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IN VITRO ANTI-PROLIFERATIVE AND ANTIOXIDANT ACTIVITIES OF CHLOROFORM EXTRACT OF *CHRISTIA VESPERTILIONIS* ON MCF 7 AND MDA-MB-231 BREAST CANCER CELLS

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ABSTRACT

Cancer continues to be a formidable adversary in global public health, exacting a devastating toll on millions of lives each year. Among the most prevalent forms of cancer, breast cancer stands out as a significant contributor to mortality rates worldwide. Despite considerable advancements in medical science and the development of various treatment modalities such as chemotherapy and radiotherapy, these interventions often bring about debilitating side effects, ranging from nausea and fatigue to hair loss. Consequently, there has been a growing interest in exploring alternative and complementary therapies, including herbal medicines, for their potential anticancer properties. One such herbal remedy under investigation is *Christia vespertilionis* (L.f.) Bakh. F, commonly referred to as "Daun Rerama" or "Butterfly wing" in Malaysia due to the shape of its leaves. Traditionally, *C. vespertilionis* has been used to treat various ailments, including tuberculosis, snakebites, bronchitis, and muscle weakness, among others. Recent scientific studies have delved into the pharmacological properties of *C. vespertilionis*, revealing its potential as an anticancer agent. The present study aimed to investigate the cytotoxic properties of *C. vespertilionis* extract against breast cancer cell lines, particularly estrogen receptor-positive (MCF7) and estrogen receptor-negative (MDA-MB-231) cells. Additionally, the study sought to explore the antioxidant properties of the extract, considering the crucial role of oxidative stress in cancer development and progression. The research methodology involved the extraction of *C. vespertilionis* leaves using chloroform, followed by phytochemical screening to identify bioactive compounds present in the extract. Flavonoids and saponin were among the compounds detected, indicating the potential pharmacological significance of the plant. Further analysis quantified the total phenolic and flavonoid contents of the extract, providing insights into its antioxidant capacity. Subsequently, the antiproliferative effects of the *C. vespertilionis* extract were evaluated using the MTT assay, a widely used method for assessing cell viability and proliferation. The results revealed a concentration-dependent cytotoxic effect of the extract on both MCF7 and MDA-MB-231 breast cancer cell lines, with decreasing IC₅₀ values observed over time. While the potency of the extract was lower compared to the standard anticancer drug tamoxifen, the findings underscored the potential of *C. vespertilionis* as a natural therapeutic agent against breast cancer. Overall, the study sheds light on the promising anticancer properties of *C. vespertilionis* and highlights the need for further research to elucidate its mechanisms of action and potential clinical applications. Additionally, the findings contribute to the growing body of evidence supporting the use of herbal medicines as adjunctive or alternative therapies in cancer treatment, emphasizing the importance of exploring nature's pharmacopeia in the fight against cancer.

Keywords: Anti-proliferative, antioxidant, MCF 7, MDA-MB-231, *Christia vespertilionis*

INTRODUCTION

Cancer is a leading cause of death globally and was responsible for 10 million deaths in year 2020. The most frequent cancer types in year 2020 in term of new cases of cancer were breast cancer (2.26 million cases), lung cancer (2.21 million cases), colon and rectum (1.41 million cases), skin (1.20 million cases) and stomach (1.09 million cases) [1]. Among 2.3 million women diagnose with breast cancer in year 2020, 685,000 were deaths globally. In Malaysia, as reported by The Global Cancer Observatory, a total number of 8 418 new breast cancer cases were diagnosed in the year 2020., of which 3 503 were reported deaths due to breast cancer [2].

Nowadays, there are many types of cancer treatment like chemotherapy, radiotherapy, cryosurgery and many more. Although all these treatments aim to kill the cancer cells, they also have their own side effects like nausea and vomiting, fatigue, sleep disorder, delirium and hair loss. Other than that, alopecia or hair loss from areas of the body is a common side effect of chemotherapies used in the treatment of breast cancer [3].

One of the herbs that was believed to possess anticancer activity and is currently under investigating in our laboratory is *Christia vespertilionis* (L.f.) Bakh. F belongs to the family of Fabaceae). Among Malaysian, this herbs commonly known as “Daun Rerama” or “Butterfly wing” due to the shaped of it leaf [4]. Generally, the whole plant has traditionally used to treat tuberculosis and snake bites. Besides that, the decoction of the leaves also has been used to treat bronchitis, colds muscle weakness, inflamed tonsils and to improve blood circulation [5,6,7].

Several scientific studies have been carried out on different part of *C vespertilionis* (L.f.) Bakh. Based on the recent study, the leaves of *C. vespertilionis* were shown to exhibit anti-malarial, anti-inflammatory, anti-proliferative and anti-cancer against various cancer types [8,9,10]. Apart from that, the plant was also reported to contain a variety of phytochemicals including polyphenols, alkaloids, and terpenoids which may be constituents that are responsible for the antioxidant and anti-cancer activities (Osman et al., 2017).

However, there is yet any report regarding the cytotoxic properties of the different parts of the plant extract of *C. vespertilionis* specifically against breast cancer cell lines and their antioxidant properties. The present study, therefore, investigated the possible phytochemicals responsible toward the anti-cancer activities of *C. vespertilionis* through its antioxidant properties.

MATERIAL AND METHOD

Source and Identification of Plant Material

The leaves of *C. vespertilionis* were purchased from Guar Perahu Herbal Valley, Bukit Mertajam, Pulau Pinang, Malaysia in May 2018. The leaves sample was deposited in the herbarium of the Biodiversity Unit, Institute of Bioscience, Universiti Putra Malaysia with voucher no. SK3350/18.

Preparation of Plant Extract

The dried leaves were ground into a powder and soaked in chloroform (Elite Advance Material, Malaysia) in the ratio of 1:20 (w/v) for 72 hours. After that, the supernatant was filtered using Whatman No. 1 filter paper. Then, the supernatant was evaporated using a rotary evaporator at 40°C under reduced pressure. This method was carried out according to Hisam et al., 2012 [11]. The crude dried chloroform extract obtained was kept in an ointment jar and store at 4°C until used.

Phytochemical Screening

The phytochemical screening of the *C. vespertilionis* chloroform extract (CVCE) was carried out according to the method described by Hisam et. al., 2018 [12]. A stock concentration of 1% (w/v) of the extract were tested for the presence of tannins, alkaloids, triterpenoids, flavonoids, phenol, and alkaloids. The change of colors or the formation of precipitate was used as an indication of a positive response to these tests.

Total Phenolic and Flavonoids Content

The total phenolic content was determined by Folin–Ciocalteu method. Briefly, 0.1 mL of sample (1 mg/mL) was diluted with distilled water (4.5 mL) and subsequently, Folin–Ciocalteu reagent (0.1 mL) was added with shaking for 3 min. A 2% (w/v) solution of sodium carbonate (0.3 mL) was added and the mixture was stirred and left to stand for 3 h. An aliquot of the mixture (200 µL) was transferred to a 96-well plate and the absorbance was measured at 760 nm against a blank using a microplate reader (BMG POLARstar Omega, Germany). The total phenolic content was expressed as µg of gallic acid equivalent per mg of the dry weight of the sample, using an equation obtained from the gallic acid standard curve.

Total flavonoid content was determined by the aluminum calorimetric method, using quercetin as the reference standard. Briefly, 150 µL of the test sample (0.3 mg/mL) was mixed with 150 µL of 2% (w/v) AlCl₃ in a 96-well plate. After 15 min of incubation at room temperature, the absorbance was measured at 435 nm by a microplate reader (BMG POLARstar Omega (BMG POLARstar Omega,

Germany). The content of the total flavonoids was expressed as μg of quercetin equivalent per mg of the dry weight of the sample, using an equation obtained from the quercetin standard curve.

Cell Line

The cell line used in this study were estrogen receptor-positive breast cancer cells - MCF7 (ATCC no. HTB 22) and estrogen receptor-negative breast cancer cell - MDA-MB-231 (ATCC no. HTB 26) breast cancer cell line. It was obtained from Integrative Pharmacogenomics Institute (iPROMISE), Universiti Teknologi MARA. Both cells were maintained in Dulbecco's modified Eagle's medium (Biowest, France) supplemented with 10% FBS (Biowest, France) and 1% penicillin-streptomycin (Sigma-Aldrich, USA) at 37 °C in a humidified 5% CO₂ atmosphere.

Antiproliferative Assay

The antiproliferative of the CVCE extract was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Exponentially growing cells were harvested, counted, and diluted with DMEM (Biowest, France). The cells (1×10^5 cell/ mL) were prepared and seeded (100 μL) into each well of a 96-well plate. The cells were cultured overnight before treatment with the extract. Cells were treated with extracts or Tamoxifen as a positive control at concentrations of 6.25, 12.5, 25, 50 and 100 $\mu\text{g}/\text{mL}$ for 24, 48 and 72 hours. At each time point, MTT (Sigma-Aldrich, USA) solution (5 mg/mL) was added to the cultured cells and incubated at 37 °C for 3 h. Crystallized MTT was dissolved in DMSO and the optical density was measured at 570 nm using a microplate reader (BMG POLARstar Omega, Germany). The percentages of viable cells were plotted against concentrations and the IC₅₀ values were determined. All experiments were performed in triplicate. The formula used Hisam et. al., 2018 [12] to calculate percentage of cell viability.

$$\text{Percentage of cell viability (\%)} = \frac{OD \text{ sample (mean)}}{OD \text{ control (mean)}} \times 100$$

RESULTS AND DISCUSSION

Phytochemical Screening of CVCE

As shown in table 1, the phytochemical screening of CVCE demonstrated the presence of flavonoids and saponin. However, tannins, triterpenoids, phenols, steroids, and alkaloids were not detected in the extract. This result is not in line with previous study that showed the present of alkaloids, triterpenes, fatty acids, phenols, alkanes and long chained alcohols [6]. This difference is due to the use of different solvents and the method of extraction. Based on the previous report, Flavonoids have been reported to possess antioxidant and anticancer activities [13]. Saponins, which are detected in the extract, were reported to induce an apoptosis response on cancer cells through the permeabilization of the mitochondrial membranes [14].

Table 1: The phytochemical constituents of CVCE

Test	Indicator
Tannins	-
Triterpenoids	-
Flavonoids	+
Saponin	+
Phenol	-
Steroids	-
Alkaloids	-

Total Phenolic, Flavonoids Content and antioxidant activity of CVCE

Table 2: Total phenolics, flavonoids contents and antioxidant activities of CVCE.

Total Phenolic (μg of gallic acid equivalent/ mg of extract \pm SEM)	Total flavonoids (μg of quercetin equivalent/ mg of extract \pm SEM)	FRAP value (μg Trolox/ μg sample dry weight \pm SEM)	IC ₅₀ of DPPH radical ($\mu\text{g}/\text{mL}$)
54.43 \pm 1.49	24.91 24.91 \pm 0.33	43.05 \pm 0.76	54.75 \pm 1.13

Based on Table 2, the total flavonoids and phenolic contents present in CVCE extract were $24.91 \pm 0.33 \mu\text{g}$ quercetin equivalent/mg of extract and $54.43 \pm 1.49 \mu\text{g}$ gallic acid equivalent/mg of extract, respectively. The concentrations of CVCE extract that reduced free radical DPPH to about 50% (IC₅₀) is $54.43 \pm 1.49 \mu\text{g/mL}$. FRAP value was expressed as Trolox equivalents (μg Trolox/ μg sample dry weight). The FRAP value of CVCE extract was found to be $43.05 \pm 0.76 \mu\text{g}$ Trolox/ μg sample dry weight.

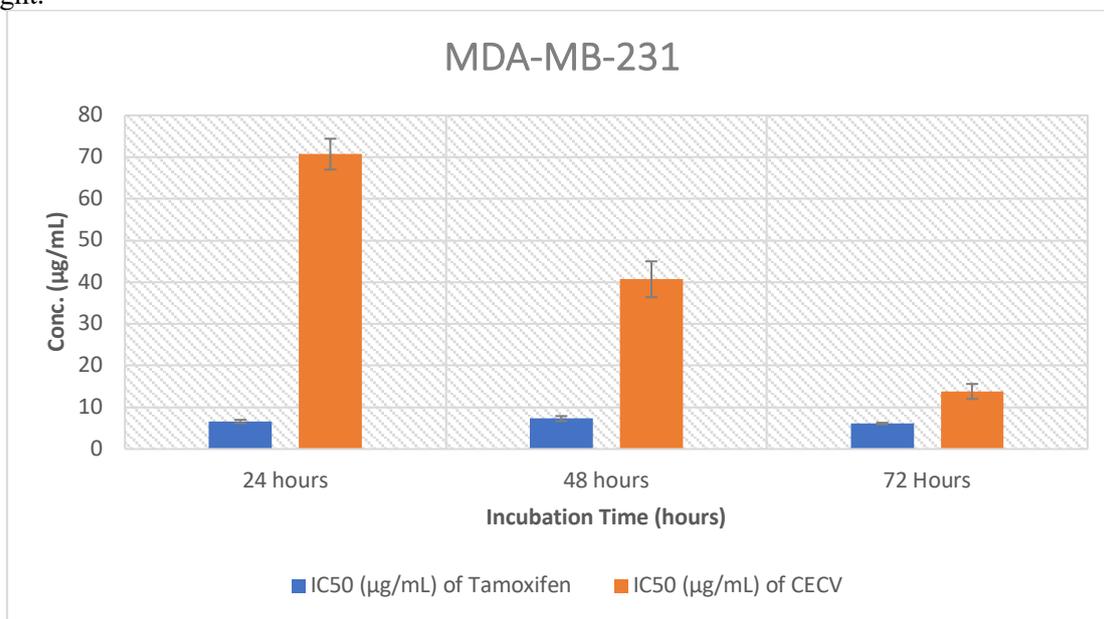


Figure 1: Inhibition concentration (IC₅₀) value of CVCE extract on MDA-MB-231

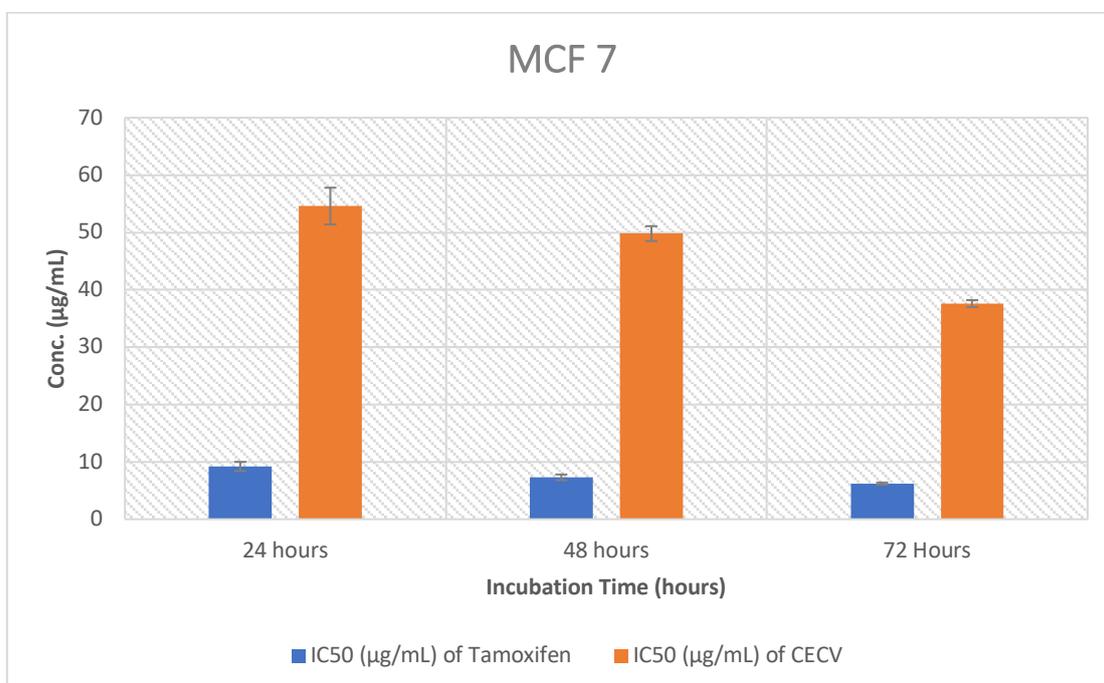


Figure 2: Inhibition concentration (IC₅₀) value of CVCE extract on MCF 7

The cytotoxic effect of CVCE extract was assessed based on the minimum concentration of extract that giving at least 50% of the cancer cell survivability (IC₅₀). Two human breast carcinoma cell lines which are estrogen receptor negative (ER-) MDA-MB-231 cells and estrogen receptor positive (ER+) MCF7 cell were used to determine the antiproliferative activity of CVCE extract. The survival

of the human breast-derived cells after treatment with the CVCE extract and tamoxifen after 24, 48 and 72 h were determined. The inhibition concentration (IC₅₀) of CVCE against MDA-MB-231 and MCF7 cell lines are shown in Figures 1 and 2, respectively.

The antiproliferative profiles of the CVCE extracts against the MDA-MB 231 cancer cell lines are shown in Fig. 1. The result show that the IC₅₀ value of the extract was decrease against time form 24 h to 72 h with IC₅₀ value of 70 µg/mL, 40 µg/mL and 15 µg/mL respectively. The activity against MCF7 cancer cell lines also showed the same pattern which are the IC₅₀ value of CVCE was decrease against time (24 h, 48 h and 72h) with the value 55 µg/mL, 50 µg/mL and 38 µg/mL, respectively.

Comparison of IC₅₀ was also performed between the CVCE extract and tamoxifen, a standard antitumor drug (Figure 1 and 2). Overall, tamoxifen was a more potent antiproliferative agent against both cancer cell lines compared with the CVCE extracts of the plant as indicated by its lower IC₅₀ values.

CONCLUSION

In conclusion, the findings of this study underscore the potential of *Christia vespertilionis* (L.f.) Bakh. F as a natural anticancer agent against breast cancer cell lines. The phytochemical screening revealed the presence of bioactive compounds, particularly flavonoids and saponin, suggesting the pharmacological significance of the plant. Moreover, the quantification of total phenolic and flavonoid contents provided insights into its antioxidant capacity, which may contribute to its anticancer effects by mitigating oxidative stress. The antiproliferative assays demonstrated a concentration-dependent cytotoxic effect of the *C. vespertilionis* extract on estrogen receptor-positive (MCF7) and estrogen receptor-negative (MDA-MB-231) breast cancer cells. While the extract exhibited promising cytotoxicity, its potency was lower compared to the standard drug tamoxifen. Nevertheless, the results support further investigation into the mechanisms of action underlying the anticancer properties of *C. vespertilionis* and its potential clinical applications.

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