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Synergistic Effects of *Clinacanthus nutans* Leaf Aqueous Extract and Paclitaxel on The Cell Cycle and Apoptosis of Breast Cancer Stem Cells Expressing $CD44^{+}/CD24^{-}$

Aldy Safruddin Rambe[1,*], Siti Syarifah[2], Adi Muradi Muhar[3], Yunita Sari Pane[2] and Alfi Khatib[4]

[1]*Department of Neurology, Faculty of Medicine, Universitas Sumatera Utara, Medan 20155, Indonesia*
[2]*Department of Pharmacology & Therapeutic, Faculty of Medicine, Universitas Sumatera Utara, Medan 20155, Indonesia*
[3]*Department of Surgery, Faculty of Medicine, Universitas Sumatera Utara, Medan 20155, Indonesia*
[4]*Department of Pharmaceutical Chemistry, Kuliyyah of Pharmacy, International Islamic University Malaysia, Pahang 25200, Malaysia*

([*]Corresponding author's e-mail: aldy@usu.ac.id)

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Abstract

We investigated the combination effect of *Clinacanthus nutans* (*C. nutans*) leaf aqueous extract and paclitaxel on breast cancer stem cells (BCSCs) cell cycle and apoptosis, and identify potential anti-cancer active compounds in *C. nutans* leaves. We performed cell cycle and apoptosis test by using flow cytometry to determine the synergistic effects of the combination. The potential anti-cancer compounds in *C. nutans* leaf aqueous extract was determined using an LC-MS/QTOF system. BCSCs expressing $CD44^{+}/CD24^{-}$ viability decreased after the administration of *C. nutans* leaf aqueous extract and paclitaxel. The combination group had a higher number of cells in S-phase compared to the control group. We found a significant difference in the percentage of number of cells between groups in G2/M phase. More early and late apoptotic cells were observed in the combination group than in only *C. nutans* leaf aqueous extract or control groups. We found the upregulation of P53 and downregulation of MDM2 in *C. nutans* leaf aqueous extract group. We found six bioactive compounds namely 3β,6beta-dihydroxynortropane, isoorientin 2"-O-arabinoside, 2-hydroxyhexadecanoic acid, 2-aminohexadecanoic acids, enigmol and spisulosine. *C. nutans* leaf aqueous extract synergized with paclitaxel, interfered with the cell cycle (mainly in G2/M phase), and induced early and late apoptosis in BCSCs. The synergistic effects are believed to be due to the presence of 3β,6beta-dihydroxynortropane, isoorientin 2″-O-arabinoside, 2-hydroxyhexadecanoic acid, 2-aminohexadecanonic acid, enigmol, and spisulosine that exert anti-cancer effects.

Keywords: *C. nutans leaf*, Aqueous extract, Breast cancer stem cells, Paclitaxel

Introduction

Breast cancer is a malignant disease that affects women worldwide, with 23% of women being diagnosed and accounting for approximately 14% of cancer deaths [1]. Technological advancements in breast cancer management, such as chemotherapy, radiation therapy, surgery, and monoclonal antibodies, have failed to eliminate metastasis, resistance, and recurrence. Previous studies have revealed that new monoclonal antibodies, such as trastuzumab emtansine (T-DM1), are unsuccessful in treating metastatic breast cancer, and T-DM1 resistance develops [2]. Metastatic breast cancer cells can develop resistance mechanisms to new drugs, such as CDK4/6 inhibitors [3]. The second issue is the recurrence of breast cancer in patients, which tends to occur in 30% of breast cancer patients who have recovered in 5 - 10 years [4]. Previous research has

indicated that HER2 (+) and triple-negative breast cancer (TNBC) subtypes have greater recurrence rates than luminal types A and B [5].

BCSCs contribute to metastasis, resistance, and recurrence of breast cancer cases [6]. BCSCs are a small proportion of cells found in the tumor that can self-renew, start, and differentiate into new cells of varying types. BCSCs are widely found in TNBC-type breast cancer that elicits resistance to chemotherapy and metastatic tumors [7]. Paclitaxel is a commonly used chemotherapeutic agent in cases of TNBC; however, paclitaxel is also beginning to experience resistance and cause severe adverse drug reactions, such as hematological toxicity, peripheral neuropathy, and hypersensitivity reactions [8,9].

The use of natural products is quite popular among patients with breast cancer because of their effectiveness and far fewer side-effects compared to chemotherapeutic agents [10]. *Clinacanthus nutans (C. nutans*) is a plant having anti-cancer properties that grows widely in Malaysia, China, Indonesia, and Thailand [11]. *C. nutans* leaves have been proven to have anticancer properties [12,13]. *In vitro*, the combination of *C. nutans* leaf ethanol extract with doxorubicin increased the apoptosis of MCF-7 and T47D breast cancer cells. However, T47D and MCF7 cells are hormone-dependent cell lines, specifically estrogen-dependent. An excellent model of luminal A subtype breast cancer is the T47D cell line. Given the chemotherapeutic sensitivity of T47D and MCF7 cells, the synergistic effect of *C. nutans* and doxorubicin is not particularly surprising [14]. Prior research has also demonstrated that the combination of ethanol extract from *C. nutans* leaves and Cisplatin worked synergistically in reducing viability and proliferation of MDA-MB231 cells [13]. Despite the promising findings of this study, Cisplatin is not a chemotherapy agent commonly given to breast cancer patients, either as neoadjuvant or adjuvant therapy. The commonly used chemotherapy agent is a combination of Paclitaxel-based chemotherapy [15].

Several studies have shown that the anti-cancer activity of *C. nutans* leaves is due to the presence of phenolic components such as vitexin, isovetexin, orientin, and isoorientin, as well as palmitic acid, 1.2 benzenedycarboxyllic acid-mono (2-ethylhexyl) esters (BMEH), and 19-oxo-all-trans-retinoic acid [11,16-18]. Amazingly, *C. nutans* leaves were shown to be harmless for normal cells [18]. Previous examinations on *C. nutans* leaves tended to employ solvents such as ethanol, n-hexane, and methanol, while studies on *C. nutans* leaves used aqueous solvents, which tend to be safer. Our previous study showed the cytotoxic activity of *C. nutans* leaves aqueous extract to breast cancer stem cells [19]. The combination of *C. nutans leaf* aqueous extract with paclitaxel and targeting BCSCs, has not yet been conducted. Therefore, we analyzed the synergistic effects of *C. nutans* leaf aqueous extract and paclitaxel on BCSCs.

Materials and methods

C. nutans leaf preparation and extraction

C. nutans leaves were obtained from local farmers in Medan, Indonesia, at coordinates 3.519131 °N, 98.642584 °E. The plants were identified in Bogor, Indonesia (voucher no. B-612/V/DI.05.07). Extraction was performed by the maceration method using aqueous solvent. The process was repeated three times; the solvent was applied using a rotary evaporator at 60 °C, and was freeze-dried for 24 h at –4 °C until no solvent remained [20].

Isolation and culture of BCSCs

MDA-MB231 cells line, acquired from European Collection of Authenticated Cell Culture (ECACC), was cultured in Dulbecco's Modified Eagle's medium (DMEM), which is a high-glucose medium (Sigma-aldrich). The DMEM was supplemented with 10% Fetal Bovine Serum (FBS) (Gibco Invitrogen, NY, USA), 1% penicillin-streptomycin, and 0.25% Amphotericin-B (Gibco Invitrogen). Cells and media were placed in T25 flasks and cultivated at 37 °C and 5% CO_2. After reaching 80% confluence, cells were harvested, incubated, and isolated using the gradual magnetic-activated cell sorting (MACS). MDA-MB 231 cells were incubated with antibody-labeled beads (CD24 (Elabscience®, TX, USA) first, then CD44 (Elabscience®, TX, USA), alternately), which were then separated using magnets to obtain four different populations: $CD24^+$, $CD24^-$, $CD24^-/CD44^-$, and $CD24^-/CD44^+$ [21].

BCSC Validation through $CD44^+$ and $CD24^-$ flow cytometry

BCSCs were cultured in BCSC culture medium in 6-well plates at a density of 1×10^5 cells/well and then incubated for 24 h at 37 °C and 5% CO_2. They were then passaged and incubated with 10 µL fluorescein isothiocyanate-labeled CD44 (Elabscience®, TX, USA) antibody and 10 µL phycoerythrin (PE)-labeled CD24 (Elabscience®, TX, USA) antibody for 20 min at 22 - 26 °C. Subsequently, the incubated cells were washed with phosphate-buffered saline (PBS) and centrifuged at 300 × *g* for 5 min. They were resuspended in 300 µL PBS and counted using flow cytometry. The isolated BCSCs were characterized using APC-CD44 and PE-CD24 (APC and PE antibodies). APC-CD44 and PE-CD24 were irradiated with a laser such that they fluoresced; then, the detector captured them during flow cytometry to form dots [22].

Cytotoxic test of combination *C. nutans* leaf aqueous extract and paclitaxel

Based on previous cytotoxic tests, we obtained an IC_{50} value of 16.16 µg/mL for only *C. nutans* leaf aqueous extract and an IC_{50} of 16 nM for only paclitaxel [19]. Then, we performed a cytotoxic test with an MTT assay to evaluate the effects of combining *C. nutans* leaf aqueous extract with paclitaxel [23]. The dose combination was $1\times IC_{50}$, 1/2 IC_{50}, 1/4 IC_{50}, and 1/8 IC_{50} for *C. nutans* leaf aqueous extract and paclitaxel [21].

Dose determination of aqueous extract *C. nutans* leaves and paclitaxel combination

After the MTT test for combination preparation, we conducted an advanced analysis by determining a combination index to determine the synergistic relationship between the aqueous extract and paclitaxel. The following phase of the cell cycle test on BCSCs was used to corroborate the isobologram results [21].

Flow cytometry for cell cycle assays

Cells (4×10^5 cells/mL) were disseminated in 6-well plates and incubated until normal return. After normal cells were returned, the medium was removed, cells were washed with PBS, and *C. nutans* leaf aqueous extract was added in various concentrations. It was then incubated for 24 h in a 5% CO_2 incubator at 37 °C. After 24 h of incubation, live and dead cells were harvested. The culture medium was transferred into a conical flask, the cells were washed twice with 500 µL PBS, and PBS was added the same flask. Thereafter, the cells were harvested, supplemented with 200 µL 0.25% EDTA trypsin, incubated for 3 min in an incubator, and given the culture medium to inactivate 1 mL trypsin. The culture medium was then transferred into the initial conical flask. The well was washed again with 500 µL PBS, and PBS was added into the conical. The conical flask now contained the harvested cells, which were centrifuged at 2000 RPM for 3 min. PBS was removed from the conical flask, cold ethanol was added, and the cells were resuspended for cell fixation and incubated for a while in a refrigerator. In the re-centrifugated suspension, the cell deposits obtained in the centrifugation tube closed with aluminum foil dissolved with 400 µL Propidium Iodide (PI) reagent containing 1 mg/mL PI, 10 mg/mL RNAse, and 0.1% (v/v) Triton-X 100. The cells were suspended and incubated for 5 min; then, the suspension was transferred into a cytometry flow tube and analyzed [24].

Apoptosis test with flowcytometry

Cells (2×10^5 cells/mL) were distributed into 6-well plates and incubated until the regular state returned. After the normal cells returned, the medium was removed, the cells were washed with PBS, and various concentrations of *C. nutans* leaf aqueous extract were added. It was then incubated for 24 h in a 5% CO_2 incubator at 37 °C. After 24 h of incubation, live and dead cells were harvested. The culture medium was transferred into a conical flask, and the cells were washed twice with 500 µL PBS and PBS was added into the same flask. We harvested the cells by adding 200 µL 0.25% EDTA trypsin, incubated for 3 min in an incubator, and given the culture medium to inactivate 1 mL trypsin. The culture medium was then placed in a conical flask. The conical flask now contained harvested cells, which were centrifuged at 2000 RPM for 3 min. PBS was removed from the flask and 120 µL binding buffer was added. Five microliters each of Annexin V reagent and propidium iodide (BD annexin V-FITC-Apoptosis Detection Kit) were added and incubated for 15 min, and then 300 µL binding Buffer was added. Subsequently, we analyzed the apoptosis by using the flow cytometer DB C6 [25,26].

Statistical analysis

Statistical analyses were performed using SPSS (version 26.0) and GraphPad Prism (version 10.0). We analyzed the differences in the number of cells expressing $CD44^+/CD24^-$ before and after isolation using an independent T-test. Differences in cell viability between groups were analyzed using the Kruskal–Wallis test. We used one-way ANOVA test to evaluate the effects of combining the aqueous extract of *C. nutans* leaves and paclitaxel on BCSC cell cycle and apoptosis. Statistical significance was set at $p < 0.05$.

Determination of active compounds with LC/MS-QTOF

C. nutans leaf aqueous extract was replicated into triplicates at a concentration of 100 mg each. We detected the active compounds by using liquid chromatography-mass spectrometry (LC-MS). The samples were analyzed using the LC/MS-QTOF system [26]. The LC/MS-QTOF system used was the Agilent 1200 liquid chromatography system (Agilent Technologies, Santa Clara, California) equipped with a binary pump, a vacuum degasser unit, an automatic sampler, and a 6520-quadrupole mass time spectrometer, with an electrospray ionization source (ESI). The column used was an Agilent ZORBAX Eclipse Plus C18 Quick Resolution HT (2.1×100 mm) 1.8 µm. The chromatography separation was performed at a temperature of 40 °C using the Agilent zorbax eclipse plus C18 quick resolution HT (2.1×100 mm) 1.8 µm with (A) 0.1% acid format in dH20 and (B) acid format 0.1% in acetonitrile for positive mode. Gradient elution program was 0.00 - 18.00 min, 5% - 95% (B); 18 - 23 min; 95% (B); 23.01 min, 5% (B), the total run time was 30 min. The LC conditions were rebalanced for 2 min before starting a new injection. The injection sample volume was 2 µL, and the movement phase flow rate was 0.25 mL/min. The mass spectrometer was operated in positive electrospray ionization mode (ESI) with an optimal gas temperature of 325 °C, gas flow of 11 L/min, and nebulizer at a pressure of 35 psi. Data analysis was performed using Agilent Mass Hunter Qualitative Analysis Software B.05.00 (Agilent Technologies, Santa Clara, CA, USA) for data analysis (MS (d) data). We used METLIN database to analyze the chromatographic [26,27]. For chemical structure of bioactive compound, we created using Marvis JS by Chemaxon (https://www.rcsb.org/chemical-sketch).

Results and discussion

Isolation and culture of BCSCs from MDA-MB 231 cell line

We isolated BCSCs that had been successfully grown and expressed $CD44^+/CD24^-$. Culture was performed up to passage three, and a morphological picture of BCSCs was obtained in spindle-like cells with the presence of actin filament extension (**Figure 1**).

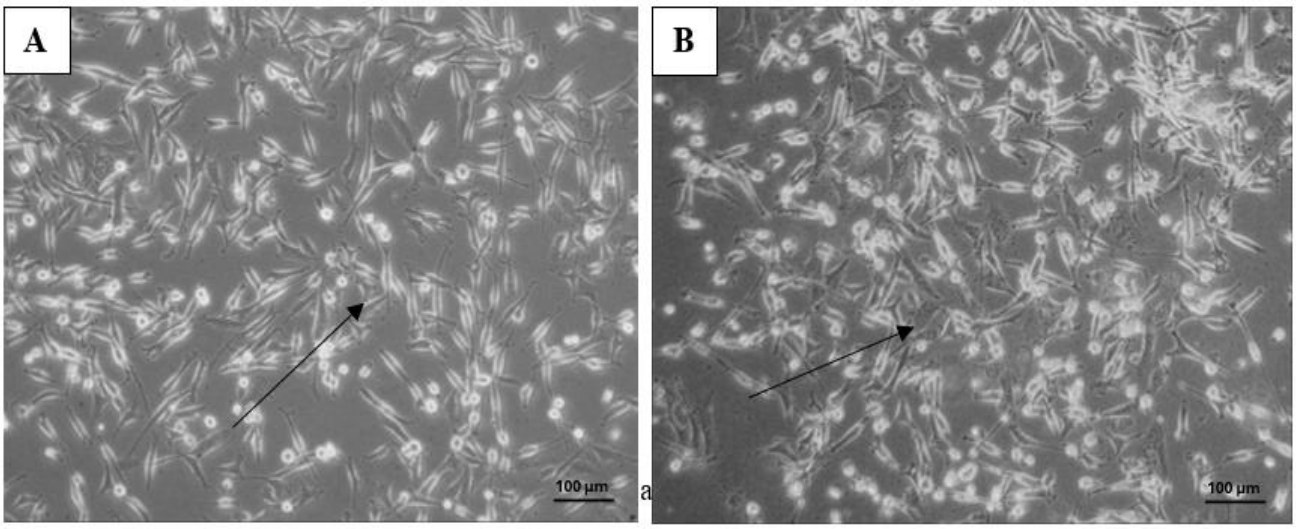


Figure 1 Cell-adherent morphology of MDA-MB231 and BCSCs clone characterization.

Black arrows indicate similarities in the actin morphology (100x magnification). (A) Morphology of actin in MDA-MB231 cells (B) actin morphology in BCSCs.

BCSC Validation Through CD44$^+$ and CD24$^-$ Flow Cytometry

We analyzed CD44$^+$/CD24$^-$ markers located on the cell surface of BCSC via flow cytometry. In this study, 79.2% of MDA-MB231 cells expressed CD44$^+$/CD24$^-$ before sorting; after isolation, the percentage of isolated BCSCs that expressed CD44$^+$/CD24$^-$ increased to 92.4% (**Figure 2**). In breast cancer, the percentage of CD44$^+$/CD24$^-$ cells is higher in malignant lesions than in non-malignant lesions [28]. CD44 is a BCSC biomarker that is used as a diagnostic, therapeutic, and prognostic indicator [30]. A high CD44$^+$/CD24$^-$ ratio correlates with strong proliferative capacity and tumorigenicity [31].

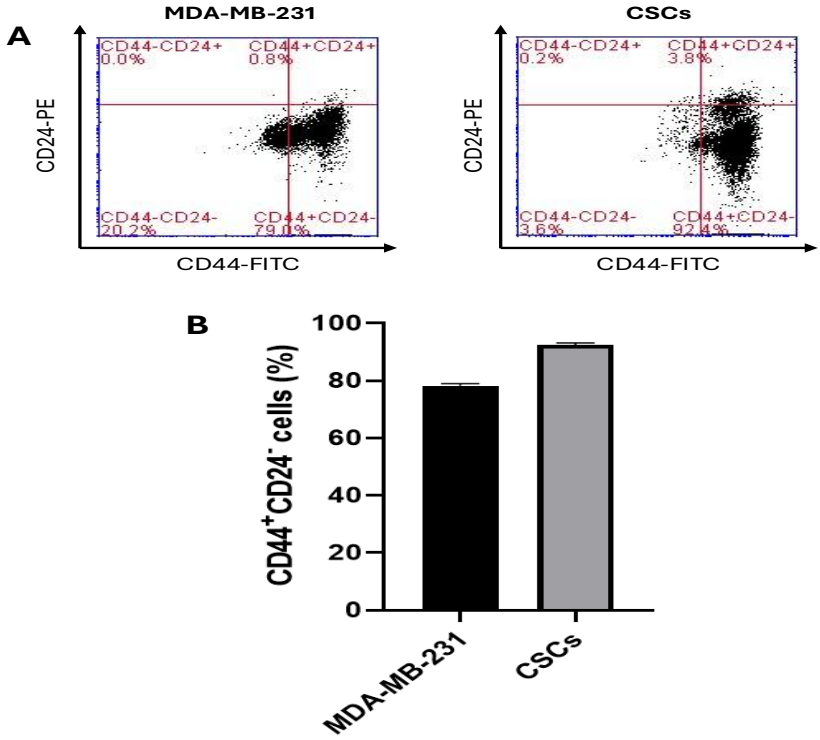


Figure 2 Differences in the number of cells expressing CD44$^+$/CD24$^-$ before and after isolation. Data are expressed as mean±SEM (n=3). (A) The percentage of MDA-MB231 cells that expressed CD44$^+$/CD24$^-$ was 79.2%. (B) The percentage of BCSCs that expressed CD44$^+$/CD24$^-$ was 92.4%. The difference was significant according to independent T-test, ***$p < 0.001$.

In this study, the number of MDA-MB231 cells expressing CD44$^+$/CD24$^-$ before and after isolation from BCSCs was significantly different ($p < 0.05$). Our findings are consistent with earlier research demonstrating that CD44 is a BCSC biomarker linked to enhanced metastasis, proliferation, and self-renewal [32]. When compared to non-malignant tissues, the percentage of CD44$^+$/CD24$^-$ in malignant lesions of breast cancer rises [29,30]. BCSCs are a tiny subset of tumor cells with the ability to self-renew, endure treatment, be highly differentiable, and exhibit the CD44$^+$/CD24$^-$/ALDH1$^+$ phenotype [33].

Cytotoxicity test combination of *C. nutans* leaf aqueous extract and paclitaxel

The IC_{50} values of *C. nutans* leaf aqueous extract and paclitaxel were previously determined in a prior study, at 16.16 µg/mL and 16 nM, respectively. For the cytotoxic assay, dose combination of 1×IC_{50}, 1/2 IC_{50}, 1/4 IC_{50}, and 1/8 IC_{50} were used for both *C. nutans* leaf aqueous extract and paclitaxel [20]. **Table 1** shows significant differences between the group of combined doses of aqueous extract of *C. nutans* leaves (EA CN) and paclitaxel (PAX) concerning the viability of BCSCs ($p < 0.05$).

Table 1 Relationship of combination doses of *C. nutans* leaf aqueous extract and paclitaxel to the viability of BCSCs. Data represent the mean ± standard deviation (SD) of three independent replicates, each performed in triplicate. ns: $p > 0.05$, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$.

No.	Group (dose combination)	Cell viability (Mean ± SEM)	*p*-value
1	1/8 IC_{50} EACN–1/8 IC_{50} PAX	51.83 ± 14.95	0.010*
2	1/8 IC_{50} EA CN–1/4 IC_{50} PAX	65.27 ± 2.74	
3	1/8 IC_{50} EA CN–1/2 IC_{50} PAX	60.05 ± 5.34	
4	1/8 IC_{50} EA CN–IC_{50} PAX	113.84 ± 23.07	
5	¼ IC_{50} EA CN–1/8 IC_{50} PAX	71.41 ± 14.07	
6	¼ IC_{50} EA CN–1/4 IC_{50} PAX	82.77 ± 13.90	
7	¼ IC_{50} EA CN–1/2 IC_{50} PAX	77.42 ± 20.86	
8	¼ IC_{50} EA CN–IC_{50} PAX	97.38 ± 12.38	
9	½ IC_{50} EA CN–1/8 IC_{50} PAX	67.49 ± 22.94	
10	½ IC_{50} EA CN–1/4 IC_{50} PAX	70.10 ± 10.81	
11	½ IC_{50} EA CN–1/2 IC_{50} PAX	62.14 ± 11.57	
12	½ IC_{50} EA CN–IC_{50} PAX	69.54 ± 9.08	
13	IC_{50} EA CN–1/8 IC_{50} PAX	65.30 ± 2.74	
14	IC_{50} EA CN–1/4 IC_{50} PAX	50.52 ± 3.70	
15	IC_{50} EA CN–1/2 IC_{50} PAX	66.45 ± 10.53	
16	IC_{50} EA CN–IC_{50} PAX	65.53 ± 18.48	

*Kruskal–Wallis test.

According to **Table 1**, a majority decrease was noted in the viability of BCSCs after the administration of various doses of EA CN–PAX combination up to 50% (IC_{50} EA CN–1/4 IC_{50} PAX group) and statistically significant differences in viability of BCSCs between groups ($p < 0.05$). In the group of 1/8 IC_{50} EA CN–IC_{50} PAX, we found the viability cells were above 100%, it demonstrated the presence of a bioactive chemical that exhibits no toxicity towards the cell lines.

Dose determination of a combination of aqueous extract *C. nutans* leaves and Paclitaxel

Advanced analysis was performed to identify a combination index for determining the synergistic relationship between EA CN and Paclitaxel. The potential application in combination treatment was analyzed using the combination index (CI). The CI was determined using formula 1, and the CI values are listed in **Table 2** [34].

$$CI = \frac{C_{Ax}}{IC_{xA}} + \frac{C_{Bx}}{IC_{xB}} \quad (1)$$

Drug concentrations A and B combined to produce x% of the maximum combination effect are denoted by C_{Ax} and C_{Bx}. IC_{xA} and IC_{xA} are the concentration of a drug needed to generate x% of the maximum effect when taken alone. The equation used to determine the two combinations of compounds in the form of synergistic (CI < 1), additive (CI = 1) or antagonistic effects (CI > 1) [35].

Table 2 Combination index values of aqueous extract of *C. nutans* leaves–Paclitaxel.

Group Aqueous extract (µg/mL)	Paclitaxel (nM)			
	1/8 IC_{50} (2)	**¼ IC_{50} (4)**	**½ IC_{50} (8)**	**IC_{50} (16)**
1/8 IC_{50} (2)	0.32	0.29	0.01	–0.11
¼ IC_{50} (4)	2.82	–0.61	–1.43	–0.29
½ IC_{50} (8)	1.87	3.37	0.84	–0.38
IC_{50} (16)	6.29	1.19	3.12	2.63

Table 2, reveals that the lowest combination index value was found at 1/4 IC_{50} EA CN-1⁄2 IC_{50} Paclitaxel, followed by 1/2 IC_{50} EA CN-1×IC_{50} Paclitaxel. Since there were presence of negative CI values in isobologram test, we underwent cell cycle testing corroborated the isobologram findings in this investigation. In the cell cycle test, the most optimum result was achieved precisely at a combination of 1/2 IC_{50} EA CN (8 µg/mL)–1×IC_{50} Paclitaxel (PAX) (16 nM) and combination of IC_{50} EA CN (16 µg/mL)–IC_{50} PAX (16 nM). For the cell cycle and apoptosis test, six groups were created, namely, the negative control group (control), EA CN 8 µg/mL (EA CN-8), EA CN 16 µg/mL (EA CN-16), Paclitaxel (PAX), the combination of EA CN 8 µg/mL–PAX (EA CN 8–PAX), and EA CN 16 µg/mL–PAX (EA CN 16–PAX).

Cell cycle testing with flowcytometry

We observed the number of cells in the sub-G1, G0/G1, S, and G2/M phases. There was a decrease in the number of cells in the sub-G1 group in the EA CN group (both single preparation and combination) when compared with the negative control group ($p < 0.05$). Significant decreases in the number of BCSCs occurred after administration of EA CN-8 and EA CN-16 (0.37 ± 0.12% and 0.47 ± 0.12%, respectively) compared to PAX group (0.80 ± 0.10%) and EA CN–PAX (0.97 ± 0.15%) ($p < 0.05$) (**Figure 3(A)**). In phase G0/G1 observations, a significant reduction was found in the EA CN 8-PAX (31.13 ± 0.74%) and EA CN 16-PAX (30.53 ± 0.87%) compared with the control group (57.03 ± 0.93%) ($p < 0.0001$). A significant decrease in cells was observed after administration of the EA CN–Paclitaxel combination compared to the only EA CN group ($p < 0.0001$) (**Figure 3(B)**).

During phase S, no significant difference was found in the number of cells in the EA CN groups (EA CN-8 and 16) compared with the negative control group; however, after the administration of EA CN 8–PAX and EA CN 16–PAX, there was a significant increase in cell number compared to the control and EA CN groups ($p < 0.0001$). Notably, in the S phase, there were increases in the percentage of BCSCs in the combined group by 27.67 ± 0.25% (EA CN 8–PAX) and 27.90 ± 0.70% (EA CN 16–PAX) in comparison with Paclitaxel alone (27.47 ± 0.45%); however, the difference was not statistically significant ($p > 0.05$) (**Figure 3(B)**). In G2/M phase, we found a significant difference in the percentage of the number of cells between the groups ($p < 0.0001$). The number of cells increased in the EA CN 8, PAX, and combination groups. Remarkably, a percentage increase was noted in the number of cells in the EA CN 16–PAX group (40.43 ± 0.49%) compared to the EA CN 8 (20.47 ± 0.78%), EA CN 16 (23.00 ± 1.31%), and control groups (20.00 ± 0.61%) ($p < 0.0001$). The difference in the percentages of the cell number was also statistically significant compared with the paclitaxel group (37.93 ± 0.83%) ($p < 0.05$) (**Figure 3(B)**).

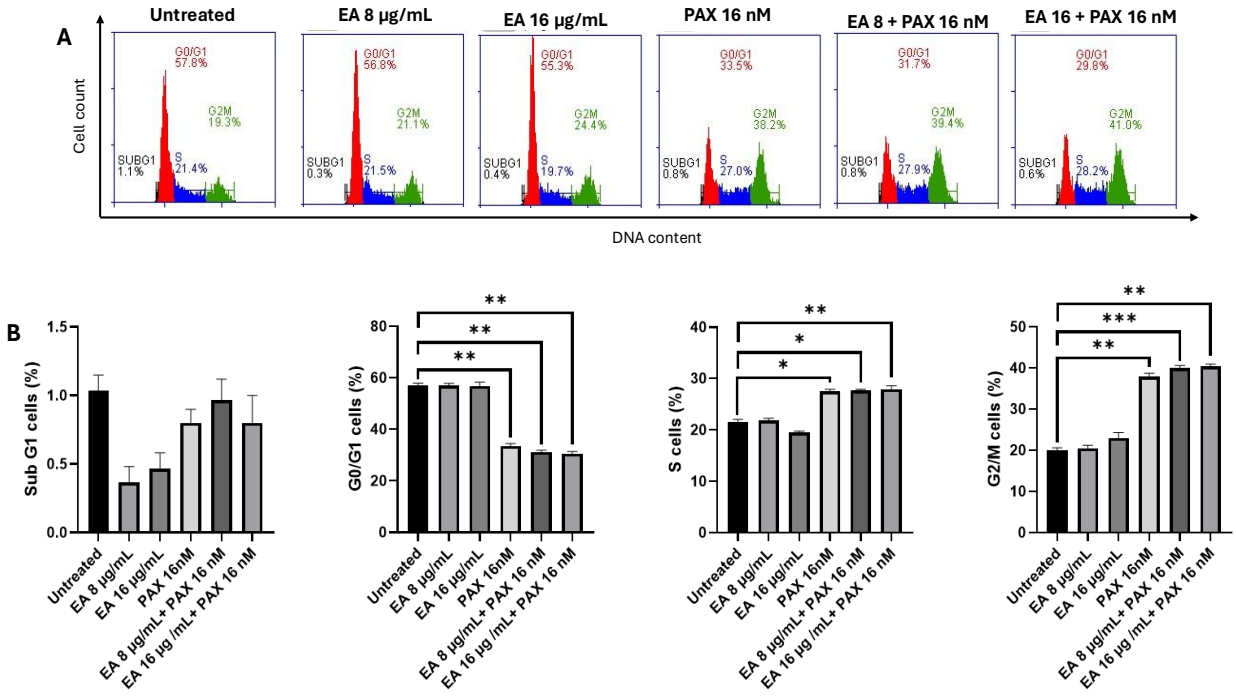


Figure 3 Cell cycle analysis by flow cytometry represents cell population of sub-phase G1, phase G0/G1, Phase S and Phase G2/M (A). Graphical representative showed significancy compared to control (B). * $p < 0.05$; ** $p < 0.01$; *** $p <$ 0.001. Data represent the mean ± standard deviation (SD) of three independent replicates, each performed in triplicate. EA: Extract aqueous, PAX: Paclitaxel.

In comparison to the control groups, this study found that the single and combination *C. nutans* aqueous extract groups had less cells in the sub-G1 phase. These results could not be compared with those of previous studies involving direct analysis of the G0/G1 phase, but the researchers argue that this may be due to an increase in the number of cells in the G2/M phase in either only *C. nutans* leaf aqueous extract group or a combination, thereby affecting a decrease in the cell count in sub-G1-phase.

We observed a drop in the number of cells in G0/G1 phase in the EA CN 8 and EA CN 16 groups, followed by a decrease in the S phase in the EA CN 16 group. Our result was inconsistent with previous studies showing that the DNA content was high in G0/G1 phases, and then decreased in the post-S phase after the administration of hexane and chloroform *C. nutans* leaf extract [36]. It was also inconsistent with previous studies on the cell cycle test; the ethyl acetate fraction: *C. nutans* leaf hexane extract inhibited cell proliferation in the G1 cycle. This contradictory result was due to an increase in the DNA content of G1 cycle and a decrease in the amount of DNA during S and M phases [26]. Previous research also showed that *C. nutans* leaf dichloromethane extract trapped HeLa cells in the S phase [20]. In this study, the amount of DNA increased in the G2/M phase. Our results differed owing to the different solvents used to produce the different active compounds that affect the phase of the cell cycle.

This study showed an increase in the DNA content in the G2/M phase after dose administration in EA CN 8 and EA CN 16 groups compared to that in the control group. An increase in the number of cells in the G2/M phase also occurred after the administration of Paclitaxel [37]. Our result is logical because Paclitaxel acts as a microtubule-stabilizing agent by inhibiting the microtubule depolymerization in the mitosis phase [38]. Stability in the microtubules causes cells to be incapable of mitosis, thus triggering the activation of P53 as the initiator of the apoptosis process. The cells trapped in the G2/M phase undergoing apoptosis can be seen during the increase in early apoptosis that occurs after

Paclitaxel administration [37]. Paclitaxel inhibits cell viability and proliferation in dose-dependent patterns (dose-dependent manner). Previous research has shown that the administration of Paclitaxel leads to an increase in the number of cells trapped in the G2/M phase of the cell cycle and increased expression of Caspase-3 and Bax, whereas decreases were observed in the Bcl-2, MMP-9, VEGF, Akt protein, and PI3K/Akt signaling pathways [39].

In this study, a significant increase was noted in the number of cells trapped in the G2/M phase after administration of EA CN doses of 8 and 16 µg/mL. However, the number of trapped cells was still below that of the Paclitaxel group, but at the time of combination treatment, there was a significant increase in the number of trapped cells in the G2/M Phase compared with the administration of only aqueous extract of *C. nutans* leaves or Paclitaxel. Our study suggested a synergistic effect between the aqueous extract of *C. nutans* leaves and Paclitaxel. After the administration of EA CN 16–PAX, the number of cells trapped in the G2/M phase increased to 40.43%, whereas that of the Paclitaxel group was only 37.93% ($p < 0.001$). Combination therapy is one of the most effective means of implementing cancer, and the treatment approach to target cancer stem cells is the latest pharmacological approach toward the development of anti-cancer drugs [40].

Apoptosis test with flowcytometry

We evaluated the percentage of cells undergoing early, late, and necrotic stages of apoptosis by using flow cytometry. The results of the apoptosis tests are shown in **Figure 4**. In this study, the proportion of cells that underwent early apoptosis between the groups were statistically significant ($p < 0.001$). An increase in the number of cells undergoing early apoptosis was found in the EA CN 8 and 16 groups (8.12 ± 0.75% and 8.47 ± 1.17%, respectively) compared to the PAX group (7.23 ± 0.15%), although the difference was not statistically significant ($p > 0.05$). Greater number of early apoptosis cells were observed in the combination groups EA CN 8–PAX and EA CN 16–PAX (8.80 ± 0.40% and 16.13 ± 1.62%) compared with the PAX group, and a statistically significant difference in the cell number was found between the EA CN 16–PAX and PAX groups ($p < 0.001$) (**Figure 4(A)**).

There was a significant difference in the percentage of cells in late apoptosis between the groups ($p < 0.001$). Greater numbers of late apoptosis cells were found in the EA CN 8 and EA CN 16 groups (21.23 ± 0.95% and 23.40 ± 1.40%, respectively) compared to the control group (2.57 ± 0.42%); however, the percentage of the cell count in PAX group (39.63 ± 1.40%) was still greater than those in the EA CN 8 and EA CN 16 groups ($p < 0.001$). A higher number of late apoptosis cells were found in both the EA CN 8–PAX and EA CN 16–PAX groups (40.83 ± 0.25% and 48.10 ± 1.82%, respectively), and the difference was statistically significant when compared between the EA CN 16–PAX and PAX group ($p < 0.001$). In this study, more early and late apoptotic cells were found in the combination group than in the EA CN 8 and EA CN 16 groups ($p < 0.0001$) (**Figure 4(B)**).

Significant differences were found in the percentage of cells experiencing necrosis between the groups ($p < 0.0001$). Compared to the control group, greater numbers of necrotic cells were observed in EA CN 8, EA CN 16, PAX, and combination groups. An increase in the number of necrotic cells was observed in the EA CN 8 and EA CN 16 groups, although the percentages of necrotic cells was still lower than that in the Paclitaxel group ($p < 0.0001$). Greater numbers of necrotic cells were observed in both the combination groups of EA CN 8–PAX and EA CN 16–PAX compared to the EA CN 8 and EA CN 16 groups ($p < 0.001$); however, no statistically significant difference was found between the combined and paclitaxel groups ($p > 0.05$) (**Figure 4(B)**).

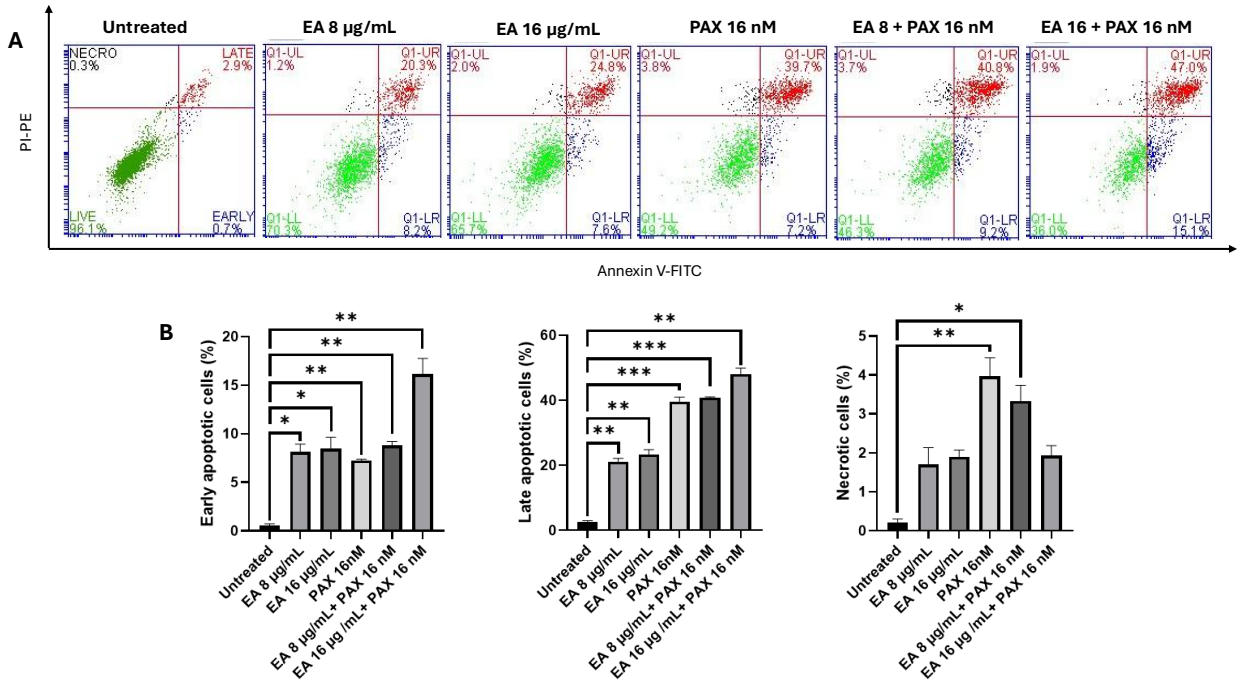


Figure 4 Apoptosis assay by flow cytometry represents cell population of sub-phase G1, phase G0/G1, Phase S and Phase G2/M (A). Graphical representative showed significancy compared to control (B). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Data represent the mean ± standard deviation (SD) of three independent replicates, each performed in triplicate. EA: Extract aqueous, PAX: Paclitaxel.

In this study, significant differences were found in the percentage of cells undergoing early apoptosis between the groups and in the number of cells that underwent early apoptosis after the administration of EA CN 8 and EA CN 16 doses. Apoptosis is the process of programmed cell death, and early apoptosis is the preliminary process for the occurrence of apoptosis. A variety of biochemical signals are conveyed to the cell during early apoptosis to prepare it for the process [30]. During early apoptosis, some caspase enzymes move from the cytoplasm to the mitochondria or nucleus, while cytochrome c is released from the mitochondria's intermembrane gap into the cytoplasm [41,42]. The increased number of cells experiencing early apoptosis following administration of *C. nutans* leaf aqueous extract demonstrates that it can trigger BCSCs apoptosis.

We found an increase in the number of cells undergoing late apoptosis after administering aqueous extract of *C. nutans* leaves [43]. The study also found an increase in the number of cells undergoing necrosis, although the number is much lower than the number that undergoes late apoptosis. Necrosis is a toxic process in which cells become passive and undergo death without energy dependency. This theory distinguishes apoptosis from necrosis, where apoptosis is a programmed and energy-dependent cell death process and necrosis is a passive and uncontrolled cell degeneration process [41].

In this study, an increase in the number of cells undergoing early and late apoptosis was observed after the administration of the combination group was found, and the difference was statistically significant when compared between the EA CN 16–PAX group and the paclitaxel group. Our results suggest a synergistic effect between the aqueous extract of *C. nutans* leaves and paclitaxel in inducing apoptosis. Previous studies have shown that the administration of paclitaxel can increase the expression of proteins involved in the intrinsic pathway of apoptosis, namely Bax and Caspase-3 expression [39]. Previous studies also show that the active fraction of *C. nutans* leaves extract causes upregulation of P53 [36].

Apoptosis is essential for shielding organisms from the development of tumors. Many anti-cancer agents work by triggering apoptosis, which removes genetically damaged or improperly divided cells. The

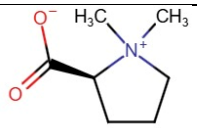
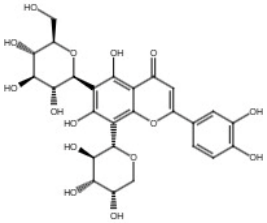
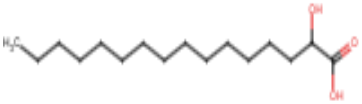
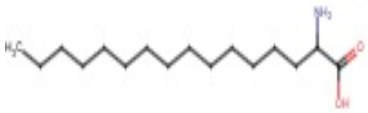
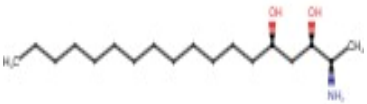
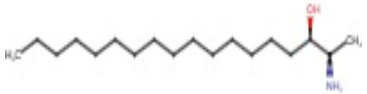
two main pathways of apoptosis in mammalian cells are the intrinsic pathway, which controls apoptotic cascades by combining signaling in the mitochondrion, which results in the activation of caspase cascades, the release of C into the cytosol, and the alteration of the mitochondrial membrane potential (MMP). The extrinsic pathway is triggered by pro-apoptotic receptor signals on the surface [44]. Downregulating the expression of the apoptosis inhibitor Bcl-2 and upregulating the expression of the apoptosis-promoting protein Bax are two methods of activating the apoptosis machinery [45].

P53 is another crucial protein in the machinery of apoptosis. P53 is a tumor suppressor gene that interacts with other genes and regulatory factors to participate in a range of cellular stress and response mechanisms. Inhibiting tumor angiogenesis, inducing cell death, maintaining genome stability, and mediating cell cycle arrest are just a few of the roles that P53 plays [46].

Determination of active compounds using LC/MS-QTOF

In this study, we identified active compounds in the aqueous extract of *C. nutans* leaves with LC/MS-QTOF. Six compounds with anti-cancer potential were identified (**Table 3**).

Table 3 Potential anti-cancer active compounds in aqueous extract of *C. nutans* leaves.

No	Compound name	Formula	*Chemical Structure*	*Retention Time (RT)*	m/z	Mass (g/mol)
1.	3β,6beta-Dihydroxynortropane	$C_7H_{13}NO_2$		1.077	144.1	143.18
2.	Isoorientin 2''-O-arabinoside	$C_{26}H_{28}O_{15}$		7.171	581.15	580.5
3.	2-Hydroxyhexadecanoic acid	$C_{16}H_{32}O_3$		12.529	290.62	272.42
4.	2-Aminohexadecanoic acid	$C_{16}H_{33}NO_2$		12.684	272.25	271.44
5.	Enigmol	$C_{18}H_{39}NO_2$		13.786	302.3	301.5
6.	Spisulosine	$C_{18}H_{39}NO$		13.817	286.31	285.5

The screening technique for discovering putative anti-cancer active chemicals in *C. nutans* was based on using LC/MS in positive ionization mode to detect bioactive metabolites. Our study aligned with previous research that found orientin in *C. nutans* methanol extract with HPLC-IT/MS chromatogram examination with a retention time of 20.55 min [11]. According previous research has shown that the important metabolites found in *C. nutans* leaves are flavonoids, stigmasterol, B-sitosterol, lupeol, botulin, C-glycosyl

flavones, vitexin, isovitexin, shaftoside, isomollupentin, 7-gluco pyranosides, orientin, isoorientin, and sulfur-containing glucosides [47]. Isoorientin is a group of flavonoids. Previous studies have revealed that isoorientin (ISO) promotes apoptosis in several ways, activating the apoptotic pathway through the Fas receptor and lowering the NF-kb signaling pathway by blocking the PI3K/Akt pathway [48]. The identification of isoorientin in our research indicated that aqueous extract of *C. nutans* leaves induced apoptosis in BCSCs by inhibiting the proliferation (PI3K/Akt) pathway.

Another molecule later identified, 2-hydroxyhexadecanoic acid (2-hydroxy palmitic acid), is a long-chain fatty acid [45]. 2-hydroxyhexadecanoic acid was thought to be a component that improves the synergistic effects of *C. nutans* leaves and cisplatin on MDA-MB 231 and 468 cells [49,50]. The two compounds discovered in this study were consistent with previous research demonstrating the presence of palmitic acid, 1.2 benzenedycarboxyllic acid-mono (2-ethylhexyl) esters (BMEH), and 19-oxo-all-trans-retinoic acid in *C. nutans* leaves, indicating that this plant has anti-cancer action [16,17].

The next chemical was 2-aminohexadecanoic acid, a member of the palmitic acid group. Previous studies have shown that hexadecanoic acid can inhibit the proliferation of human colon cancer cells HT-29 [51]. Previous studies using a molecular docking approach to *C. nutans* leaf dichloromethane extract showed a high affinity between palmitic acid and the Mouse Double Minute 2 Homologue (MDM2) protein [16].

The compounds found in this study but not in previous studies were enigmol, spisulosine, and 3β,6beta-dihydroxynortropane. Enigmol is a sphingolipid analog that has anti-cancer potential in several cancer cell lines and effectively suppresses the proliferation of cancer cells in animal models of prostate and intestinal cancers [52]. Previous research also showed that enigmol added with fluorine led to an increased anti-cancer activity of enigmol in mice models of prostate cancer and was non-toxic in *in vivo* studies [53]. A previous study showed that the 4-hydroxy enigmol compound, an enigmol analog, can bind and inhibit the protein Sphingosine Kinase I (SPHK-I), which leads to stimulation of the apoptosis pathway in prostate and colon cancer cells. SPHK-1 is a novel target for triple-negative breast cancer [54].

The next most abundant compound was spisulosine. Spisulosine is a sphingolipid analog with anti-cancer potential. Recent studies show that the derivative spisulosine can increase the autophagic response to cancer cells [55]. Previous studies have shown that the antiproliferative effect on prostate cancer cells occurs by inhibiting the path of peroxisome proliferator-activated gamma receptor (PPAR-γ), phosphatidylinositol 3-kinase/(PI3K/Akt), and Jun N-terminal kinase (JNK) [56].

Enigmol and spisulosines are sphingolipids. Sphingolipids are lipids that have many functions; in addition to acting as a component of cell membranes, sphingolipids also play an important role in processes related to cancer biology, including, among other things, proliferation, migration, differentiation, and apoptosis [57].

Enigmol and spisulosine in the *C. nutans* leaves aqueous extract differed from those reported in previous studies. Previous research indicated that phytosterols have been found in the petroleum extracts of *C. nutans* leaves. Combining phytosterols with other bioactive components is believed to have a cytotoxic effect on cancer cell lines [58]. Another study that identified the active compound in *C. nutans* leaves extract fraction using LC-MS revealed that most of the peaks detected on the chromatogram were believed to have anti-cancer activity. Based on prior research, we conclude Enigmol and spisulosine worked synergistically as anticancer by inhibiting proliferation and apoptosis of BCSCs.

Compounds with negative polarity include 19-oxo-all-trans-retinoic acid and (2S)-2-({2-[1-(propane-2-yl)-1H-1,2,4-triazol-5-yl]-5,6dihydroimidazo[1,2d] [1,4]benzoxazepin-9yl-oxy) propanamide, pregnenolone sulfate, and 9,13-di-cis-retinosic acid while tetradecaethyleneglycol and elaidamide have positive polarity [59,60]. Other investigations have found that 19-oxo-all-retinoic acid is a component of a *C. nutans* leaf extract that has anti-cancer activity [18]. According to other studies, the alkaloid group, which includes methysergide, calycanthine, eburnamonine, and dextromethorphan, has anti-cancer activity [36]. A GCMS analysis of *C. nutans* leaves extract identified β-amyrenol and campesterol as potential anti-cancer components. The results of the molecular docking test

show that β-amyrenol has stretched affinity with MDM2-P53, MCL1-BAX, MCL1BID, and Caspase-9, whereas campesterol has the highest affinities with caspase-8 and caspase-3 [16]. Differences in the isolation results of active compounds from previous studies may be due to differences in solvents and methods of isolation of active substances. Previous research has looked into combining paclitaxel with other chemicals to increase its anti-cancer potency. This study showed that paclitaxel, combined with aqueous-based *C. nutans* extract, increase its anti-cancer properties.

Conclusions

C. nutans leaves aqueous extract synergized with Paclitaxel, interfered with the cell cycle mainly in the G2/M phase, and induced early and late apoptosis of BCSCs. The synergistic effects are believed to be due to the presence of 3β,6beta-dihydroxynortropane, isoorientin 2″-O-arabinoside, 2-hydroxyhexadecanoic acid, 2-aminohexadecanonic acid, enigmol, and spisulosine, which have anti-cancer activity, in the aqueous extract of *C. nutans* leaves.

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Declaration of Generative AI in Scientific Writing

The authors acknowledge the use of Grammarly in the preparation of this manuscript to improve language and grammar. No content generation or data interpretation was performed by AI. The authors take full responsibility for the content and coclusions of this work.

CRediT Author Statement

Aldy Safruddin Rambe: Conceptualization, Methodology, Supervision, Validation, Funding acquisition, and Writing-original draft. **Adi Muradi Muhar:** Data curation, Formal analysis, Investigation, Validation, and Visualization. **Yunita Sari Pane:** Data curation, Formal analysis, Investigation, Validation, and Visualization. **Alfi Khatib:** Data curation, Formal analysis, Investigation, Validation, and Visualization. **Siti Syarifah:** Methodology, Project administration, Resources, Supervision, Validation, and Writing–original draft.

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