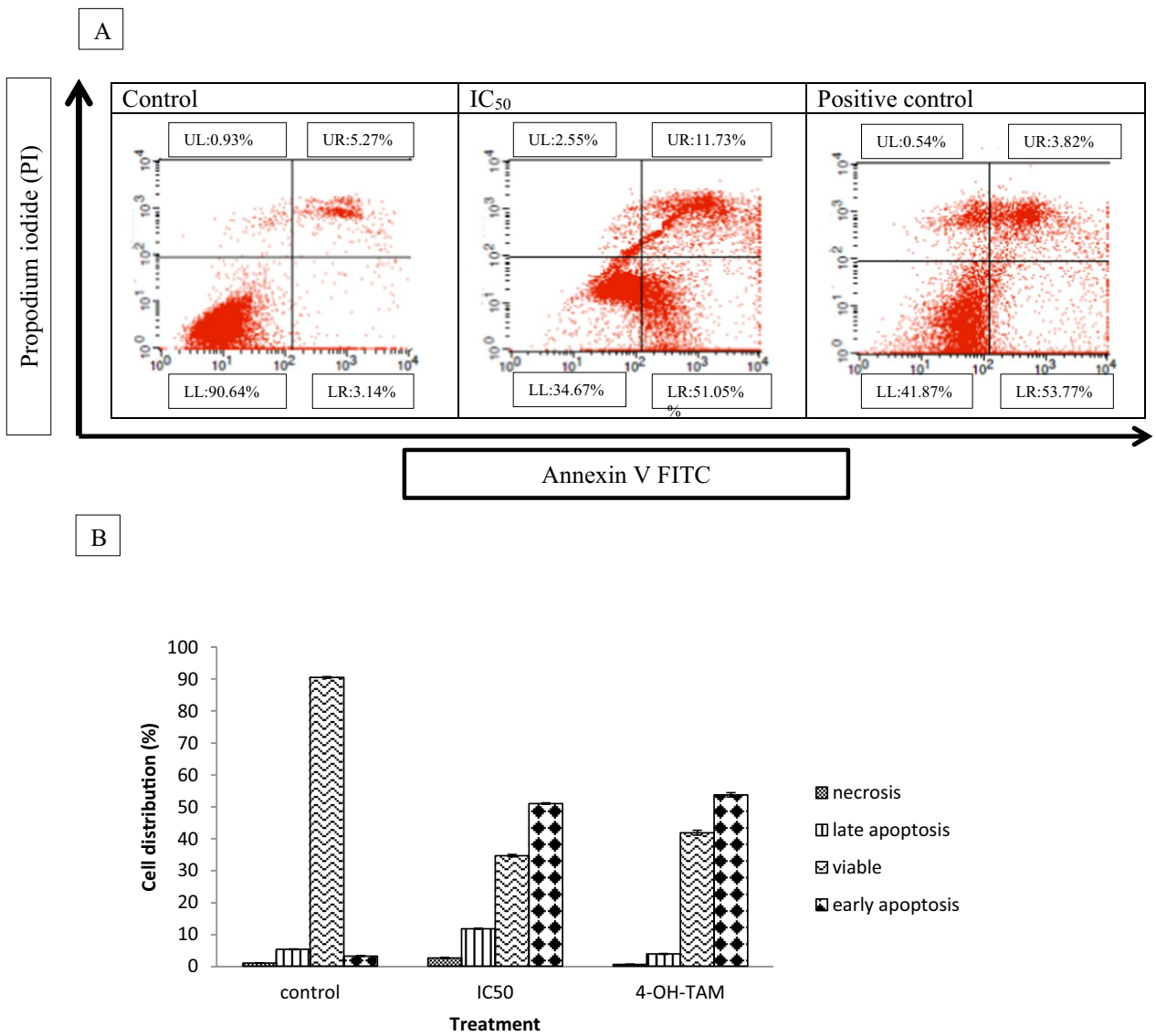

Fig. 7 Dot plots for flow cytometry analysis of the apoptosis effects MCF-7 cells treated with IC_{50} CNDCM for 72 h. MCF-7 cells were exposed to vehicle control, IC_{50} CNDCM and positive control. **A** Dot plots and **B** histogram for apoptosis analysis. **A** Quadrant location for the representative dot plots: UL (upper left)—$FITC^-/PT^+$ (necrotic cells); UR (upper right)—$FITC^+/PI^+$ (late apoptotic cells); LL (lower left)—$FITC^-/PI^-$ (viable cells); LR (lower right)—$FITC^+/PI^-$ (early apoptotic cells). **B** Percentage of cell distribution of MCF-7 cells as determined by annexin V-FITC and PI staining. Asterisks signify significance statistically at $p < 0.05$ compared to control (untreated MCF-7 cells) and positive control (4-OH-TAM). The percentage of the MCF-7 cell population was increased significantly for both IC50 of CNDCM extracts and positive control of treatment when compared with the control group

22, 23-dihydro and beta sitosterol. The potential compounds with anticancer property were reported as stigmasterol, 22, 23-dihydro and beta sitosterol. Other compounds that have the potential of being anticancer were identified as hexadecanoic acid, 9, 12-octadecadienoic acid and 9, 12, 15-octadecatrienoic acid and methyl ester (Z,Z,Z). Many compounds from the methyl ester group have shown anti-tumour and cytotoxic effects, in addition to have demonstrated evidence that suggests the ability to produce high cytotoxic effects on MCF-7 cells [33, 34]. *Clinacanthus nutans* is in the family of Acanthaceae that has the largest medicinal plant species and is well known to provide effective traditional benefits against human health [35]. The antiproliferative compounds found in the GC–MS analysis of CNDCM fractions extracts can

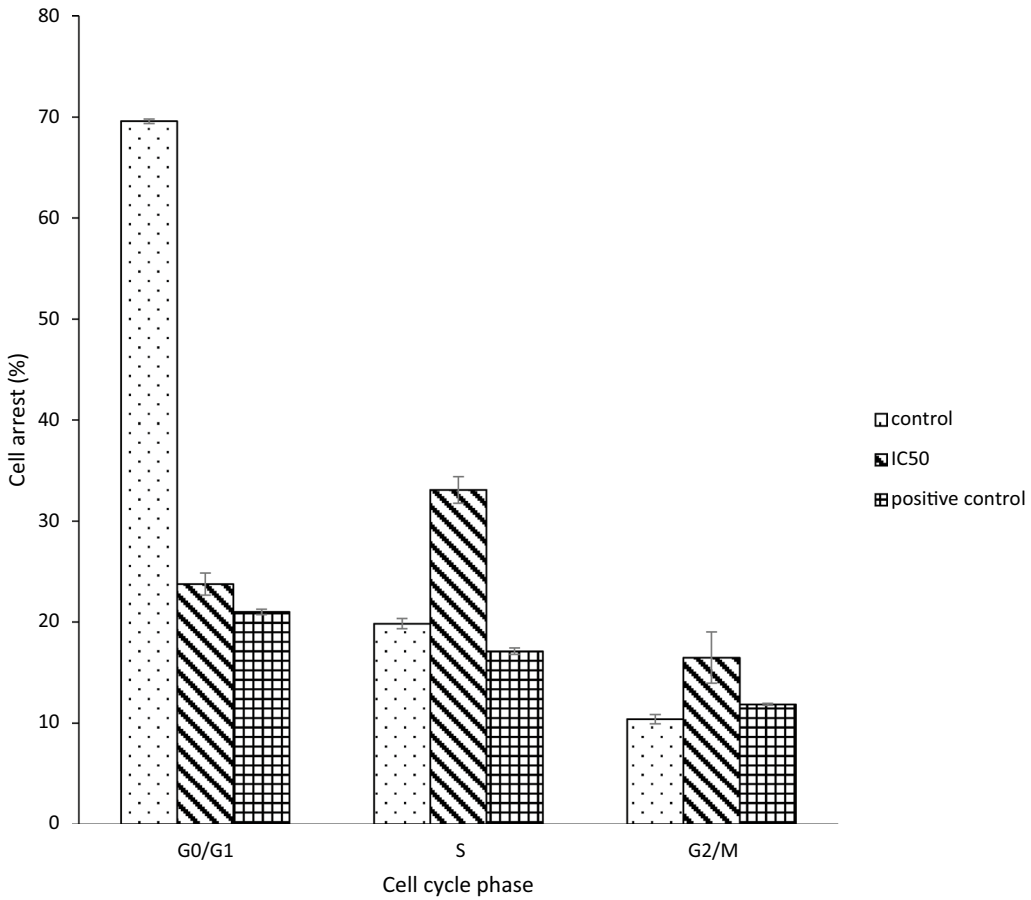

Fig. 8 Effects of the IC_{50} CNDCM leaf extracts on the flow cytometric cell cycle analysis of the MCF-7 cells. The cells were treated for 72 h. The statistical significance at $p < 0.05$ compared to control

inhibit the growth of cells and can be potentially used in the treatment of cancer [36]. These results implied that the CNDCM fractions extracts from the leaves of this plant have major compounds that contribute to the antiproliferative effects.

The function of cancer therapy is to inhibit and kill cancer cells. Chemotherapy is a therapy that used chemotherapy drugs that destroy whole cells, normal and cancer cells [37]. Thus, the development and discovery of breast cancer treatments need to be safe as well on normal cells [38]. In this study, all the extracts exposed to the human normal breast cancer cell lines (MCF-10A) for 72 h did not show any effects or inhibition (Fig. 4). These results showed that all the extracts tested at various concentrations (31.25, 62.5,125, 250 and 500 µg/mL) are safe to normal cells, when compared with the control group. Similar findings have supported a study by Teoh (2021) that had observed that the CN hexane and dichloromethane extracts exhibited less inhibitory effects on normal breast cells [28]. In addition, another similar study that used aqueous and methanol CN extracts for the treatment of normal kidney cells (Vero) also showed no cytotoxicity effect [18].

A long-term cell assessment of the proliferation growth survival after treatment with CNDCM extracts was determined by the cells colony-forming ability (clonogenic assay). The principle of colony formation assay is based on the surviving ability of the single cell to form a colony that contains at least 50 cells [39]. The CNDCM extracts treatments carried out in our study appeared to inhibit the colony-forming ability of human breast cancer cells in a dose-dependent manner (Fig. 5). The cells treated with > 500 µg/mL of extract were unable to generate colonies and were not able to take up the dye, thus being more likely to represent cell death.

A simple and effective experiment of wound healing assay was continued to determine the effects of treatment on the cell proliferation and migration, the vital role in the wound healing process. The confluence monolayer of cells was scratch to make a wound, and

then, the growth and migration of the cells were monitored after the treatment with IC_{50} of CNDCM fractions extracts. The images of wound scratch on the monolayer were captured the beginning and after regular intervals to study the cells proliferation and migration abilities [25]. The results from this wound study reported that the cells treated with CNDCM fractions extract were not able to promote cellular migration and proliferation in vitro, as compared to the control cells group that successfully proliferated and migrated towards closing the wound (Fig. 6). These migration effects provide a deeper understanding on the importance to explore the metastasis effects of CNDCM fractions extracts on the human breast cancer cells. The metastasis process is made possible by the ability of the cancer cells to migrate through the bloodstream and subsequently causes the worsen of cancer and reduce the chances of life survivability considerably [40]. The results of CNDCM fractions extracts indicated the antimigration and antiproliferation effects of the compounds at the IC_{50} dosage concentration. The roles of cell migration include the development and maintenance of the function of multicellular organisms, while the aberrant of cell migration is proven to cause many pathological disorders, not only in cancerous diseases but also in atherosclerosis [41].

Natural programmed cell death or apoptosis assessment was based on annexin V- FITC and PI double staining using a flow cytometer. The apoptosis process involved loses of plasma membrane integrity resulting in phosphatidylserine (PS) residues exposed to the outer membrane, detectable with annexin V, while PI, a DNA binding dye, is permeable in dead cells. Both dye combinations are recommended for the identification of early and late apoptotic cells [42]. The apoptosis result indicated the IC_{50} CNDCM fractions extracts were induced MCF-7 cells compared with control and positive control, 4-hydroxytamoxifen at 72 h (Fig. 7). The percentage of cell distribution of CNDCM fractions extracts showed the highest result in early apoptosis (51.5%) and the same result with the 4-hydroxytamoxifen group (53.77%). 4-Hydroxytamoxifen (4-OH-TAM) is an oxidative metabolite of tamoxifen which is important as a chemotherapeutic agent for the treatment of breast cancer. These showed IC_{50} CNDCM fraction extract exhibits antiproliferative effects and proapoptotic activities on human breast cancer cell lines (MCF-7). The previous investigation used CN root extracts showed the same effect of antiproliferative and apoptotic on breast cancer cells [28]. The information of CNDCM fractions phytochemical constituent potentials may contribute to the antiproliferative and apoptosis activity that useful for novel development of anticancer treatment.

Apoptosis is a mechanism where the cells undergo death through the response to DNA damage or a mechanism to control cells proliferation and death through the cell cycle [43]. Thus, the results of IC_{50} on the antiproliferative effects of CNDCM fraction extract revealed that the death of cells was mainly due to the induction of apoptosis and cell cycle arrest, as compared to the cells treated with 4-hydroxytamoxifen as the positive control. The result showed that the IC_{50} of CNDCM fraction extract had induced a high percentage of cell cycle arrest at all the cell cycle phases (Fig. 8). The findings indicated that the IC_{50} of CNDCM fraction extract treatment was able to inhibit the cell growth at G1 phases, induced the DNA replication at the S phase and decreased the process of mitosis or cell division at the M phase. The previous study on liquid partitioning of CN leaf extract through methanol extraction also observed the proliferation inhibition of cervical cancer cells treated with the dichloromethane fraction, possible by inducing apoptosis and cell cycle arrest [44]. The important part of the GI cell cycle phase is that the signal to allow the cells to enter the cell division stage is given in this phase. Many of the traditional plants were reported to confer cell cycle arrest to various cancer cells lines during the G1/S phase transition [40]. The results from the flow cytometric analysis in the current study also demonstrated the accumulation of treated MCF-7 cells in the G1 phase. A study on HeLa treated with IC_{50} of 70 µg/mL methanol dichloromethane fraction at 48 h reported the induction of apoptosis and cell cycle arrest at the S phase by decreasing the DNA synthesis activity [10]. The lacking of normal cancer cell growth controls is potentially caused by the exhibiting loss of cell cycle control and growth signal self-sufficiency [45]. Data from this preclinical study suggested that the antiproliferative activity of CNDCM fraction extract is most probably linked to cell cycle arrest and the induction of cell apoptosis.

Conclusions

In conclusion, the CNDCM leaf extracts fraction was shown to contain biochemical active compounds that are important to prevent and treat breast cancer. The presence of active compounds in this extract may provide synergic effects that could contribute to the short- and long-term antiproliferative effects against the human breast cancer cell lines. The antiproliferative property of CNDCM fraction extract was revealed by causing rapid cells death in the MCF-7 cells via the induction of apoptosis and cell cycle arrest mechanisms in all the phases. Furthermore, CNDCM fraction extract was observed to suppress the migration of MCF-7 cells, but not in normal cells. An additional experiment can be conducted to identify the

mechanism and pathway of breast cancer. Further studies are warranted for the identification, separation and isolation of the active single compounds found in the CNDCM fraction extract that are responsible for antiproliferative activities of breast cancer cells.

Future studies involving the identification, separation and isolation, as well as purification techniques, can be conducted in order to reveal the phytochemicals in all the extracts, focusing on the potential anticancer compounds for breast cancer. The mechanistic study can also be carried out to identify novel apoptosis marker further, while pathway analysis study within silico research can also be done. To complete the study on CN leaves, in vivo animal study can also be performed as this would be important in providing evidence for anticancer interaction and can further lead to a clinical trial to confirm the safety of CN extracts for human consumption.

Abbreviations

CN: *Clinacanthus nutans*; CNDCM: Dichloromethane leaves extract; MCF-7: Michigan Cancer Foundation 7; GC–MS: Gas chromatography–mass spectrometry; WHO: World Health Organization; NIST: National Institute of Standard and Technology; RT: Retention time; SRB: Sulforhodamine; MTT: MTT,3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DCM: Dichloromethane.

Acknowledgements

The authors warmly acknowledge support and facilities provided by Advanced Medical and Institute, Universiti Sains Malaysia.

Author contributions

ZMT was involved in data collection and analysis and manuscript writing. NHH was involved in data collection and analysis. NNSNMK and MK were involved in manuscript writing—editing and conceptualization. . HA was involved in supervision, manuscript structure and editing. All authors have read and approved the manuscript.

Funding

This research was funded by a FRGS grant (203/CIPPT/6711340) provided by the Ministry of Higher Education, Malaysia, and a USM Bridging Grant (304/CIPPT/6316239), Universiti Sains Malaysian, which was awarded to Hasni Arsad.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Declarations

Ethics approval and consent to participate

Clinacanthus nutans plants were used in this study. The plants were collected and harvested from a farm located at Pongsu Seribu (HERBagus), 13200 Kepala Batas, Seberang Perai Utara, Pulau Pinang, Malaysia (GPS longitude 100.4457376 and latitude 5.4959299). The plant was identification by Dr Rahmad Zakaria and deposited at Unit Herbarium, School of Biology, Universiti Sains Malaysia, under voucher number 111536.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Received: 5 July 2022 Accepted: 2 November 2022
Published online: 02 December 2022

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