ORIGINAL PAPER

Water Extract Have Superior Cytotoxic Effect Than Ethanolic Extract of Clinacanthus Nutans Leaves in Breast Cancer Stem Cells

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ABSTRACT

Background: The rapid development of medical technology in managing breast cancer patients still cannot solve the problem of recurrence and resistance. One of the causes of recurrence and molecular resistance is the presence of breast cancer stem cells (BCSCs). Clinacanthus nutans (C.nutans) is a plant found in Medan, North Sumatra, Indonesia. This plant is believed to have anticancer activity in community. **Objective:** Our study aimed to assess phytochemical of C.nutans leaves, isolate breast cancer stem cells and determine the cytotoxic effects of the ethanolic extract and water extract of C.nutans leaves on breast cancer stem cells at 24, 48, and 72 h of observation. **Methods:** We underwent the cytotoxic test by using MTT assay and isolated breast cancer stem cells by using MACS and validated them by mammosphere test. **Results:** We found alkaloids, flavonoids, glycosides and tannins in simplicia and all extracts. BCSCs was valid with the diameter of the mammosphere BCSCs was > 60 µm. The IC50 values of 100%, 60%, 40%, 20% EE, and WE of C.nutans leaves were 227.30; 46.05; 31.12; 98.54, and 16.16 µg/ml respectively in the first 24 hours. In administering WE of C.nutans leaves, BCSCs viability was decreased at 24,48 and 72 hours of observation, namely 69.29±26%; 75.82 ± 21.02% and 38.94±9.34 % ($p < 0.0001$). **Conclusion:** The WE of C.nutans leaves had more substantial cytotoxic potential against BCSCs than the EE. The capability of WE C.nutans leaves to suppress BCSC's viability was time-dependent. The anticancer activity were believed originate from alkaloid and flavonoid group.

Keywords: Clinacanthus nutans leaves, cytotoxic, breast cancer stem cells.

1. BACKGROUND

The number of patients with breast cancer is increasing worldwide, with the number of new cases reaching 2 261 419 and ranking first; in Indonesia, the number of new cases of breast cancer has reached 396 914, accounting for 16.6% of cancer cases (1). Although the development of medical technology, including chemotherapy, radiotherapy, surgery, and hormonal therapy, for the management of patients with breast cancer is advancing rapidly, these treatments have been unable to solve problems such as recurrence and resistance (2-5). Of the patients with breast cancer at Dr. Soetomo Hospital, Indonesia, 30% experienced recurrence and 19.5% received adjuvant therapy (6). This situation indicates that alternative therapies are needed to treat resistant and recurrent breast cancer.

Breast cancer has heterogeneous cells; approximately 15%-20% of breast cancer cases are triple-negative breast tumors (TNBCs). TNBCs contain a population of breast cancer stem cells (BCSCs) (7). The molecular causes of resistance and recurrence include the presence of BCSCs. BCSCs have the ability to self renew, resist chemotherapy and radiotherapy, and initiate new tumor formation and metastatic processes (8). Clinacanthus nutans is a plant with anticancer activity commonly found in Malaysia, China, Indonesia, and Thailand. Traditionally, its leaves are widely used by being boiled in water (9). Several previous studies have reviewed the anticancer effects of this plant by using its methanolic extracts (10, 11). The methanolic extract of C. nutans leaves inhibited MCF7 cell proliferation (9). The combination

of the ethanolic extract (EE) of C. nutans leaves and doxorubicin also had synergistic effects on the apoptosis of MCF7 and T47D cells in vitro (12). Interestingly, the ethyl acetate extract of C. nutans leaves did not exhibit its cytotoxic effect when administered to the subcutaneous connective tissue cell line L929 and normal embryonic fibroblast cells (NIH 3T3) in vitro (13). The leaf extract of C. nutans has higher phenolic and terpenoid components than the stem extract, indicating that bioactivity is higher in the leaves than in other tissues (14). Previous studies have shown that C. nutans leaf extract has the potential to suppress the growth of cancer cells. However, its ability to target BCSCs specifically is still unknown. We aim to analyze and compare the cytotoxic effect of various concentrations of the EE and water extract (WE) of C. nutans leaves on BCSCs. Moreover, this is the first study conducted in Indonesia in relation to this topic.

2. OBJECTIVE

The aim of the study were threefold: a) to asses non-spesific and specific parameters of C.nutans leaves; b) to isolate BCSCs and validate with mammosphere test, and c) to analyze and compare the cytotoxic effect of various concentrations of the EE and water extract (WE) of C. nutans leaves on BCSCs.

3. MATERIAL AND METHODS

Materials

C. nutans leaves were obtained from a local farmer in Medan City, North Sumatera, Indonesia with coordinate 3.519131ºN 98.642584ºE. The tools needed for cell culture included a 96-well plates (Iwaki), micropipettes, an analytical balance (Santorius), a water bath (Heidolph), a microscope, filter paper, a CO2 incubator (Binder), a biological safety cabinet, capillary tubes, and a magnetic activated cell sorting (MACS) system. Plant identification was carried out at Badan Riset dan Inovasi Nasional (BRIN), Bogor, Indonesia (No: B-612/V/DI.05.07). Extract preparation were carried out in Faculty of Pharmacy, Universitas Sumatera Utara, Medan, Indonesia. The MTT test was conducted at the Stem Cell and Cancer Research Laboratory in Semarang, Indonesia. The study groups consisted of a negative control group; groups treated with the EE of C. nutans leaves of various concentrations, namely, 20%, 40%, 60%, and 100%; a group treated with WE; and a group treated with paclitaxel as the positive control for a total of seven groups. Each group consisted of three repetitions (n=3).

Extract preparation

In this study, the wet weight of C. nutans leaves was 5 kg and the weight of the simplicia powder was 500 g. C. nutans leaf powder was macerated by using ethanol and water as a solvent. The ethanol solvent used had several concentration gradients, namely 100% (v/v), 60% (v/v), 40% (v/v), 20% (v/v), and water. Soaking was carried out for 24 h;. The macerate was separated through filtration. Maceration was repeated three times. The extract was evaporated by using a rotary evaporator at 60ºC and freeze dried until no solvent remained.

Simplicia characterization

Examination of water content, ethanol soluble extract content, water-soluble extract content, total ash content, and acid insoluble extract content was carried out on C.nutans leaves simplicia.

Phytochemical qualitative test

Phytochemical qualitative tests were conducted on C. nutans simplicia and leaf extracts to identify alkaloids, steroids, triterpenoids, tannins, flavonoids, saponins, and glycosides (13).

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

MDA-MB 231 cells were cultured in T25 flasks. The culture medium was high-glucose Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin, and 0.25% amphotericin-B. Cell culture was conducted at 37ºC and 5% CO2. After they reached 80% confluence, the cells were passaged for 4 min at 37°C by using 1 mL of trypsin. The passaged cells were then incubated with CD24 and CD44 microbead-labeled antibodies. The population of CD44+/CD24– BCSCs was isolated through gradient MACS.

BCSC Validation Through Mammosphere test

BCSCs were cultured in MammoCultTM basal medium consisting of 10% MammoCultTM Proliferation Supplement, 1% penicillin–streptomycin, 0.25% ampho-B, 4 µg/mL heparin solution (Stemcell Technologies), and 0.48 µg/mL hydrocortisone solution (Stemcell Technologies) in ultralow attachment well plates. The morphology and number of cell clusters (diameter > 60 µm) in each well were evaluated under an inverted phase-contrast microscope on days 0, 3, and 7.

Cytotoxicity test with MTT assay

The MTT assay was performed on the basis of a prior study with certain modifications (15). EEs of various concentrations were administered to BCSCs cultured with BCSC culture medium in 96-well plates at 37ºC and 5% CO2. Observations were made at 24, 48, and 72 h. The medium was discarded, and 100 µL of the MTT reagent was added to the BCSC culture. The cells were incubated for 3 h at 37ºC and 5% CO2. The MTT reaction was stopped by adding the stopper reagent (100% DMSO). Furthermore, the 96-well plates were incubated for 15 min in the dark at room temperature. An enzyme-linked immunosorbent assay reader was used to read the absorbance at a wavelength of 595 nm. The IC50 value for each group was calculated by converting the absorbance data into the percentage of cell viability.

$$\% \text{ of viable cells} = \frac{\text{Cell absorbance under treatment} - \text{blank}}{\text{Cell absorbance under control treatment} - \text{blank}} \times 100.$$

Statistical analysis

Data were analyzed statistically by using SPSS v26.0 and GraphPad Prism v10.0.3 software.). The relationship between various concentrations of the EE and WE of C. nutans leaves and the percentage of cell viability was analyzed through One-way Anova if the data were normally distributed and homogeneous and with the Kruskall-Wallis test otherwise, $p < 0.05$ was considered statistically significant.

4. RESULTS

Non-specific parameter test results

Our study showed the water content of the C.nutans leaves simplicia of 4.98%. This result met the general requirements of below 10%. Moisture content greater than 10% can be a mold

No.	Parameter	Results					
		Simplicia	EE CN 100%	EE CN 60%	EE CN 40%	EE CN 20%	WE CN
1	Alkaloids	+++	-	+++	+++	+++	+++
2	Flavonoids	+	+	+	+	+	+
3	Glycosides	+	+	+	+	+	+
4	Tannins	+	+	+	+	+	+
5	Saponins	+	-	+	+	+	+
6	Steroids	+	+	+	-	+	-
7	Triterpenoids	-	-	-	+	-	-

Table 1. Phytochemical screening results

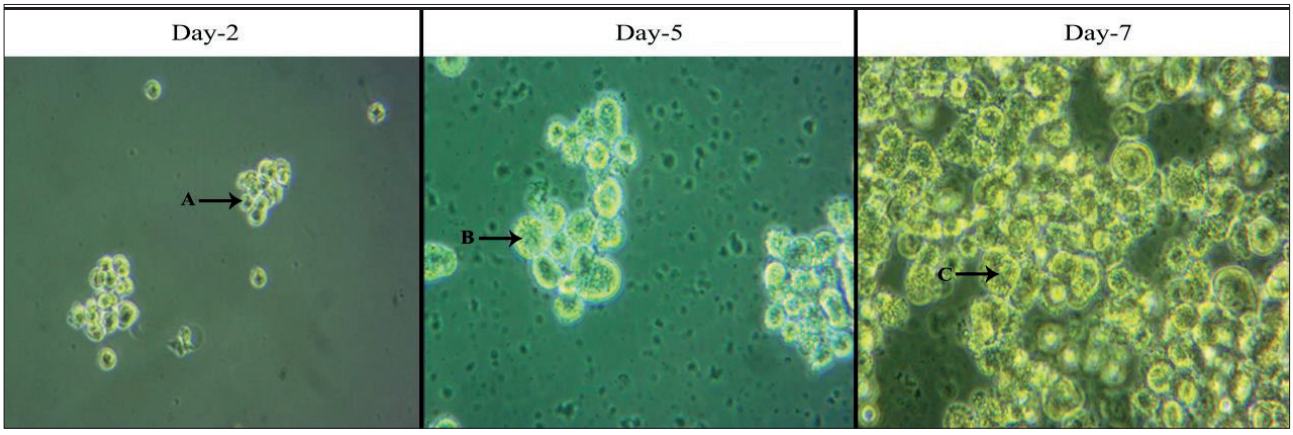

Figure 1. The mammosphere formed from the second to the seventh day as a marker for BCSCs (Magnification 100x).(A) The diameter of the mammosphere BCSCs formed was still <60µm, the cell confluency was rare, (B) The diameter of the mammosphere BCSCs which was formed was already 60µm, the cell confluency was increasing, (C) The diameter of the mammosphere BCSCs which was formed was > 60µm, the cell confluency was dense.

and other microorganisms' growth medium. The water-soluble essence content also met the requirements of at least 18% (26.91%), the ethanol-soluble essence content was not less than 6.3% (6.52%), and the ash content was not more than 9% (4.49%) and 3.49%) (16).

Phytochemical qualitative test

Table 1 shows that alkaloids were found in all extracts of C. nutans leaves except in the 100% EE.

Sorting results of BCSCs

MDA-MB231 cells that had been successfully grown and expressed CD44+/CD24– were isolated to obtain BCSC clones. The BCSC clones were extracted by using the MACS CD44/CD24 microbead technique and cultured in T75 flasks. The culture medium used was specific for the growth and proliferation of BCSCs, namely low-glucose DMEM:MammoCultTM medium (3:1).

The culture was conducted until the third passage and a morphological picture of BCSCs in the form of spindle-like cells with elongated actin filaments was obtained.

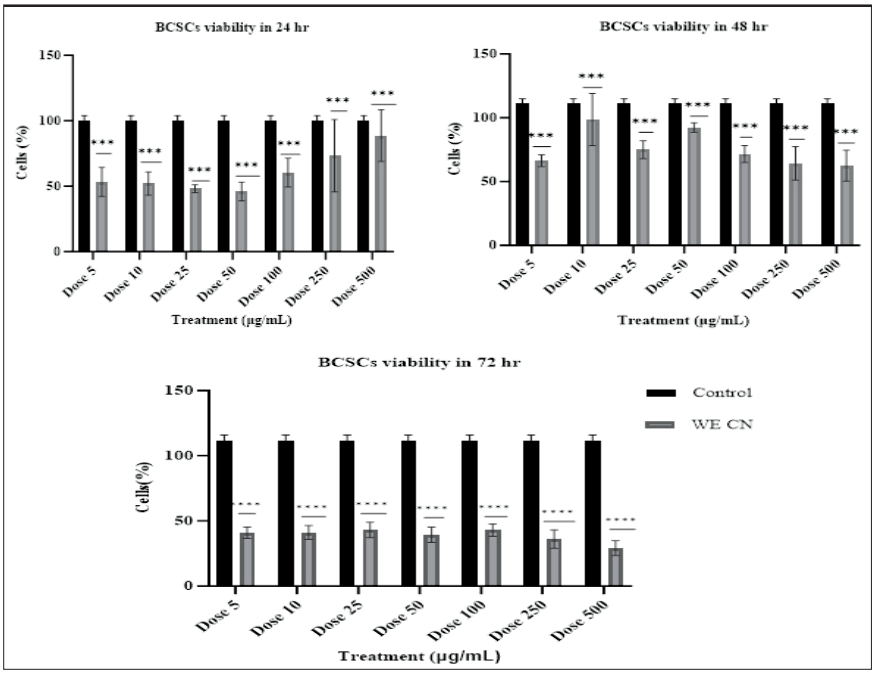

Figure 2. Comparison of BCSCs viability in water extracts of C.nutans leaves in 24,48 and 72 hr observation. A) As the dose increased, there was no decrease in the linear viability of BCSCs in the first 24 hours (*** p <0,001 vs control).B) There has not been a linear decline in viability of cells with increased doses (* p<0.001 vs control).C) Cells viability has decreased as the dosage of WE C.nutans leaves increases at 72 hr (**** p < 0,0001 vs control). The error bars represent the mean ± SEM of triplicate results.**

Validation results of BCSCs

The validation test was conducted to determine the ability of BCSC clones to form mammospheres when cultured with MammoCultTM medium in ultralow attachment six-well plates. Mammospheres initially formed with a diameter of more than 60 µm on day 3 and grew in size until day 7 of observation (Figure 1).

Extract	Doses (µg/mL)	Average of BCSCs viability (%, ±SEM) n = 3	IC50 (µg/mL)	p-value
EE 100%	5	46.64±17.85	227.30	0.009a
	10	36.01±8.38		
	25	40.11±10.64		
	50	91.79±22.88		
	100	68.66±17.94		
	250	60.26±5.34		
	500	63.43±17.63		
EE 60%	5	42.35±5.89	46.05	0.112a
	10	41.60±5.94		
	25	46.64±15.15		
	50	54.10±16.31		
	100	49.81±9.86		
	250	52.61±3.64		
	500	71.27±17.67		
EE 40%	5	53.36±10.95	31.12	0.134b
	10	60.63±6.84		
	25	52.05±19.23		
	50	49.44±13.26		
	100	47.57±7.76		
	250	81.34±20.32		
	500	66.05±1.62		
EE 20%	5	47.95±17.59	98.54	0.081b
	10	69.03±2.12		
	25	58.58±11.32		
	50	75.75±15.84		
	100	49.45±4.34		
	250	70.34±22.90		
	500	84.51±8.69		
Water extract	5	53.36±19.28	16.16	0.413a
	10	52.05±15.48		
	25	48.14±5.05		
	50	46.08±12.33		
	100	60.45±19.11		
	250	73.33±47.78		
	500	88.62±34.19		

Table 2. The relationship of various doses extract of the C.nutans leaves to BCSCs viability and IC50 at 24 hours of observation

BCSCs viability and IC50 value in the first 24 h

In this study, cytotoxicity tests were performed on the EE of C.nutans leaves at various concentrations and doses (5–500 µg), and BCSC viability was assessed and IC50 values were determined. The administration of various concentrations of EE (5–500 µg/mL) caused a reduction in BCSC viability (Table 2). The lowest IC50 value of 16.16 µg/mL was found under the administration of WE.

BCSCs viability and IC50 value at 48 h of observation

The results of the assessment of BCSC viability and determination of IC50 at 48 h of observation can be seen in Table 3. The decrease in BCSCs viability was still not linear with increasing doses of each C.nutans extract of various concentrations, but increasing doses of EE of various concentrations, especially the 100% EE and WE caused a significant decrease in BCSCs viability, although not significantly statistically ($p > 0.05$) (Figure 3). Interestingly, in just 48 h of observation, BCSCs were able to proliferate. The average cell viabilities under treatment with 250 and 500 µg of the 60% EE exceeded 100% were 109.54% ± 30.44% and 118.84% ± 41.55%, respectively (Table 3).

BCSCs viability after WE C.nutans leaves At 24, 48, and 72 h of Observation

The cell viability after the administration of the WE of C. nutans leaves were also compared. No linear relationship was found between the increase in the dose of the WE of C. nutans leaf and decrease in the viability of BCSCs in the first 24 h of observation. However, over time, especially in the 72-hour observation, there has been a linearity between increasing the dose of water extract and inhibiting BCSCs viability. The average total cell viability of BCSCs in 24,48 and 72 hr were 69.29±26.00; 75.82±21.02 and 38.94±9.34 % respectively ($p < 0.0001$) (Figure 2).

BCSCs viability after Paclitaxel Administration

In this study, paclitaxel was used as the positive control. Data on BCSC viability after paclitaxel administration can be seen in Figure 3. The BCSC viability was 56,53±6,95 % (dose 10 nM) in the first 24 h of observation, increased to 57,75±6,35 (dose 10 nM) at 48 h of observation, and decreased again to 33,11±2,64 % (dose 10 nM) at 72 h of observation. Therefore, the cytotoxicity test results demon-

Extract	Doses (µg/mL)	Average viability BCSCs (%,± SEM), n = 3	IC50 (µg/mL)	p value
EE 100%	5	73.41±7.54	230.09	0.948a
	10	80.67±11.22		
	25	86.38±20.59		
	50	83.61±20.64		
	100	88.01±26.18		
	250	76.92±17.99		
	500	78.55±14.21		
EE 60%	5	73.98±47.62	97.16	0.152b
	10	77.32±28.49		
	25	66.31±3.29		
	50	59.46±5.37		
	100	71.94±23.18		
	250	109.54±30.44		
	500	118.84±41.55		
EE 40%	5	70.06±11.40	195.48	0.283a
	10	61.91±8.11		
	25	58.24±6.75		
	50	72.27±8.11		
	100	74.63±20.07		
	250	72.52±3.92		
	500	86.70±23.53		
EE 20%	5	88.74±29.14	32.49	0.570a
	10	80.42±15.29		
	25	56.77±1.12		
	50	71.94±19.72		
	100	74.63±13.48		
	250	75.77±20.85		
	500	87.36±29.57		
Water extract	5	66.39±7.84	65.73	0.269b
	10	98.78±35.38		
	25	75.04±12.20		
	50	92.33±6.52		
	100	71.54±11.44		
	250	64.27±22.65		
	500	62.39±20.98		

Table 3. The relationship of various doses extract of the C.nutans leaves to the viability of BCSCs and IC50 at 48 hours of observation. aOne-way Anova test; bKruskal-wallis test

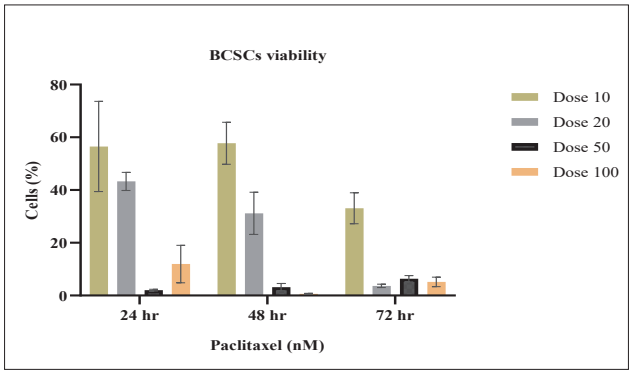

Figure 3. BCSCs viability after paclitaxel administration at 24, 48 and 72 hours of observation

strate the strong cytotoxic potential of paclitaxel. Notably, paclitaxel was not able to kill BCSCs as a whole (100%); this can be seen in the cell viability column; at doses of 100 and 200 nM, there were still surviving BCSCs (1.30% and 0.37%).

The comparison of BCSC morphology after treatment

The morphology cells of BCSCs pre and post treatment with extract C.nutans leaves and paclitaxel can be seen in Figure 4.

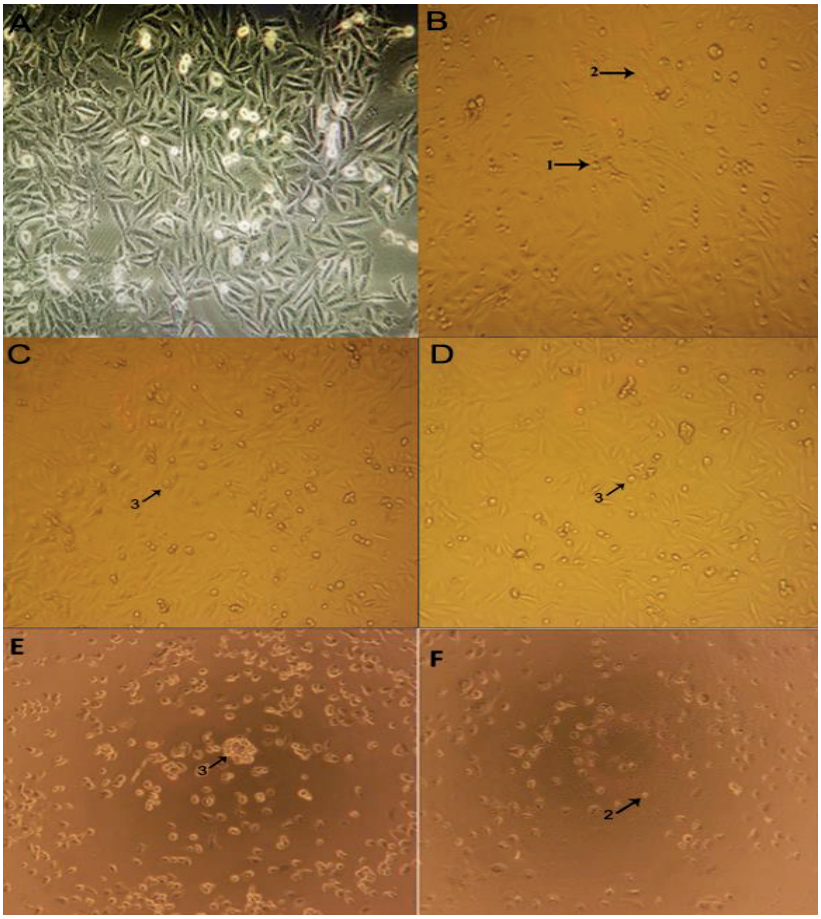

Figure 4.(A) Pre-treatment BCSCs appearance, dense confluence was found, typical spindle-like cells. (B) BCSCs after administration of WE C.nutans leaf dose of 5 µg/mL, slight confluency was found, (1) Cells undergoing apoptosis (bubble-like cells) (2) Microscopic appearance of BCSCs became blurred (3) Bubble-like cells (C) After the administration of WE dose 10 µg/mL (D) WE C.nutans dose 25 µg/mL (E) Paclitaxel 10 nM (F) Paclitaxel 20 nM (40x magnification)

5. DISCUSSION

Our study found alkaloid in WE of C.nutans leaves. Previous studies have shown that alkaloids are traditionally found in plants (16). Alkaloids have pharmacological activities that include antihypertension, antiarrhythmic, antimalarial, and anticancer. Many researchers believe alkaloids, flavonoids, and triterpenoids have anti-inflammatory and anticancer activities (10, 17). This study found that WE C.nutans leaf has the smallest IC50 value compared to EE, which is believed to have anticancer potential. The results of this study were also in line with previous studies that showed WE C.nutans leaf has a high cytotoxic potential against HeLa line cells (18). In this research, WE C.nutans leaf only has alkaloids and flavonoids, while in the previous study, it only has terpenoids and flavonoids (18). Another study found that the active compounds in C.nutans leaf that have anticancer activity came from the alkaloid group with amines as a functional group (19). The results of this study were in accordance with those of several previous works, which stated that mammospheres could be used to identify the presence of BCSCs in cell lines and primary tumor cells (20).

We found the lowest IC50¬ in WE of C.nutans leaves (16.16 µg/mL). Our finding was accordance with the American National Cancer Institute with IC50 values ≤ 20 µg/mL = high cytotoxic potential (21). This classification indicates that various concentrations of C. nutans extract have cytotoxic potential against BCSCs. The EE 100% had weak cytotoxic potential; the 20%, 40%, and 60% EEs had moderate cytotoxic potential; and the WE had high cytotoxic potential. There was no previous study which evaluate the effect of C.nutans leaves with BCSCs viability, but our finding is in accordance with previous results with other cancer cells, which showed the WE of C. nutans leaves had an inhibitory effect on the viability of Hela cells (cervical cancer) with an IC50 value of 13 ± 0.82 µg/mL in the first 24 h of observation (18).

The nonlinear relationship between dosage and BCSC viability was likely due to the high survivin expression in BCSCs. Increased survivin expression was found in the pre-metastatic intermediate phase, causing BCSC to survive because survivin inhibits the process of apoptosis (22). Survivin overexpression is believed to cause resistance to chemotherapy and radiotherapy in various cancers (23). Based on Table 4, in just 48 h of observation, BCSCs were able to proliferate. This finding was also due to the unique characteristics of BCSCs. Although BCSCs commonly overexpress survivin and CD44, they cannot work alone but require particular intercellular signaling pathways, such as the Notch, PI3K/Akt/mTOR, Wnt-β catenin, and Hedgehog signaling pathways (24).

Based on Figure 2, brief exposure to WE C. nutans leaf extracts did not inhibit BCSC proliferation. However, cell proliferation could be inhibited with the prolongation of exposure to increase concentrations of the WE of C. nutans leaves. The decrease in BCSC viability indicated that apoptosis had

occurred after the administration of the WE of C. nutans leaves. No previous studies have examined the dose linearity of WE C.nutans leaves with BCSC viability. Previous studies only showed that in cervical cancer cell lines (SiHa), apoptosis was induced by a gradual increase in P53 levels at 24-72 h after the administration of the fraction of the C. nutans leaf extract (24). Our study also found that prolonging the exposure of BCSCs to paclitaxel still cannot kill the entire BCSCS. This finding shows that BCSCs have unique characteristics, namely, the ability to proliferate without limits and quickly develop resistance to chemotherapy (24).

This study found that the WE of C. nutans leaves had higher cytotoxic potential than the EE. The morphological picture of BCSCs after the administration of the WE of C. nutans leaves can be seen in Figure 4. In this study, the simplicia and extract of C. nutans contained flavonoids. Flavonoids are secondary metabolites in plants with biological activity(10). Another study demonstrated that the active compounds in C. nutans leaves with anticancer activity originate from the alkaloid group and possess amines as functional groups(19).

Our study found the BCSCs had the capacity to proliferate so fast. The ability of BCSCs to proliferate very quickly is because the highly complex BCSC signaling path includes Wnt/β-Catenin, Hedgehog (Hh), Notch, HER2, Nf-Kβ, TGF-β, JAK-STAT and PI3K/Akt/mTOR(24).

6. CONCLUSION

The WE of C.nutans leaves had more substantial cytotoxic potential against BCSCs than the EE. The capability of WE C.nutans leaves to suppress BCSC's viability was time-dependent. The anticancer activity were believed originate from alkaloid and flavonoid group.

- **Authors' contributions:** All S.S., A.S.R., A.P, and M.I made substantial contributions to the conception and design; S.S, A.S.R., A.P., M.I., and A.K had responsibility for the acquisition of data or analysis and interpretation of data; Y.S.P., A.M.M., A.K., D.M., M.R., M.M.A took part in drafting the article or revising it detail and critically. All authors approved to submit to the current journal, allowed final approval of the manuscript to be published, and agreed to have responsibility for all aspects of the work.
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