ORIGINAL ARTICLE

Effects Of *Clinacanthus nutans* Leave Extracts on the Functional Roles of Drug-Resistant HER2 Expressing Breast Cancer Cells Using *In Silico* And *In Vitro* Assays

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ABSTRACT

Introduction: *Clinacanthus nutans* have increasingly been associated with anticancer properties. This can be attributed to myricetin an active compound present in the leaves of *C. nutans*. Therefore, this study aims to determine whether the binding of *myricetin* to HER2 receptors is associated with the anticancer effect of extracts from *C. nutan*. **Methods:** The docking simulation method was performed with AutoDock software and the extraction process utilized ethanol and aqueous methods. The extracts were tested by functional assays in the UACC732 cells. **Results:** The results revealed that *myricetin* exhibited binding affinity to the active site of HER2 receptors. Cytotoxic assay of ethanol extract on parent and resistant UACC 732 cells achieved IC_{50} at 837.98 µg/mL and 923.98 µg/m, respectively. However, the aqueous extract did not cause a cytotoxic effect on UACC 732 cells. Wound closure was significantly reduced with ethanol extract, particularly in parent HER2 cells (p=0.03) than resistant cells (p=0.3). The aqueous extract did not exhibit any difference in both cells. **Conclusion:** Therefore, the anticancer effects of ethanol extract can be attributed to the postulated binding of *myricetin* to HER2 receptors.

Keywords: *Clinacanthus nutan*, myricetin, HER2 receptor, breast cancer, *in silico*

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INTRODUCTION

Growth factor receptors play an important function in the transduction of extracellular signals. The epidermal growth factor family of the receptor tyrosine kinases (RTKs) has been extensively studied for its role in cancer treatment. These receptors are associated with the expression HER1, HER2, and HER3 in breast and squamous cancer. The RTKs are primary mediators that regulate the biological process such as proliferation, differentiation, migration, and cell death (1). According to previous studies, HER2 overexpression has been linked with metastases and resistance in breast cancer (2). Additionally, the epidermal growth factor receptor 2 (*ERBB2*, which encodes HER2) was found to be mutated in breast cancer cells, primarily via gene amplification or overexpression of HER2 receptors. Furthermore, tumors with overexpression of HER2 confer resistance to anticancer therapies (3). HER2 was found to drive proliferation of breast tumor cells (4) and stimulate the migration and intravasation of breast tumor cells during metastasis (5). One of the prognostic markers of cancer is the proliferation of tumor cells (6). The HER2 expression has been frequently associated with the development of resistance towards tamoxifen treatment due to the estrogen receptor in breast cancer. Despite existing therapies like Trastuzumab and Lapatinib, a significant percentage of patients with primary HER2-positive breast cancer succumb to the progression of the disease due to drug resistance (7). Overall, there has been continuous effort to discover new strategies for the treatment of cancer. A common approach is resorting to plant-based products or treatments by tapping into the diversity of tropical plants. Different types of natural therapies have been gaining interest related to cancer treatment due to their lower toxicity (8, 9). Moreover, they have been used widely for centuries by local communities as a cure for various ailments (10).

The Acanthaceae is one of the largest sources of medicinal plants with effective natural properties and *C. nutans* is derived from this family of flowering plants (11). Its origins are in South China and South East Asia, where such species of plants are generally found in countries like Thailand, Malaysia, and Indonesia (12). It grows widely in tropical countries and is used as a medicinal herb in China, Thailand, and Malaysia (13). In Malaysia,

it is referred to as the Sabah snake grass or *belalai gajah* (12). This plant is classified under the phylum of magnoliophyta and the class of magnoliopsida. The Acanthaceae family consists of 250 genera and 2500 species from the taxon of dicotyledonous flowering plants. The genus Clinacanthus consists of two species, namely, *C.* (Burm. f.) Lindau and *C. siamensis* (14). This species of *nutans* and *siamensis* have no discrete differences in their distribution and medicinal values (14). It can grow up to 1m and its stem is slender with circular transverse section edges. It is striated and has a smooth surface. The leaves of this plant are either oval-shaped or lance-like measuring 2.5 cm to 13 cm in length. The widths of the leaves are between 0.5 to1.5 cm with either apex acute or acuminate. Most of the species from this family are used for various ailments and act as an analgesic (15). Its medicinal properties have been exhibited in treating diabetes (16) whereas its anti-inflammatory effects were reported in cases of insect bites, herpes, and allergic reactions (17, 18). In Thailand, *C. nutans* has been used for treating conditions that relate to rashes, snake poisoning, and viral lesions (19). In a clinical trial (NCT03359187), a comparison was made between the effects of *C. nutans* with benzydamine to reduce oral mucositis after radiation in patients with head and neck cancer. The study suggests that the average time required for the onset of oral mucositis in patients treated with *C. nutans* was significantly lower than those treated with benzydamine.

The anticancer effects of *C. nutans* are associated with its active chemical compounds. Myricetin is a plant-derived flavonoid compound that is well-known for its nutraceutical value (20). Furthermore, it is one of the compounds present in the aqueous and ethanol extracts that have been associated with anticancer properties (21, 22). A previous study in colorectal cancer cells reported its antiproliferative effects (23). To date, there is limited information with regards to its binding effect on HER2 receptors in breast cancer cells. The effectiveness of targeting these receptors is an important aspect of the HER2 treatment. Therefore, this study aims to perform in silico analysis on molecular interaction between myricetin with HER2 receptors and to correlate such findings with *in vitro* cell proliferation and cell migration studies.

MATERIALS AND METHODS

HER2-Myricetin Docking Simulation

The simulation of HER2 receptors as to its binding effect was performed using myricetin, one of the active compounds present in the ethanol extract. The protein model of HER2 (PDB ID 3WSQ) was used and the molecular docking simulation on this protein was performed with the AutoDock 4.2.6 software package (24), whereas files for the docking simulation were prepared with the AutoDockTools (ADT) 1.5.6 RC3 packages. Next, a 100 x 100 x 100 points grid map was placed at the center of the macromolecule along with a 0.375 E spacing between the grid points. In this analysis, the Lamarckian genetic algorithm (LGA) was selected for the ligand conformational search and 100 independent docking runs were performed on the control and myricetin. The docked conformations of the ligands were grouped into clusters according to the increasing level of binding energy. The chemical interactions of myricetin and control were analyzed using the Accelryls® Discovery Studio Visualizer 3.5 (Accelrys, Inc., San Diego, CA, USA). These include the hydrogen bonding and hydrophobic interactions with protein residues, the free energy of binding (FEB), estimated inhibition constant (Ki) value, and bond distance. A further comparison was undertaken on the control.

Plant Material and Sample Preparation

C. nutans are grown locally in the district of Kepala Batas located in Penang, Malaysia. It was purchased from a local herbal product supplier (i.e. Herbagus, Malaysia). The leaves were air-dried for up to four hours at 55°C. After air-drying, coarse grinding of the leaves measuring 2-3 mm followed by fine grinding (45-60 mesh) was performed. The powder was placed in a sealed bag and stored at 25°C before the extraction process.

Soxhlet Extraction of Ethanolic *C. nutans*

Solvent extraction was done with a Soxhlet tool for 48 hours. Absolute ethanol (250 ml) was mixed with 25g powder derived from the *C. nutans* leaves. The heating temperature of ethanol extraction was fixed at 60°C for approximately 48 hours. Subsequently, the extracts were filtered using the rotary vacuum evaporation method (Heidolph, Germany) at 65°C with 145 hPa until it was waterless followed by freeze-drying.

Heating Extraction of Aqueous *C.nutans*

Solvent extraction was performed in a beaker for 72 hours. The powder weighing 25g was dissolved in 250 ml of deionized water and heated at 60°C on a hot plate in a fume hood chamber. The samples were mixed with a magnetic stirrer to ensure complete mixing. This method caused the diffusion of the solvent into the sample and led to the movement of bioactive compounds into the solvent. Upon completion of the heating process, the extract was then filtered with a rotary vacuum (Heidolph, Germany) at 65°C with 160 hPa.

Percentage of Yield

The extracts were weighed and stored at 4°C before use. The percentage of yield derived from dried aqueous and ethanol extracts were calculated as follows:

$$\text{Yield (\%)} = \frac{(W1 \times 100)}{W2}$$

W1 represents the weight of the extract after lyophilization of the solvent and W2 represents the weight in powder form.

Cell Lines
The UACC 732 human breast cancer cell line with overexpression of HER2 used in this study was purchased from the American Type Culture Collection (ATCC, Manassas, Virginia, USA).

Cell Culture
The incubator was sustained at 37°C with 5% CO2 atmosphere and 95% humidity. Parental UACC 732 cells were retrieved from liquid nitrogen storage and were grown with RPMI-1640 medium in a T25 culture flask (Gibco Thermo Fisher Scientific, USA). The complete media contained 10% fetal bovine serum (FBS), penicillin (100 IU/mL), and streptomycin (100µg/mL) that were purchased from Gibco/Life Technologies (MA, US). The cell culture medium was replaced with a fresh complete media at every 3-days interval.

UACC 732 TAM-R Cell Line Culture
The parental cells were treated with tamoxifen to create a multidrug-resistant cell line model. Studies have shown that tamoxifen has been prescribed to HER2 subtype patients experiencing a recurrence (25). The UACC 732 cell line resistant to tamoxifen was generated by exposing the parent UACC 732 cells to increasing concentrations of tamoxifen as previously reported (26). Stable UACC 732 cells resistant to tamoxifen were used for *in vitro* experiments. The UACC 732 Tam-R cells were cultured in phenol RPMI (Gibco Thermo Fisher Scientific, USA) with 10% FBS and antibiotics Gibco (Thermofisher Scientific, US).

Cell Proliferation Assay
Cell proliferation assay was analyzed for the determination of half-maximal inhibitory concentration (IC_{50}) of *C. nutans* extracts with an MTT reagent kit. The CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega, Madison, USA) contained 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) that produced formazan that developed color intensity directly proportional to the number of viable cells. As to the assay in this study, parental UACC 732 cells were grown in a 96-well cell culture plate (Corning, NY, USA) at a density of 4.4 x10^6 cells with 200 µL of complete culture media. The cells were incubated overnight to allow cell adhesion to the surface of a 96-well plate. The cells were then treated with different concentrations of the *C. nutans* extracts (0-1000 µM) for 24 hours, then the medium in the 96-well plate was discarded. 100 µL of complete culture medium and 15 µL of CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega, Madison, USA) were pipetted into each well and incubated at 37°C for two hours with 5% CO_2 atmosphere and 95% humidity. Subsequently, 100 µL of the solubilization reagent was placed in each well. The level of absorbance of samples was recorded at 570 nm using the ELISA microplate reader analysis (Biotek, Winooski, US). Each treatment was done in triplicates. The absorbance readings were directly proportional to the number of viable cells in the samples. As to control, the absorbance of control cells was measured and blank represents the absorbance of media without cells. The percentage of cell viability was determined with the formula provided below.

$$\text{Cell viability (\%)} = \frac{(\Delta \text{ Treatment} - \Delta \text{ Blank}) \times 100}{(\Delta \text{ Control} - \Delta \text{ Blank})}$$

Δ represents absorbance.

Dose-response graph was plotted and each data point represent mean of triplicates. The 50% inhibitory concentration (IC50) for each extract is defined as the concentration producing 50% decrease in cell viability(27). This method for determining the IC50 is similar to those reported previously (28, 29).The formula for calculating the IC50 was estimated using a non-linear regression equation in excel as provided in the results section.

Cell Migration Assay
In the present study, the assay was carried out to determine the migration and interactions of the cancer cells. Cell migration has been studied through the wound healing assay due to its simplicity and capability of revealing the complexity of collective cell migration. The parent and resistant UACC 732 cell lines were seeded on 24 well-plates. They were grown at a density of 2 x 105 cells in each well with 200 µL of complete RPMI-1640 medium (Gibco Thermo Fisher Scientific, USA). The media was supplemented with 10% FBS and antibiotics (Gibco/Life Technologies). The plates were left for at least 24 hours to allow cell adhesion to the surface of the 24 well plates before treatment with 838 µg/ml of ethanol extract and similar concentration 838 µg/ml of aqueous extract for parent cells to compare its effect. While in resistant cells, 924 µg/ml of ethanol extract and similar concentration of aqueous extract were tested. Next, the medium in the 24 well plates was discarded and 100 µL of complete culture medium was placed into each well. Then, the 10 µg/mL of colcemid (Demecolcine) solution was mixed with the medium and incubated for 2 hours. After 2 hours of incubation, 200 µL microliter tips were used to scratch at the center of each well. The pipette tips were kept under an angle of 45°C to ensure the scratch widths were similar. The wells were washed with PBS to remove any remaining debris. Finally, the cells were treated with *C. nutans* extracts. Movement of cells was captured with a phase contrast microscope at 200 x magnification of the normal cell cycle in hours as reported previously (30). The migration rate is expressed as the percentage of wound closure, where the closure percentage increased with the migration of cells over time. ImageJ software was used to measure the difference in the percentage of the open area that is unoccupied by the cancer cells (31). The wound closure percentage was determined at the scratch area in each of the wells. Each test was performed in triplicates for control and

treatment at 40 hours. The wound area was outlined using the freehand selection tab in the ImageJ software. Next, the parameter setting was fixed by selecting the area and limit threshold functions under the analyze tab. Subsequently, the measure function was chosen to obtain readings based on the formula below.

$$\text{Wound closure (\%)} = \frac{(At0h - At\Delta h) \times 100}{At0h}$$

At0h represents the wound area after the scratch (t0h) whereas AtΔh represents the wound area h hours after the scratch. Images were captured at 200x magnification using a phase contrast microscope (Zeiss, Germany).

Statistical Analysis

IBM SPSS Statistics 26 was used to perform the statistical analysis. An independent T-test was undertaken to compare the values between the two groups. The P value < 0.05 was considered statistically significant.

RESULTS

Docking Simulation of HER2 Receptor with Myricetin and Lapatinib

To understand the molecular interaction of compounds in the drug-binding site of HER2 receptors, chemical interactions based on the free energy of binding (FEB) and estimated inhibition constant (Ki) were studied. The docking simulation study indicated that the FEB and Ki of myricetin were comparable to the control inhibitor (lapatinib), which suggested that myricetin could be a potential inhibitor of HER2 receptors. Docking simulation of myricetin and lapatinib with HER2 receptors produced 32 and 36 clusters, respectively. Based on the compound of HER2 complex interactions, myricetin (– 6.21 kcal/mol) appeared to have similar interactions within the same binding pockets as compared with lapatinib (– 8.51 kcal/mol) (Table I). The Ki value of myricetin (27.93 µM) was found to be higher than the positive control inhibitor, lapatinib (582.49 nM). From the findings of the docking simulation, both lapatinib and myricetin formed interactions within the same binding pockets. Myricetin formed hydrogen bonds with Cys293, Glu299, and Ala317 residues (Fig. 1A), whereas lapatinib had interactions with Asn280 and Gly442 residues (Fig. 1B). As for hydrophobic interactions, π-π T-shaped was observed to form between the pi-orbitals group of myricetin and Phe349 residue of HER2 receptors. In addition, a hydrophobic

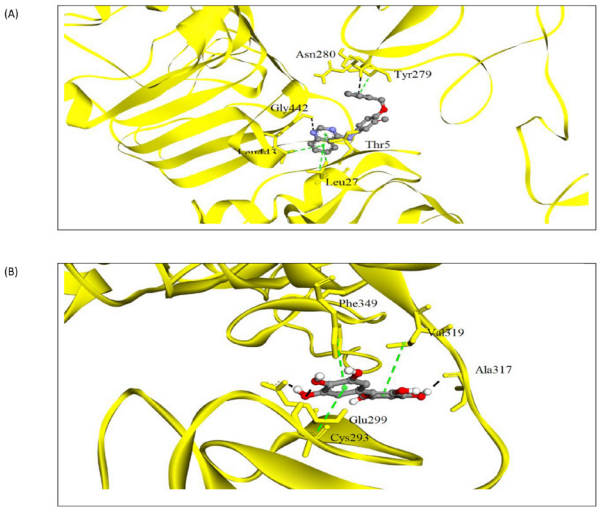

Figure 1: Molecular interaction between the compound and bindingsite of HER2 receptors. (A) Lapatinib (B) Myricetin. The line color represents the type of interactions as follows: black (hydrogen bond); green (hydrophobic interaction). The HER2 protein and compound were represented as yellow ribbon, ball, and stick, respectively. Atoms were colored as follows: dark grey for carbon, blue for nitrogen, brown for sulfur, red for oxygen, and light grey for hydrogen.

interaction was observed between Val319 and Cys293 residues which formed ϖ-alkyl interactions with the alkyl group. As for lapatinib, interactions between the π-orbitals of lapatinib and the alkyl group of Leu27 and Leu443 residues were observed by the formation of π-alkyl, respectively. Hydrophobic interactions (π-sigma) were also present between the pi-orbitals of lapatinib, and carbon-hydrogen atoms of Thr5, Leu27 and Tyr279 HER2 residues. The bond distance of myricetin and lapatinib was between 1.93–1.99 and 2.81–3.09, respectively, which suggested a strong binding affinity of myricetin within HER2 binding pockets. Interestingly, based on the molecular docking simulation, both compounds did not exhibit any similar interactions despite having interactions within the same binding pockets. Therefore, based on the FEB, Ki, bond distance, and binding pockets' affinity, it is postulated that myricetin may act as an inhibitor of HER2 receptors, similar to lapatinib.

Extraction Yield

A total of 0.68g of *C. nutans* aqueous extract was acquired from the leaves of this plant. Therefore, the

Table I: Summary of molecular interaction of compounds within the HER2 model and the complexation energies calculated using AutoDock 4.2 software

DRUG		HYDROGEN BOND	HYDROPHOBIC INTERACTIONS	FREE ENERGY BINDING, FEB (KCAL/MOL)	ESTIMATED INHIBITION CONSTANT, K_i (µM)
Known inhibitor	Lapatinib	Asn280(√), Gly442(√)	Thr5 (π-sigma), Leu27 (π-sigma, π-alkyl), Tyr279 (π-sigma), Leu443 (π-alkyl)	-8.51	582.49 nM
Natural compound	Myrecetin	Cys293(√), Glu299(√), Ala317(√)	Phe349 (π-π T-shaped) Val319 (π-alkyl), Cys293 (π-alkyl)	-6.21	27.93

extraction method produced a yield of 2.7% from 25g of freeze-dried *C. nutans* powder. A similar amount of yield was obtained with 25g of air-dried powder using the ethanol extraction method (Table II).

Table II: Percentage of yield from aqueous and ethanol extracts

PLANT	PERCENTAGE OF YIELD W/W (%)	
	Ethanol extract	Aqueous extract
Clinacanthus nutans (Dried Powder Leaves) 25 g	2.8 %	1.7 %

Effects of *C. nutans* extracts on cell proliferation

In the present study, the proliferation of cancer cells was assessed with the MTT assay. Both extracts caused a proliferation of the UACC 732 parent and resistant cells at lower concentrations. The percentage of cell viability increased to 200% in parent cells when treated with aqueous extracts (300 µg/mL). Meanwhile, in resistant cells, the percentage of cell viability increased to 180% when treated with ethanol extract treatment (100 µg/mL). The result of the parent cell viability with ethanol extract is provided in Fig. 2A. The IC50 was derived from the equation of y = -32.056x2 + 80.894x + 89.977 and the IC_{50} value for a treatment of 24 hours was 838 µg/mL. A further comparison was made using resistant cells and the percentage of resistant UACC 732 cell viability with ethanol extract treatment is provided in Fig. 2B. The IC50 was estimated from the y = -49.987x2 + 128.83x + 94.471 equation and the IC50 value of 924 µg/mL at 24 hours was achieved, which indicated that a high dose was required for resistant UACC 732 cells. In the next experiment, the percentage of cell viability was compared with the aqueous *C. nutans* treatment. Results for the parent UACC 732 (Fig. 2C) and resistant UACC 732 cells (Fig. 2D) are provided. The findings suggest that the aqueous extract did not exhibit cytotoxic effects after a 24-hour treatment even with the highest dose.

Cell Migration Assay

Effects of ethanol C. nutans extract on cell migration

A further experiment was undertaken using the migration assay to compare the effects of ethanolic extract of *C. nutans* on parent and resistant UACC 732 cell lines. For parent cells (Fig. 3A), there was no significant difference between the control and treatment groups (p=0.11). The wound was still visible at 40 hours post-treatment. Next, the C.nutans ethanol extract was tested in resistant UACC 732 cells. The results of resistant cell migration are provided in Fig. 3B. The upper panel represents the migration of untreated resistant UACC 732 cells and the lower panel represents the migration of resistant UACC 732 cells following the treatment with *C. nutans* ethanol extracts. Similar to parent cells, there was an inhibition of resistant cell migration at 40 hours of treatment. There was a marginal yet significant difference between the control and resistant UACC 732 cells treated with ethanol extract (p=0.057). The findings gathered revealed a lower percentage of wound closure in both cell types than control after 40 hours of treatment (Table III).

Effects of aqueous C. nutans extract on cell migration

In another experiment, the aqueous extract was tested in the parent and resistant UACC 732 cells. As for parent cells, there was a significant difference between the control and treatment groups (p=0.03). There was a lower migration of the parent cells compared to the control cells (Fig. 3C). However, the resistant UACC 732 cells treated with aqueous *C. nutans* extracts exhibited a higher percentage of migration than the control cells (p=004) (Fig. 3D). Wound closure was measured for both parent and resistant UACC 732 cells and the percentage of wound closure with aqueous extract is provided in Table IV.

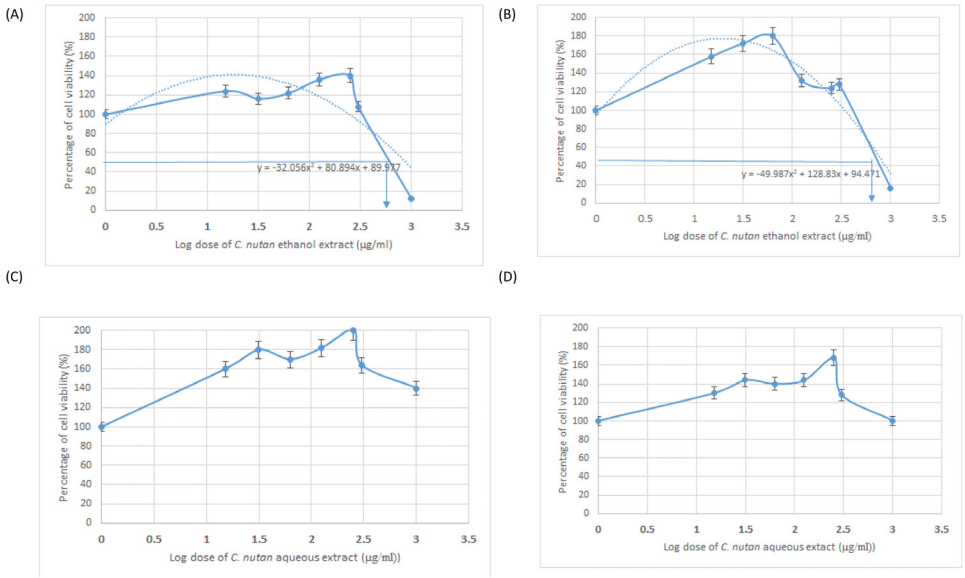

Figure 2: Cytotoxic effects of *Clinacanthus nutans* leave extract (A) ethanol extract on the viability of parent UACC 732 cell (B) ethanol extract on the viability of resistant UACC 732 cell (C) aqueous extract on the viability of parent UACC 732 cell (D) aqueous extract on the viability of resistant UACC 732 cell. Data are expressed as a means of triplicates experiments.

Table III: Comparison in percentage of wound closure with *Clinacanthus nutans* ethanol extract

CELL TYPE	PERCENTAGE OF WOUND CLOSURE (%)		P VALUE
	Control	Treatment	
PARENT UACC 732	84.2 ± 21.8	39.4 ± 7.3	0.11
RESISTANT UACC 732	85.7 ± 19.9	27.7 ± 4.9	0.057

Table IV: Comparison in percentage of wound closure with *Clinacanthus nutans* aqueous extract

CELL TYPE	PERCENTAGE OF WOUND CLOSURE (%)		P VALUE
	Control	Treatment	
PARENT UACC 732	94.2 ± 8.0	60.3 ± 1.4	0.03
RESISTANT UACC 732	53.5 ± 10.5	95.9 ± 5.1	0.04

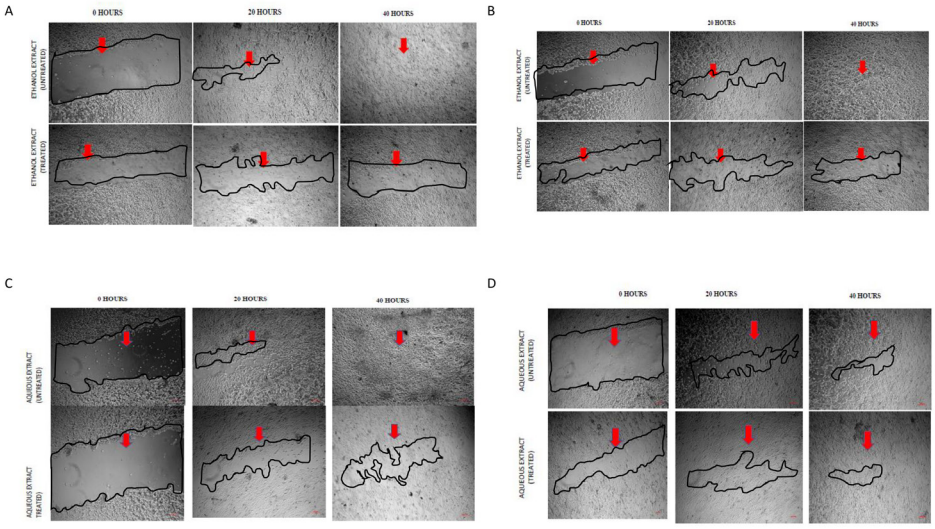

Figure 3: Wound healing assay for effects of *Clinacanthus nutans* extract (A) ethanol extract on the migration of parent UACC 732 cells (B) ethanol extract on the migration of resistant UACC 732 cells. (C) aqueous extract on the migration of parent UACC 732 cells (D) aqueous extract on the migration of resistant UACC 732 cells. The upper panel represents untreated cells and the lower panel represents cells treated with Clinacanthus nutans ethanol extract. The duration of treatment was 0 hours, 20 hours and 40 hours. Images were captured at 200x magnification using a phase contrast microscope.

DISCUSSION

The extraction process was performed using water and ethanol as solvents. The percentage of yield from aqueous extracts was 2.7% from 25g of freeze-dried powder of *C. nutans*. A similar percentage of yield was derived from ethanol extracts (2.8%). This indicated that the soxhlet method and heating method produced similar quantities of yield. The comparison between ethanol and aqueous extracts of *C. nutans* was made in the traditional form by which plant extracts are consumed. However, a low percentage of yield may be contributed by factors like slow rate, decomposition, or evaporation (32). The anti-proliferation effect of these extracts was tested on HER2 overexpressed resistant and parent cells. In this study, dose-dependent experiments were performed using cytotoxicity assay. The data obtained suggested if the extracts were lower than 300 µg/mL, then the aqueous extracts were able to cause UACC 732 cell proliferation. Previous studies found that the C. nutan aqueous extract has no or weak antiproliferative effect in cancer cells (33). Another study also indicated a low concentration (100 µg/mL) of leaf extracts using water resulted in the proliferation of human embryonic kidney cells (34). Despite increasing the treatment dose to more than 300 µg/mL of aqueous *C. nutans* extract, there was no cytotoxic effect on the parent and resistant UACC 732 cells. These findings were in line with previous research by Kow et al. (35), which stated that the aqueous extract was not cytotoxic to rat basophilic leukemic cells even at the highest concentration of 8 mg/ml. In contrast, the aqueous extract was cytotoxic to MDA-MB 231 cells (IC50= 191 µg/mL)(36) and HeLa cells with an IC50 value of 13 µg/mL (37). These results indicated that the cancer types differ in their level of sensitivity to aqueous extract treatment.

Further analysis was on the effects of treating cancer cells with ethanol extracts. Similar to the aqueous extract, less than 300 µg/m of ethanol extract was able to stimulate UACC 732 cell proliferation. Present study suggests that the C nutan ethanol extract has dose-dependent effect in HER2 breast cancer cells. This could be associated with the increased sensitivity of HER2 cells to the extract at high concentrations. Similar finding was reported previously in which low dose of retinoid acid was able to induce the proliferation of cells by increasing the EGF signalling, while higher concentration caused inhibition of cell division by decreasing the ERK1 activation (38). In another report, low doses of genistein, a natural compound from soy product exhibited mitogenic effect in MCF-7 breast cancer cells (39). Treatment of B16-BL6

cells with low dose of the *Uncaria tomentosa* extract also did not significantly inhibit B16-BL6 cell viability but treatment with a high dose inhibited growth of the B16-BL6 cells (40). This is supported by previous research in which low doses of C. nutan ethanol extract induced the proliferation of lymphocytes attributed to the activation of interleukin 4 (41). Based on the present findings, the IC50 value of parent cells was lower than resistant UACC 732 cell viability with the IC50 of 923.98µg/mL after 24 hours of treatment. This indicated that higher doses of ethanol extract were needed for the treatment of resistant cells.. According to previous research on triple-negative breast cancer cell line (MDA-MB-231), ethanol extract had low cytotoxicity whereas aqueous extract did not demonstrate cytotoxic activity (42). On the contrary, the aqueous extract was more cytotoxic on human umbilical endothelial cells than the ethanol extract from leaves (43). The modulatory properties of *C. nutans* ethanol extract on IFN-γ/TNF-α also induced apoptosis. This may be attributed to the presence of phenolic content (44). Similar findings were obtained when methanol and chloroform extracts of *C. nutans* were tested for their antiproliferative and antioxidant properties (45). There was substantial antiproliferative effect on K-562 and the Raji cells at 72 hours with 91.3% (IC50:100 µg/mL) and 89.0% (IC50: 47.31 µg/mL), respectively. On the contrary, another study reported that *C. nutans* ethanolic extract had no cytotoxic effect on RBL-2H3 cells after 24 hours of treatment (35). In a mice model study, high dose of 1000 mg/kg *C. nutans* leave extract was required to inhibit the growth of tunour (46).

In cancer biology studies, resistance provides the ability for cancer cells to migrate and evade tissues. Therefore, a wound-healing assay was performed to mimic the tissue microenvironment. The migratory characteristics of the cells were monitored after treatment with C. nutan extracts in parent and resistant UACC 732 cells. In the present study, the ethanol extract caused significantly lower migration of parent and resistant UACC 732 cells compared to control cells. The aqueous extract also had a migratory inhibitory effect in parent UACC 732 cells. However, it promoted the migration of resistant UACC 732 cells. The response by the resistant cells was supported by the increase in the proliferation of resistant cells. These findings suggest that the resistant UACC 732 cells responded differently upon exposure to aqueous C. nutan extracts. This can be attributed to chemical compounds present in the aqueous extract that may promote the wound repair process based on a previous study that reported that the aqueous extract is rich in flavonoids and phenolic compounds. (47, 48). Consequently, the migratory mechanism was associated with an increase in cytokine production, generation of vascular endothelial growth factor, platelet-derived growth factor, and epidermal growth factor which contributed to the migration of cells (49). Recent studies show that chloroform extract of *C. nutans* leaves induced wound closure in macrophage cells (50) and human gingival fibroblast cells. Chemical compositions present in *C. nutans* extract may have regulated this function. According to previous research, extracts of *C. nutans* exhibited anticancer characteristics in numerous cancer cells. *In vivo* studies have indicated there was an anti-tumorigenic activity of the *C. nutans* ethanolic extract (51) that prevented the proliferation of HeLa cells (45). However, it did not have antiproliferative activity on primary human gingival fibroblasts (52). The crude chloroform leaf extract promoted an antiproliferative effect in human lungs (NCI-32) and cervical cancer after 72 hours of treatment (45). A lower dose of crude petroleum ether of *C. nutans* leaf extract was sufficient to induce a cytotoxic effect on HeLa and K-562 cells (52). In another research, the crude aqueous leaf extract exhibited high potency against the growth of the K-562 cells and the Raji cells. Nevertheless, identification and quantification of the chemical compositions present in the extracts are necessary. Further studies on the methods of harvesting the plant parts and storage will reduce the loss of vital phytochemicals and conserve their activities.

The extraction methods were found to play an important role in the phytochemical yield (53). The extract contained multiple bioactive compounds such as C-glycosyl flavones, phytosterols, triterpenoid, stigmasterol, glycoglycerolipids, lupeol, b-sitosterol, belutin, sulfur-containing glycosides, and chlorophyll. The other types of compounds present in the methanol extract comprise vitexin, iso-vitexin, schaftoside, isomollupentin 7-Obd-glucopyranoside, orientin, and iso-orientin. In addition, the ethanolic leaf extract contained myricetin, orientin, iso-orientin, vitexin, iso-vitexin, isookanin, apigenin, and ferulic acid. A recent study associated sulphur containing compound in methanol extract with its anticancer effect (54). Furthermore, geographical differences may contribute to the difference in the composition (21). The leaves used in the present study were collected from the northern region of Malaysia that may differ in their chemical composition compared to those found in the southern region. The modeling study suggested that myricetin binds to the active site of HER2 receptors as the control drug. This finding was in agreement with the results obtained using *in vitro* assay. Myricetin exhibits inhibitory effects in HER2 subtype breast cancer cells. It is postulated that myricetin may have an antiproliferative effect on cancer cells. Further research is warranted to confirm these findings.

CONCLUSION

The findings in the present study indicated that the ethanol extract of *C. nutans* exhibited higher inhibitory of breast cancer cells than the aqueous extract. Parent cells were more sensitive to the ethanol extract than the resistance cells. Myricetin is postulated to have an anticancer effect exhibited by *C. nutans*. This suggests that further analysis with the bioactive compound

is necessary to unravel the underlying molecular mechanisms that underpin drug resistance in HER2 breast cancer cells.

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