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Assessment of Cytotoxicity Potency of Paclitaxel in Combination with *Clinacanthus Nutans* Extracts on Human MDA-MB-231 Breast Cancer Cells

(Penilaian Potensi Sitotoksisiti Paclitaxel secara Kombinasi dengan Ekstrak *Clinacanthus Nutans* terhadap Sel Kanser Payudara Manusia MDA-MB-231)

NUR HASNIEZA MOHD ROSLI, CHAN KOK MENG, FARIZA JULIANA NORDIN, LEONG LEK MUN, NUR SYAZWANI ABDUL AZIZ & NOR FADILAH RAJAB

ABSTRACT

Clinacanthus nutans (C. nutans) leaf extracts have been widely used by cancer patients in Malaysia and local practice claims a cure to cancer. There were several studies done to determine the cytotoxicity potency of C. nutans extracts on various types of cells. However, there is still lacking on the knowledge regarding the combination effect of C. nutans with anticancer drugs. Thus, the study was carried out to determine the cytotoxicity potency of C. nutans extracts and paclitaxel (PTX) alone and, in combination on MDA-MB-231 cells. The cells were treated with 100% ethanol extract of C. nutans (CNE) and water extract of C. nutans (CNA), PTX and combination of both extracts and PTX for 72 hours and the cytotoxic activity was determined using SRB assay. Result showed that CNE had little cytotoxic activity, whereas CNA showed no cytotoxic activity on MDA-MB-231 cells. For combination treatment of C. nutans extracts and PTX, only CNE showed significant enhanced PTX-induced cytotoxicity ($p < 0.05$), meanwhile CNA inhibited PTX-induced cytotoxicity significantly ($p < 0.05$). As a conclusion, CNE was able to increase PTX potency to inhibit the viability of MDA-MB-231 cells.

Keywords: Clinacanthus nutans; paclitaxel; MDA-MB-231 cells; cytotoxicity activity

ABSTRAK

Ekstrak daun Clinacanthus nutans (C. nutans) telah digunakan secara meluas oleh pesakit kanser di Malaysia dan pengamal tempatan mendakwa ia dapat mengubati kanser. Beberapa kajian lepas telah dijalankan untuk menentukan potensi sitotoksisiti ekstrak C. nutans terhadap pelbagai jenis sel. Walau bagaimanapun, pengetahuan mengenai kesan kombinasi C. nutans dengan dadah antikanser adalah masih kurang. Oleh itu, kajian ini telah dijalankan untuk menentukan kesan potensi sitotoksisiti ekstrak C. nutans, paclitaxel (PTX) dan kombinasi di antara ekstrak C. nutans dengan PTX ke atas sel MDA-MB-231. Sel MDA-MB-231 telah dirawat dengan ekstrak 100% etanol C. nutans (CNE) dan esktrak air C. nutans (CNA), PTX dan kombinasi kedua-dua ekstrak dan PTX selama 72 jam dan aktiviti sitotoksik telah ditentukan menggunakan asai SRB. Hasil kajian menunjukkan CNE mempunyai aktiviti sitotoksik yang sedikit, manakala CNA tidak mempunyai aktiviti sitotoksik. Bagi rawatan kombinasi ekstrak C. nutans dan PTX, kombinasi CNE dan PTX menunjukkan peningkatan kesan sitotoksik secara signifikan ($p < 0.05$). Kesimpulannya, CNE berkebolehan untuk meningkatkan potensi PTX untuk merencat viabiliti sel MDA-MB-231.

Kata kunci: Clinacanthus nutans; paclitaxel; sel MDA-MB-231; aktiviti sitotoksisiti

INTRODUCTION

Breast cancer is the commonest cancer among women (Omar & Tamim 2011). It is estimated that age-standardized rate (ASR) for breast cancer in Malaysia was 38.7 per 100000 with 5410 new cases in 2012, according to IARC (GLOBOCAN) in 2012 (IARC 2016). Breast cancers are heterogenous in nature consisting of a few major subtypes including luminal A, luminal B, human epidermal growth factor receptor (HER-2) positive and basal cell-like (BCL) or triple negative breast cancer (TNBC) (Griffiths & Olin 2012; Schnitt 2010; Spitale et al. 2009).

Current chemotherapeutic approach includes specific antihormonal therapies that are ready for use against the hormonal receptors such as estrogen receptor (ER) and progesterone receptor (PR) along with human HER-2 (Griffiths & Olin 2012). For metastatic HER-2 positive cancer patients, trastuzumab is suggested as first-line treatment which can be administered as a single agent or in combination with endocrine treatment or chemotherapy, including in adjuvant setting (Awada et al. 2012). For TNBC, the only choice of treatment for TNBC is conventional systemic cytotoxic chemotherapy such as taxanes and anthracyclines. Furthermore, TNBC is characterized as higher aggressive behavior, earlier relapses, different patterns or metastases and greater rates of mortality compared to other breast tumors, thus very potent treatment is required (Elias 2010; Griffiths & Olin 2012).

PTX, a class of taxane drugs is a natural diterpenoid pseudoalkaloid that target microtubules. It interrupts the

cell cycle in the G_0/G_1 and G_2/M phase which can lead the cells undergo apoptosis (Zhang et al. 2014; Kampan et al. 2015). For breast cancer, it is used as a first-line treatment and it can be administered as a single agent, but it is commonly administered in combination with an anthracycline such as doxorubicin since it improves disease-free survival and overall survival (Sprouse et al. 2014). PTX has lots of serious side effects and great level of toxicity like majority of chemotherapy drugs (Liu et al. 2013). The side effects and toxicity of PTX at higher doses are neutropenia and neuropathy. Therefore, these significantly restrict dosage of PTX and its use in patients (Sprouse 2014; Liu et al. 2013).

Clinacanthus nutans (*C. nutans*) is a plant belongs to Acanthaceae family (Wanikiat et al. 2008). It is commonly known as Belalai gajah, Sabah Snake Grass in Malaysia, phaya yo or phaya plongtong in Thailand and Giro de flores, cocodrilo flor, e zui hua in Chinese dialect. It has been utilized as conventional medicine in Thailand and Malaysia. In Thailand, fresh leaves alcoholic extract is applied externally for treatment of skin rashes, herpes simplex and varicella-zoster virus lesions (Aslam et al. 2014). It is a popular medicinal plants extensively used in Thai conventional medicine and are classified as important medicinal plants for basic healthcare by the Thai Ministry of Public Health (Wanikiat et al. 2008). In Malaysia, the raw leaves are boiled with water and drank as herbal tea (Aslam et al. 2014). It is used for treatment of diabetes mellitus, fever, diarrhea, dysuria, skin rashes and snake bites and wounds due to herpes simplex virus (Aslam et al. 2014; P'ng et al. 2012).

Previous study has shown that *C. nutans* possessed antiproliferative activities against cancer cell lines (Yong et al. 2013). This suggested that *C. nutans* has the potential to be used as an alternative adjuvant or neoadjuvant chemotherapy treatment for cancer or can be consumed for cancer prevention. Currently, combination therapy is more favourable due to its likelihood to reduce development of resistant cancer cells and its ability to reduce the adverse effects of the drugs. However, there is no study on the combination effect of *C. nutans* with anticancer drugs to date. In the present study, MDA-MB-231 human breast cancer cells, a triple negative subtype that has aggressive behavior was used. These cells are treated with PTX in combination with *C. nutans* extracts to assess the cytotoxic potency of the combination treatment against the breast cancer cells. This study may lead to the discovery of a new combination chemotherapy regime that can be used for the treatment of breast cancer in addition to the existing treatments currently available.

MATERIALS AND METHODS

CELLS AND CELL CULTURE

MDA-MB-231 human breast cancer cell line was purchased from American Type's Tissue Culture (ATCC). MDA-MB-231 cells were cultured in DMEM, supplemented with 10% FBS, sodium pyruvate (100 mM) and antibiotic-antimycotic (100x). MDA-MB-231 cells were cultured in T75 cm^2 flask in a humidified atmosphere containing 5% CO_2 incubator at 37°C. Further maintenance and subculturing of cells were performed according to ATCC guideline.

ETHANOL AND WATER EXTRACTS OF *CLINACANTHUS NUTNAS* (*C. NUTANS*)

100% ethanol extract of *C. nutans* (CNE) and water extract of *C. nutans* (CNA) leaves crude extracts were obtained from Universiti Putra Malaysia (UPM). Prior to use, both extracts were dissolved in 100% DMSO.

CYTOTOXICITY ASSAY

Sulforhodamine B (SRB) assay was performed to determine the cytotoxic activity of the *C. nutans* extracts, PTX and combination of both on MDA-MB-231 cells. SRB assay was performed as previously described (Houghton et al. 2007). Briefly, 5×10^4 cells/ml MDA-MB-231 cells were seeded into 96-well plates and allowed to growth overnight. On the next day, the cells were exposed to serial dilutions of PTX, *C. nutans* extracts, or combination of *C. nutans* extracts and IC_{50} of PTX for 72 hours. After 72 hours, 50 µl of 50% w/v tricholoroacetic acid was added to the wells and incubated at 4°C for 1 hour. Then, the plates were washed for five times with distilled water and allowed to dry in the air. 50 µl of 0.4% SRB dye solution were added into each well of the dry 96-well plate and allowed staining at room temperature for 30 minutes. After that, the SRB solution was removed by washing the plates quickly with 1% acetic acid for five times to remove the unbound dye. The washed plates were dried in the air. The bound SRB was solubilized by adding 100 µl of 10 mM unbuffered Tris base (pH 10.5) into each well and the plates were shaken for 5 minutes on the microplate shaker. The absorbance of each well was measured by using an ELISA microplate reader at 570 nm. The viability of the cells was calculated by using the following formula:

$$\text{Percentage of Cell Viability} = \frac{\text{OD Treated Cell}}{\text{OD Untreated Cell}} \times 100\%$$

STATISTICAL ANALYSIS

All experiments were carried out in triplicates and results were expressed as mean ± standard error mean (SEM). One-way ANOVA was used to compare between the control group and the groups treated with PTX alone, *C. nutans* extracts alone and combination of PTX and *C. nutans* extracts on cell viability. A p-value of < 0.05 was considered statistically significant.

RESULTS

THE CYTOTOXIC ACTIVITY OF PTX ON MDA-MB-231 CELLS

Determination of IC_{50} was done using graphs generated from Microsoft Excel 2007 edition. The effect of PTX on MDA-MB-231 cells viability was investigated to determine the IC_{50}. The cells were cultured in the presence of increasing concentrations of PTX (0-0.05 µg/ml) for 72 hours and the cellular viability was evaluated using SRB assay. At 0.05 µg/ml, PTX significantly reduced the MDA-MB-231 cellular viability to 47.19 ± 1.73% ($p < 0.05$). The IC_{50} value obtained was 0.028 µg/ml. The percentage of viability is shown in Figure 1.

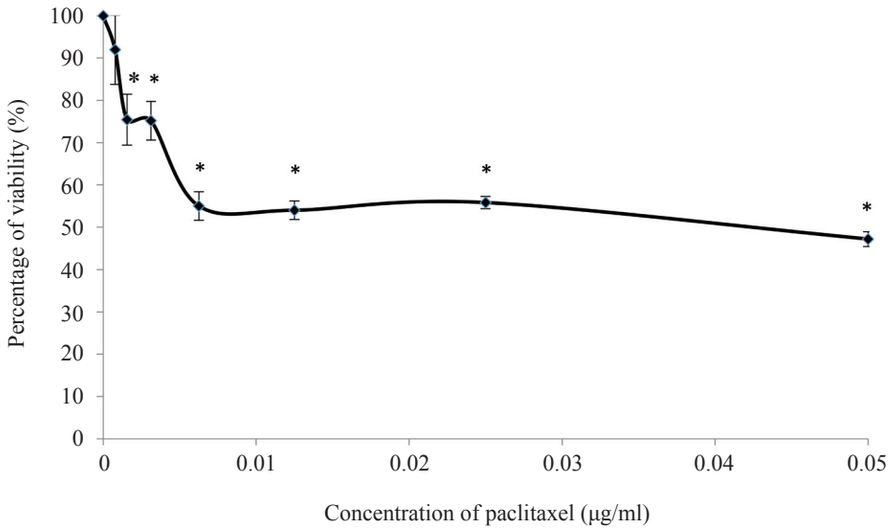

FIGURE 1. The mean of percentage of MDA-MB-231 cells viability versus the concentration of PTX treated for 72 hours. Every point represents mean ± SEM for triplicate from three different experiments (n = 3). *$p < 0.05$ compared to negative control.

THE CYTOTOXIC ACTIVITY OF *C. NUTANS* EXTRACTS ON MDA-MB-231 CELLS

The effect of CNE and CNA on MDA-MB-231 cells viability was investigated. The cells were cultured in the presence of increasing concentrations of CNE (0-0.5 mg/ml) for 72 hours and the cellular viability was evaluated using SRB assay. At 0.5 mg/ml, the CNE significantly reduced the MDA-MB-231 cellular viability to 67.70 ± 3.21% ($p < 0.05$). The percentage of viability is shown in Figure 2.

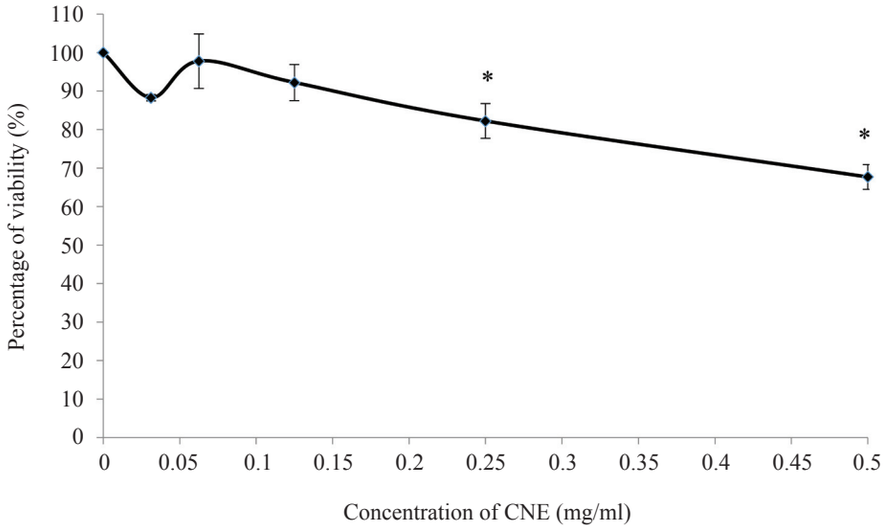

FIGURE 2. The mean of percentage of MDA-MB-231 cells viability versus the concentration of CNE treated for 72 hours. Every point represents mean ± SEM for triplicate from three different experiments (n = 3). *$p < 0.05$ compared to negative control.

The cells were also cultured in the presence of increasing concentrations of CNA (0-1 mg/ml) for 72 hours and the cellular viability was evaluated using SRB assay. At 1 mg/ml, the CNA slightly reduced the MDA-MB-231 cellular viability to 97.35 ± 0.71%. The percentage of viability is shown in Figure 3.

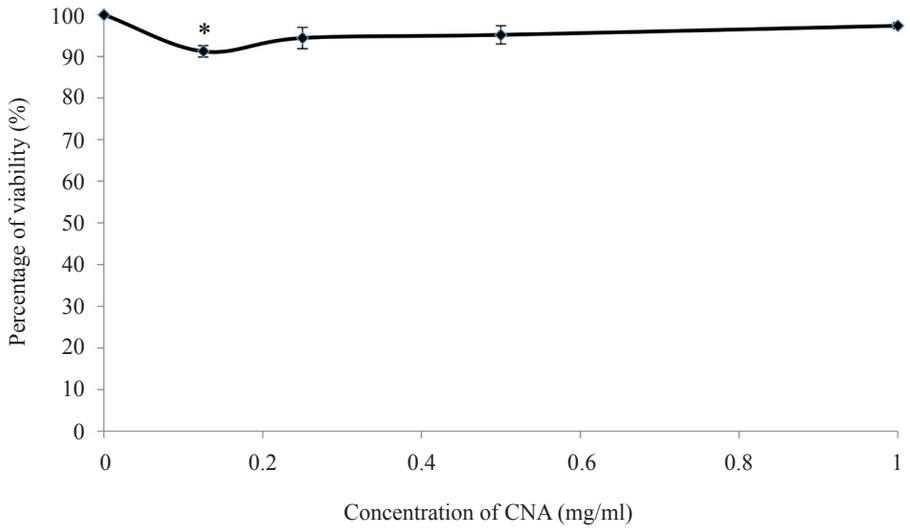

FIGURE 3. The mean of percentage of MDA-MB-231 cells viability versus the concentration of CNA treated for 72 hours. Every point represents mean ± SEM for triplicate from three different experiments (n = 3). *p < 0.05 compared to negative control.

THE CYTOTOXIC ACTIVITY OF *C. NUTANS* EXTRACTS IN COMBINATION WITH PTX ON MDA-MB-231 CELLS

The MDA-MB-231 cells were cultured in the presence of increasing concentrations of CNE (0.125-0.5 mg/ml) in combination with IC_{50} of PTX (0.028 µg/ml) for 72 hours and the cellular viability was evaluated by using SRB assay. The mean of cell viability for the MDA-MB-231 cells decreased from 82.61 ± 7.05% to 69.30 ± 4.15% with increasing doses of CNE (0.125-0.5 mg/ml) alone. The mean of cell viability for the MDA-MB-231 cells increased from 31.05 ± 1.87% to 34.02 ± 0.59% for the combination with CNE (0.125-0.5 mg/ml) and PTX (0.028 µg/ml). The mean of cell viability for PTX (0.028 µg/ml) alone was 45.33 ± 5.42%. The combination of all concentrations of CNE and PTX had significantly lower percentage of viability compared to PTX alone ($p < 0.05$). Significant decreased of cell viability at all concentrations of CNE in combination with PTX was also observed compared to CNE ($p < 0.05$). The percentage of cell viability is shown in Figure 4.

The MDA-MB-231 cells were cultured in the presence of increasing concentrations of CNA (0.5-1 mg/ml) combined with IC_{50} of PTX (0.028 µg/ml) for 72 hours and the cellular viability was evaluated by using SRB assay. The mean of cell viability for the MDA-MB-231 cells decreased from 81.97 ± 2.64% to 74.45 ± 1.22% with increasing doses of CNA (0.5-1 mg/ml) alone. The mean of cell viability for the MDA-MB-231 cells increased from 46.58 ± 1.34% to 53.06 ± 1.73% for the combination with CNA (0.5-1 mg/ml) and PTX (0.028 µg/ml). The mean of cell viability for PTX (0.028 µg/ml) alone was 41.61 ± 0.78%. Significant increased of cell viability of concentration 1 mg/ml of CNA in combination with PTX was observed compared to PTX alone ($p < 0.05$). However, significant decreased of cell viability of all concentrations of CNA in combination with PTX was observed compared to *C. nutans* extract alone ($p < 0.05$). The percentage of cell viability is shown in Figure 5.

MORPHOLOGICAL CHANGES ON MDA-MB-231 CELLS

The morphological changes of MDA-MB-231 cells were observed after 72 hours of incubation with PTX. The unaffected normal viable MDA-MB-231 cells were fibroblast-like and were attached to the surface of the 96-well microplate. However, for the treated cells, they exhibited few morphological features characteristic of apoptosis such as formation of apoptotic bodies and membrane blebbing. There were also cells that became smaller and appeared round in shape, a characteristic of dying cells. The number of cells in the treated group was obviously lesser than the control. The morphological change of MDA-MB-231 cells treated with PTX is shown in Figure 6.

The morphological changes of MDA-MB-231 cells were observed after 72 hours of incubation with CNE and CNA. The unaffected normal viable MDA-MB-231 cells were fibroblast-like and were attached to the surface of the 96-well microplate. For the treated cells, they also exhibited fibroblast-like cells and were attached to the surface of the 96-well microplate same as the normal viable cells. But the number of the cell slightly reduced and appeared round in shape. The morphological features characteristic of apoptosis such as formation of apoptotic bodies and

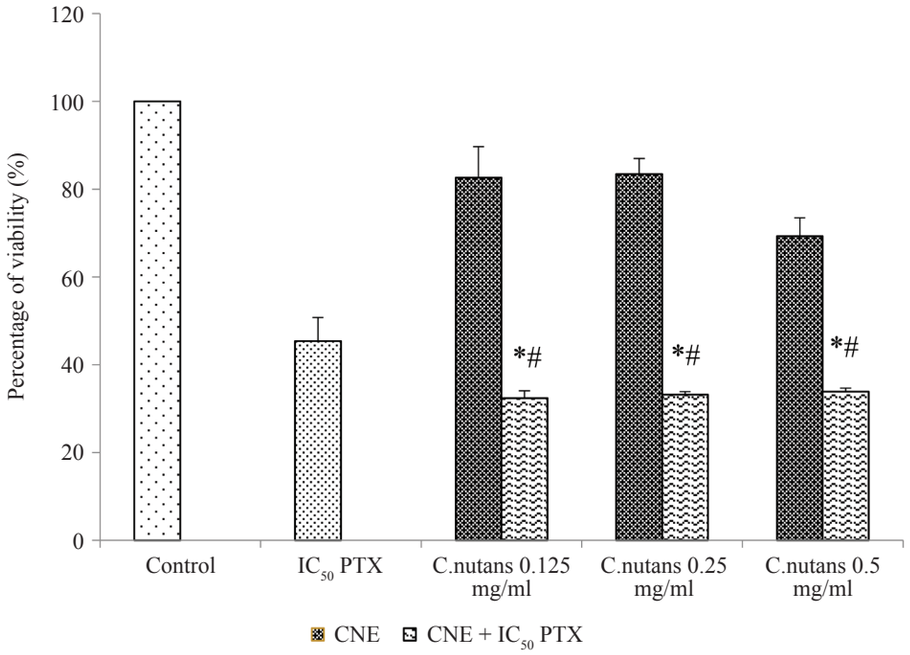

FIGURE 4. Enhancement of the PTX-induced cell death by high concentration of 100% ethanol extract of *C. nutans*. Histogram showed the percentage of viability with the indicated treatments. The MDA-MB-231 cells were kept in control condition, or treated with IC_{50} of PTX (0.028 µg/ml) alone, or with the 100% ethanol extract of *C. nutans* alone, or with the 100% ethanol extract of *C. nutans* in combination with IC_{50} of PTX (0.028 µg/ml) for 72 hours. The data are expressed as mean ± SEM for triplicate from three different experiments (n = 3). * Significant difference of cell viability when comparing the *C. nutans* extract treatment alone versus the combination of *C. nutans* extract plus PTX ($p < 0.05$). # Significant difference of cell viability when comparing the PTX treatment alone versus the combination of *C. nutans* extract plus PTX ($p < 0.05$).

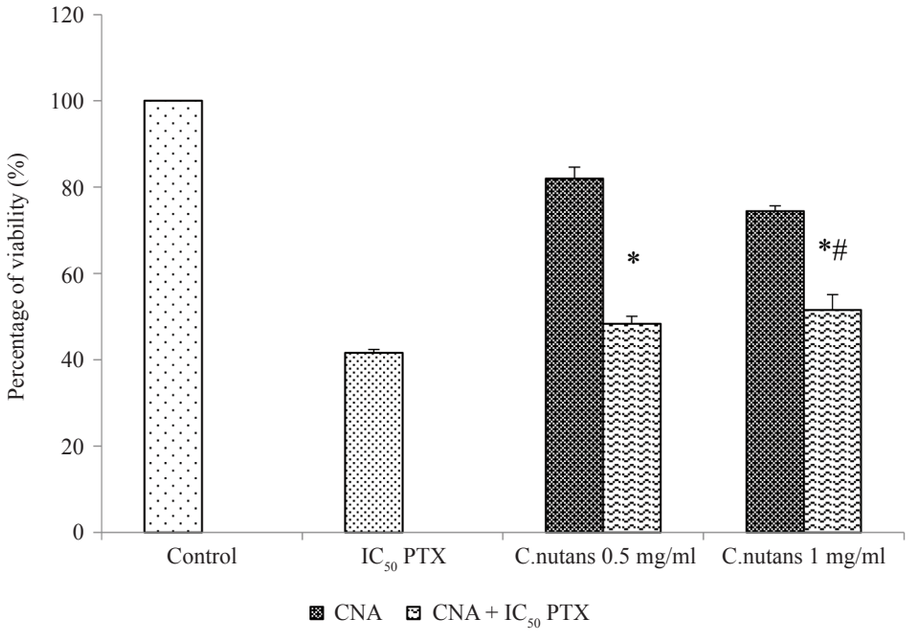

FIGURE 5. Enhancement of the PTX-induced cell death by high concentration of water extract of *C. nutans*. Histogram showed the percentage of viability with the indicated treatments. The MDA-MB-231 cells were kept in control condition, or treated with IC_{50} of PTX (0.028 µg/ml) alone, or with the water extract of *C. nutans* alone, or with the water extract of *C. nutans* in combination with IC_{50} of PTX (0.028 µg/ml) for 72 hours. The data are expressed as mean ± SEM for triplicate from three different experiments (n = 3). * Significant difference of cell viability when comparing the *C. nutans* extract treatment alone versus the combination of *C. nutans* extract plus PTX ($p < 0.05$). # Significant difference of cell viability when comparing the PTX treatment alone versus the combination of *C. nutans* extract plus PTX ($p < 0.05$).

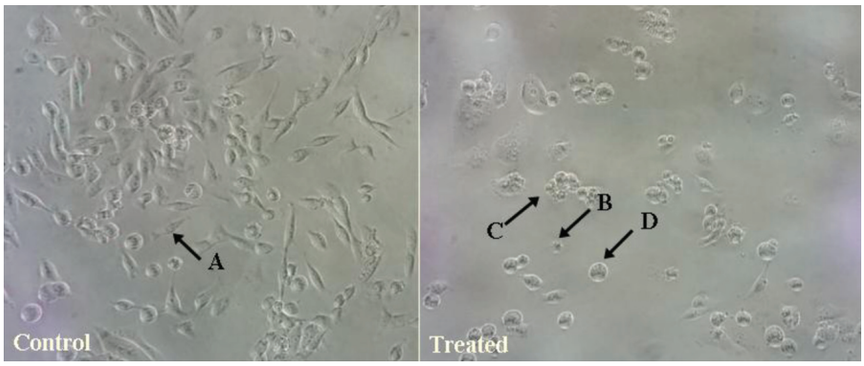

FIGURE 6. Effect of PTX on morphology of MDA-MB-231 cells for 72 hours. A: The normal viable cell with the fibroblast-like shape and was attached to the surface of 96-well microplate. B: A membrane-bound, apoptotic body that was separated from mother cell. C: Blebbing of the cell. D: Cell shrinkage and appeared round in shape. These cells were observed under inverted light microscope (20X magnification).

membrane blebbing were not observed. The necrotic cells were also not observed. The number of cells in the treated group was lower than the control. The morphological change of MDA-MB-231 cells treated with CNE and CNA is shown in Figure 7.

The morphological changes of MDA-MB-231 cells were observed after 72 hours of incubation with CNE and CNA in combination with PTX (0.028 µg/ml). The unaffected normal viable MDA-MB-231 cells were fibroblast-like and were attached to the surface of the 96-well microplate. For the treated cells, they exhibited the morphological features characteristic of apoptosis such as membrane blebbing and the cells that became smaller and appeared round in shape. The necrotic cells were not observed. The number of cells in the treated group was lower than the control. The morphological change of MDA-MB-231 cells treated with CNE and CNA in combination with PTX is shown in Figure 8.

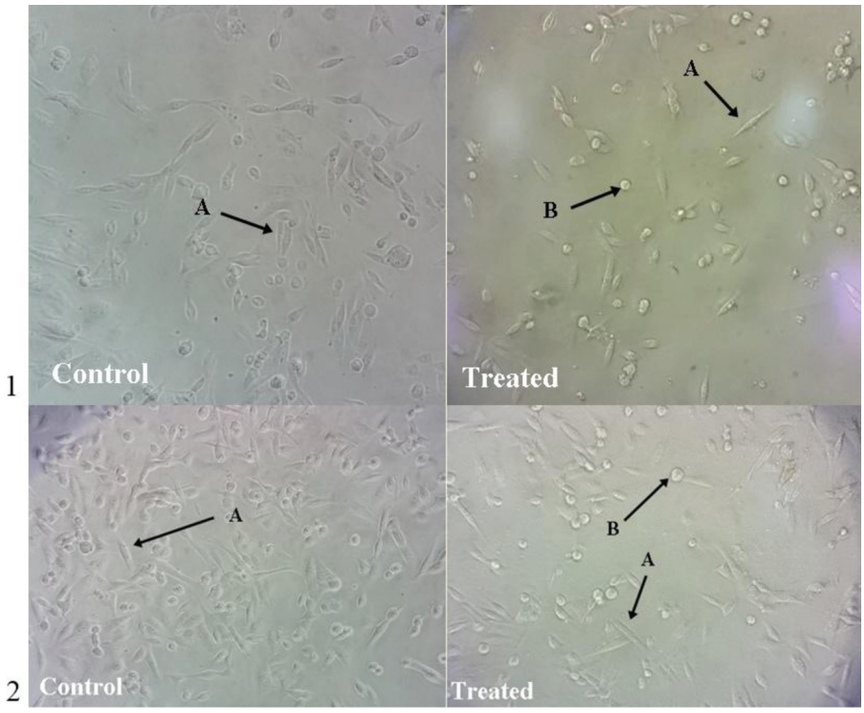

FIGURE 7. Effect 100% ethanol and water extracts of *C. nutans* extracts on morphology of MDA-MB-231 cells for 72 hours were determined under an inverted light microscope. A: The normal viable cell with the fibroblast-like shape and was attached to the surface of 96-well microplate. B: Cell shrinkage and appeared round in shape. These cells were observed under inverted light microscope (20X magnification). Images 1: Cells treated with 100% ethanol extract of *C. nutans*. Image 2: Cells treated with water extract of *C. nutans.*

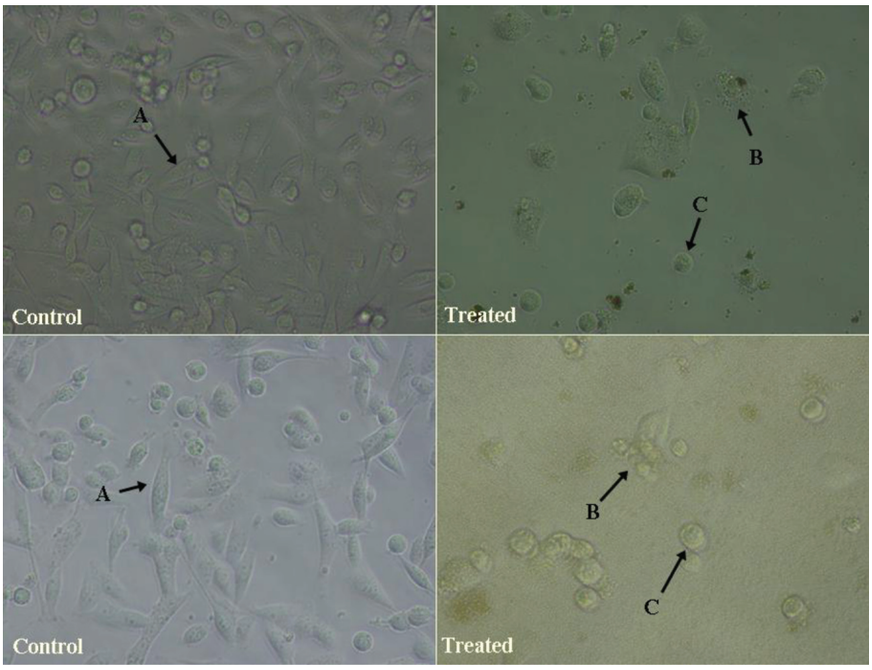

FIGURE 8. Effect of 100% ethanol and water extract of *C. nutans* in combination with PTX on morphology of MDA-MB-231 cells for 72 hours. A: The normal viable cell with the fibroblast-like shape and was attached to the surface of 96-well microplate. B: Blebbing of cells. C: Cell shrinkage and appeared round in shape. These cells were observed under inverted light microscope (20X magnification). Image 1: The cells treated with 100% ethanol extract of *C. nutans* in combination with PTX. Image 2: The cells treated with water extract of *C. nutans* in combination with PTX.

DISCUSSION

In Malaysia, the leaf extracts of *C. nutans* are broadly used on cancer patients and local practice claims a cure to cancer (Fong 2015; Farooqui et al. 2015). Various studies had been done to determine the phytochemical compounds present in *C. nutans*. Ethanol, methanol and water are polar solvents which usually extract more polar or hydrophilic phenolic compounds and flavonoid (Fong 2015). A study by Chelyn et al. (2014) had identified four known flavones C-glycosides such as shaftoside, orientin, isovitexin and vitexin from the ethanolic extract of *C. nutans* leaves. In another study done on ethanol extract of *C. nutans*, sulfur-containing compounds were isolated named clinamides A-C and 2-*cis*-entadamide A (Tu et al. 2014).

Phenolic compounds have displayed health-promoting benefits and nowadays, they become main concern because of their important biological and pharmalogical properties, particularly the anti-inflammatory, antioxidant, antimutagenic and anticarcinogenic activities (Stalikas 2007). There is also an example of flavonoid compound that has anti-cancer potential which is vitexin that can be found in *C. nutans*. Vitexin-induced antitumor effect and cytotoxic activity is exerted via proapoptotic process, where it is mediated by a reduced of Bcl-2/Bax ratio and caspases activation. Vitexin also has been shown to induce apoptotic actions on human breast cancer cell lines and have anti-inflammatory activities (Aslam et al. 2015). In this study, the cytotoxicity potency of 100% ethanol and water extracts of *C. nutans* alone on MDA-MB-231 cells was determined. The 100% ethanol extract showed little cytotoxic activity, meanwhile water extract of *C. nutans* did not show any cytotoxic activity against MDA-MB-231 cells.

Previous study by Fong (2015) also demonstrated that water and ethanol extract of *C. nutans* did not show any cytotoxic effect on MCF-7 cells at 72 hours. However, a study done by Yong et al. (2013) showed that water extract of *C. nutans* had significant inhibition cell proliferation in HeLa and K-562 cells. The different in activity of water extract between the studies might be contributed by the type of the cells used where every cell line has their own unique characteristics and susceptibility toward any treatment. Water extract did not show any cytotoxic effect against breast cancer cell lines (MCF-7 and MDA-MB-231) but showed cytotoxic effect on HeLa and K-562 leukemia cells.

We observed the discrepancies in cytotoxic activity of 100% ethanol and water extracts of *C. nutans* on MDA-MB-231 cells. Even though both ethanol and water are polar solvents which usually extract more polar or hydrophilic phenolic compounds and flavonoid, they may extract the same compounds from the same plant, but the level or amount of each compound extracted might be different for each solvent. This also suggests that every extract may yield a unique set of compounds (Fong 2015).

And this unique set of compounds may have different effects on the cells. In this study, we suggest that ethanol is a better solvent in extracting cytotoxic agent out of *C. nutans*.

In this study, the cytotoxicity potency of *C. nutans* extracts in combination with PTX was also determined. The 100% ethanol extract of *C. nutans* enhanced PTX-induced cell death significantly when combined together, meanwhile the water extract of *C. nutans* inhibited PTX-induced cell death significantly at highest concentration. It is interesting to note that there were contradictions found in the results between the treatment with extract alone and combination of extract with PTX. For example, 100% ethanol extract of *C. nutans* had only little cytotoxic effect when it was used alone against MDA-MB-231 cells but significantly enhanced PTX-induced cytotoxicity when combined together. For water extract, it did not exhibit cytotoxic activity when it was used alone and inhibited PTX-induced cytotoxocity significantly at the highest concentration when in combination with PTX.

The extract used in this study was crude extract that consisted of various substances which have not yet been purified and fully characterized. Hence, it is possible for substances with antagonistic or synergistic effects can be found within the same extract (Elias et al. 2013; Fong 2015). This may suggest that the compounds contained in the crude extract may possess opposing effects when they were used alone but exhibited synergistic effects when combined with another compounds even though the same extract was used. It is also possible for the crude extract to have compounds that inhibited another compound when combined together even though it did not exhibit any effect when it was used alone.

The significant decreased of the cell viability when the cells were treated with *C. nutans* extract in combination with PTX compared to *C. nutans* extract alone may be due to the cytotoxic effect of the PTX itself. Based on the morphology of the cells treated with combination of *C. nutans* extracts and PTX, the results showed that the cells morphology is similar with the cell morphology when treated with PTX alone.

Based on the morphology of the cells treated with *C. nutans* extracts alone, the cells exhibited normal cell morphology which is fibroblast-like shape, a few cells appeared round in shape and number of cells decreased compared to control after treated with the extracts. Compare to the morphology of the cells treated with PTX, the overall cells appeared healthier without significant morphology features characteristic of cell death such as apoptotic bodies, membrane blebbing or necrosis occurred.

MDA-MB-231 cell is the type of cell that was used in this study. It is a hormone-independent breast cancer with aggressive characteristic (Mur et al. 1998; Gluz et al. 2009). This characteristic also may contribute to resistance characteristic shown by the cells when treated with the *C. nutans* extracts. Based on the results obtained where CNA inhibited the potency of PTX, we suggest that the cancer patients under medication of PTX not to consume CNA together with PTX. However, further study is needed to confirm this finding before conclusion can be made.

CONCLUSION

Overall, this finding suggests that CNE was able to increase the PTX potency and has potential to be used as new combination chemotherapy. However, further study to understand the underlying mechanism of the observed PTX enhancement by CNE is needed to discover its potential use in cancer treatment.

ACKNOWLEDGEMENT

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Nur Hasnieza Mohd Rosli
Fariza Juliana Nordin
Leong Lek Mun
Nur Syazwani Abdul Aziz
Biomedical Science Programme
School of Healthy Aging and Wellness (H-Care)
Faculty of Health Sciences
Universiti Kebangsaan Malaysia
Jalan Raja Muda Abdul Aziz
50300 Kuala Lumpur.

Chan Kok Meng
Environmental Health and Industrial Safety Programme
School of Applied Health Sciences
Faculty of Health Sciences
Universiti Kebangsaan Malaysia
Jalan Raja Muda Abdul Aziz
50300 Kuala Lumpur.

Corresponding author: Nor Fadilah Rajab
Email: nfadilah@ukm.edu.my
Tel: +603-92897002
Fax: +603-26929032

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