IOSR Journal Of Pharmacy And Biological Sciences (IOSR-JPBS)
e-ISSN:2278-3008, p-ISSN:2319-7676. Volume 19, Issue 2 Ser. 1 (Mar. – Apr. 2024), PP 16-24
www.Iosrjournals.Org

Antiproliferative And Anti-Migration Properties, In Vivo Safety And Efficacy Evaluations Of The Ethyl Acetate Fraction Of The Ethanolic Extract From The Aerial Parts Of Centratherum Punctatum Cass.

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Abstract:
Background: *Centratherum punctatum Cass. is an ornamental plant commonly grown in tropical regions worldwide. This plant has been reported to have a variety of pharmacological effects, including anti-inflammatory, anthelmintic, antiplasmodial, antioxidant, antiproliferative, antibacterial, and antifungal activities. The purpose of this study was to identify the active substances of C. punctatum and examine their antiproliferative and anti-migration activities on a human breast cancer cell line. Additionally, the acute oral toxicity and in vivo efficacy of the plant extract were investigated.*
Materials and Methods: *In this study, the bioactive substances were isolated from the aerial parts of C. punctatum through bioassay-guided separation. The fractionated extract of C. punctatum and its active substances were examined for antiproliferative and anti-migration activities using the MTT assay and scratch assay, respectively. The acute oral toxicity study of the fractionated extract was carried out as per the OECD test guideline 423. In addition, the in vivo antitumor activity of the fractionated extract was evaluated using the MDA-MB-231 xenograft model.*
Results: *Based on the guidance of the scratch assay, six active substances were isolated from the aerial parts of C. punctatum, including centratherin (**1**), 3-O-methyl-kaempferol (**2**), apigenin (**3**), kaempferol (**4**), quercetin (**5**), and luteolin (**6**). The fractionated extract and centratherin (**1**) were found to possess strong antiproliferative effects against the MDA-MB-231 cell line, with IC_{50} values of less than 10 µg/mL. Moreover, the fractionated extract and all active substances showed potent anti-migration activity, with a percentage of wound closure less than 50% at a test concentration of 10 µg/mL after 48 hours of treatment. The acute toxicity study of the fractioned extract revealed no mortality and any toxic effects at the tested dose of 5,000 mg/kg bw, classified as category 5, which is of low acute toxicity. Furthermore, the oral administration of the fractionated extract at 100 mg/kg bw once daily for 7-9 consecutive weeks could significantly decrease in tumor growth rate as compared to the vehicle control group in the xenograft model.*
Conclusion: *The aerial parts of C. punctatum contain at least six anti-migration substances. The ethyl acetate fraction of the ethanolic extract derived from the aerial parts of C. punctatum could strongly inhibit proliferation and migration of MDA-MB-231 cells. This fractionated extract showed remarkable antitumor activity and low acute toxicity. These findings suggest that the fractionated extract from the aerial parts of C. punctatum is a promising candidate for the development of an herbal remedy within the domain of complementary medicine for cancer therapy.*
Keywords*: Antiproliferative activity; Anti-migration; Antitumor; Acute toxicity; Centratherum punctatum Cass.*

Date of Submission: 04-03-2024 Date of Acceptance: 14-03-2024

I. Introduction

Cancer is a public health and economic burden worldwide. There were an estimated 19.3 million new cancer cases diagnosed and nearly 10 million cancer deaths globally in 2020. Among these cases, breast cancer emerged as the most prevalent, with an estimated 2.3 million new cases, followed by lung and colorectal cancers[1]. The treatment of cancer typically depends on the type and stage of the cancer. Treatment for early-stage cancer generally includes surgery, radiation therapy, and chemotherapy, while treatment of advanced-stage cancer or metastasis cancer requires more advanced treatment options such as targeted therapy, hormone therapy, immunotherapy, or a combination of treatment options [2-4]. Despite current therapeutic advances in cancer

treatment, metastasis remains the majority cause of cancer death [4-5]. The treatment of metastatic cancer presents a significant challenge, and there is a need for new drugs to improve patient outcomes.

Centratherum punctatum Cass. is an ornamental plant in the family Asteraceae. It is widely distributed in tropical areas, encompassing Central and South America, Southeast Asia, India, and Australia. The plant can grow to 10-50 cm tall, stem-erect and pubescent, single- and multi-stemmed. The leaves are lanceolate to ovate or obovate, 1.5-6 cm long, 0.5-4 cm broad, and have serrated margins. The inflorescences are sessile and solitary, terminating with the branches. The flower color is purple and has a 3-4 cm diameter [6]. This plant has been reported to have a variety of pharmacological effects, including anti-inflammatory [7], anthelmintic [8], antiplasmodial [9], antioxidant [10-11], antiproliferative, and antimicrobial activities [9-11].

In the course of our search for antimigration substances from medicinal plants using the scratch assay, the aerial parts of *C. punctatum* exhibited potent anti-migration activity in various cancer cell lines, including human carcinoma cell line (HuCCA-1), hepatocellular carcinoma cell lines (PLC/PRF/5 and HepG2), cervical adenocarcinoma cell line (HeLa), and breast cancer cell line (MCF-7). In addition, the ethanolic extract of *C. punctatum* showed notable inhibition of HeLa cell migration in a transwell migration assay [12]. These findings suggest the potential of *C. punctatum* as a candidate for the development of an herbal remedy targeting cancer growth inhibition and the mitigation of metastasis. In this study, we identified the active substances of *C. punctatum* and examined their antiproliferative and anti-migration properties specifically on a human breast cancer cell line. Furthermore, we conducted an evaluation of the acute oral toxicity and *in vivo* efficacy of the plant extract to gain a comprehensive understanding of its pharmacological potential.

II. Material And Methods

Collection of Plant Materials

The aerial parts of *Centratherum punctatum* Cass. were collected from Ratchaburi Province, Thailand. It was harvested at the age of 3-6 months. The plant was authenticated by the research officer of the Forest Herbarium Division, and the voucher specimen was then deposited at the Forest Herbarium Division, Forest and Plant/Conservation Research Office, Department of National Park, Wildlife, and Plant Conservation, Bangkok, Thailand, with herbarium specimen number BKF 194728.

Preparation of the Extract

Fresh aerial parts of *C. punctatum* were washed thoroughly and air dried in the shade. The dried plants were ground and sieved through a 10-mesh sieve. The air-dried and ground aerial parts (894.5 g) were extracted with 95% ethanol (9 L, twice) at room temperature. The extract was concentrated under reduced pressure to yield the ethanolic extract. The extract was suspended in distilled water and partitioned with ethyl acetate (1.5 L, 3 times). The ethyl acetate-soluble portion was concentrated to dryness *in vacuo* to yield 53.4 g of an ethyl acetate fraction of *C. punctatum* (EAFCP).

Isolation of Active Substances

The air-dried and ground aerial parts of *C. punctatum* (228.9 g) were extracted with 95% ethanol (4 L, twice) at room temperature. The extract was concentrated under reduced pressure to yield 23.87 g of dried ethanolic extract. The extract was partitioned into a water-ethyl acetate mixture. The ethyl acetate-soluble portion (13.72 g) was further partitioned with an *n*-hexane and 90% methanol mixture. Under the guidance of a scratch assay, the 90% methanol-soluble portion (8.66 g, effective concentration 3-100 µg/mL) was subjected to silica gel column chromatography and eluted with chloroform-methanol to yield twelve fractions (Fractions A1-A12). Several fractions exhibited strong anti-migration activity and had different chemical compositions based on TLC analysis. The strong active fraction A4 (2.60 g, effective concentration 1-100 µg/mL) was fractionated by Sephadex LH-20 column chromatography using chloroform-methanol (1:1 v/v) as eluents, giving six fractions (Fractions A41-A46). The active fraction A44 (1.22 g, effective concentration 1-100 µg/mL) was further separated by C18 column chromatography, eluting with methanol and water to give 272.0 mg of centratherin (**1**). The active fraction A5 (0.55 g, effective concentration 3-100 µg/mL) was subjected to Sephadex LH-20 column chromatography and eluted with chloroform-methanol (1:1 v/v) to give 43.4 mg of 3-*O*-methyl-kaempferol (**2**), 17.3 mg of apigenin (**3**), and 12.7 mg of kaempferol (**4**). The strong active fraction A7 (0.48 g, effective concentration 1-100 µg/mL) was fractionated by Sephadex LH-20 column chromatography with chloroform-methanol (1:1 v/v) to give 25.8 mg of quercetin (**5**) and 114.5 mg of luteolin (**6**).

Structure Elucidation

The chemical structures of active substances were determined using 2D-NMR data and compared their spectral data with the published data in the literature[13-17].

Centratherin (**1**): light brown amorphous solid; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.58 (3H, s, H-14), 1.78 (3H, s, H-5'), 1.88 (3H, m, H-4'), 2.31 (1H, m, H-9), 2.49 (1H, m, H-9), 3.77 (1H, m, H-7), 4.39 (2H, br, H-15), 4.53 (1H,

m, H-8), 5.36 (1H, m, H-6), 5.45 (1H, d, *J* = 2.7 Hz, H-13), 5.81 (1H, s, H-2), 6.08 (1H, m, H-3'), 6.22 (1H, d, *J* = 3.1 Hz, H-13), 6.28 (1H, m, H-5); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 15.7 (C-4'), 20.0 (C-5'), 20.7 (C-14), 44.1 (C-9), 51.0 (C-7), 63.2 (C-15), 72.9 (C-8), 81.7 (C-6), 89.9 (C-10), 106.7 (C-2), 124.5 (C-13), 126.3 (C-2'), 133.4 (C-11), 134.4 (C-11), 135.4 (C-5), 140.9 (C-3'), 167.2 (C-1'), 168.9 (C-12), 184.4 (C-3), 204.7 (C-1); ESI-MS m/z 375 $[M+H]^+$.

3-*O*-Methyl-kaempferol (**2**): light yellow powder; ^{1}H NMR (DMSO-d6, 500 MHz) δ 3.78 (3H, s, 3-OCH_3), 6.20 (1H, d, *J* = 2.0, H-6), 6.44 (1H, d, *J* = 2.0 Hz, H-8), 6.94 (2H, d, *J* = 8.9 Hz, H-3'/H-5'), 7.93 (2H, d, *J* = 8.9 Hz, H-2'/H-6'), 12.68 (1H, s, 5-OH); ^{13}C NMR (DMSO-d6, 125 MHz) δ 59.7 (3-OCH_3), 93.7 (C-8), 98.6 (C-6), 104.2 (C-10), 115.6 (C-3'/C-5'), 120.6 (C-1'), 130.1 (C-2'/C-6'), 137.6 (C-3), 155.6 (C-2), 156.4 (C-9), 160.2 (C-4'), 161.2 (C-5), 164.1 (C-7), 177.9 (C-4); ESI-MS m/z 301 $[M+H]^+$.

Apigenin (**3**): light yellow powder; ^{1}H NMR (DMSO-d6, 500 MHz) δ 6.19 (1H, d, *J* = 2.0, H-6), 6.48 (1H, d, *J* = 2.0 Hz, H-8), 6.79 (1H, s, H-3), 6.92 (2H, d, *J* = 8.8 Hz, H-3'/H-5'), 7.93 (2H, d, *J* = 8.8 Hz, H-2'/H-6'), 12.96 (1H, s, 5-OH); ^{13}C NMR (DMSO-d6, 125 MHz) δ 93.9 (C-8), 98.8 (C-6), 102.8 (C-3), 103.7 (C-10), 115.9 (C-3'/C-5'), 121.2 (C-1'), 128.5 (C-2'/C-6'), 157.3 (C-9), 161.1 (C-4'), 161.4 (C-5), 163.7 (C-2), 164.1 (C-7), 181.7 (C-4) ; ESI-MS m/z 271 $[M+H]^+$.

Kaempferol (**4**): light yellow powder; ^{1}H NMR (DMSO-d6, 500 MHz) δ 6.19 (1H, d, *J* = 2.0, H-6), 6.44 (1H, d, *J* = 2.0 Hz, H-8), 6.92 (2H, d, *J* = 8.9 Hz, H-3'/H-5'), 8.04 (2H, d, *J* = 8.9 Hz, H-2'/H-6'), 9.42 (1H, s, 3-OH), 10.13 (1H, s, 4'-OH), 10.81 (1H, s, 7-OH), 12.48 (1H, s, 5-OH); ^{13}C NMR (DMSO-d6, 125 MHz) δ 93.5 (C-8), 98.2 (C-6), 103.0 (C-10), 115.4 (C-3'/C-5'), 121.7 (C-1'), 129.5 (C-2'/C-6'), 135.6 (C-3), 146.8 (C-2), 156.2 (C-9), 159.2 (C-4'), 160.7 (C-5), 163.9 (C-7), 175.9 (C-4); ESI-MS m/z 287 $[M+H]^+$.

Quercetin (**5**): yellow powder; ^{1}H NMR (DMSO-d6, 400 MHz) δ 6.18 (1H, d, *J* = 2.0, H-6), 6.40 (1H, d, *J* = 2.0 Hz, H-8), 6.88 (1H, d, *J* = 8.5 Hz, H-3'), 7.53 (1H, dd, *J* = 8.5, 2.2 Hz, H-2'), 7.67 (1H, d, *J* = 2.2 Hz, H-6'), 12.49 (1H, s, 5-OH); ^{13}C NMR (DMSO-d6, 100 MHz) δ 93.3 (C-8), 98.2 (C-6), 103.0 (C-10), 115.0 (C-6'), 115.6 (C-3'), 120.0 (C-2'), 121.9 (C-1'), 135.7 (C-3), 145.0 (C-5'), 146.8 (C-2), 147.7 (C-4'), 156.1 (C-9), 160.7 (C-5), 163.9 (C-7), 175.8 (C-4); ESI-MS m/z 303 $[M+H]^+$.

Luteolin (**6**): light yellow powder; ^{1}H NMR (DMSO-d6, 500 MHz) δ 6.19 (1H, d, *J* = 2.1, H-6), 6.44 (1H, d, *J* = 2.1 Hz, H-8), 6.67 (1H, s, H-3), 6.88 (1H, d, *J* = 8.3 Hz, H-3'), 7.39 (1H, d, *J* = 2.2 Hz, H-6'), 7.42 (1H, dd, *J* = 8.3, 2.3 Hz, H-2'), 12.98 (1H, s, 5-OH); ^{13}C NMR (DMSO-d6, 125 MHz) δ 93.8 (C-8), 98.8 (C-6), 102.9 (C-3), 103.7 (C-10), 113.4 (C-6'), 116.0 (C-3'), 119.0 (C-2'), 121.5 (C-1'), 145.7 (C-5'), 149.7 (C-6'), 157.3 (C-9), 161.5 (C-5), 163.9 (C-2), 164.1 (C-7), 181.7 (C-4); ESI-MS m/z 287 $[M+H]^+$.

***In vitro* Assay for Biological Activities Studies**

Cell Culture

The human breast cancer cell lines MDA-MB-231 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). MDA-MD-231 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco; Billing, MT, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone; Logan, UT, USA), 100 units/mL penicillin, and 100 µg/mL streptomycin (Gibco; Billing, MT, USA) at 37 °C in a 5% CO_2 atmosphere.

Cell viability assay (MTT assay) for analysis of antiproliferative activity

MDA-MD-231 cells were seeded on a 96-well plate at 2×10^4 cells/well and incubated. After 24 hours of incubation, the cells were treated with the samples at different concentrations (0.3, 1, 3, 10, 30, and 100 µg/mL) for 48 hours. DMSO (Sigma-Aldrich; St. Louis, MO, USA) was used as vehicle control, and gefitinib (Sigma-Aldrich; St. Louis, MO, USA) was used as positive control. After incubation, 50 µL of 2 mg/mL MTT (TCI; Tokyo, Japan) solution was added to each well and incubated at 37 °C for 3 hours. The supernatant was removed, and 100 µL of DMSO was added to dissolve the formazan crystals. The absorbance was determined at 570 nm by a microplate spectrophotometer. The percentage of cell viability was calculated relative to the control, and the half maximal inhibitory concentration (IC_{50}) value was calculated using the software GraphPad Prism 6. Cell viability assay was performed with three independent experiments.

Scratch assay for analysis of cell migration

MDA-MD-231 cells were plated on a 96-well plate at 2×10^4 cells/well and cultured for 24 hours until they reached confluence. The cells were scratched using a sterile 10 µL pipette tip across the well to create a wound and treated with the samples at different concentrations (3, 10, 30, and 100 µg/mL) for 48 hours. DMSO

was used as vehicle control, and gefitinib was used as positive control. The wounds were photographed at 0 and 48 hours with an inverted microscope. The wound area was determine using the ImageJ program and plug-in wound healing size tool [18]. The cell migration was calculated as a percentage of wound closure using the formula: Wound closure (%) = [[*Area 0h* – *Area 48h*]/*Area 0h*] ×100, where *Area 0h* and *Area 48h* represent the wound area at 0 and 48 hours, respectively. Scratch assay was performed with three independent experiments. The results were presented as the mean ± standard error of three independent experiments. Student t-test was used to analyze the statistically significant differences with a confidence level of $p < 0.05$.

***In vivo* Toxicity Study**

Animals

Female ICR mice (20-25 g) were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. The animals were housed in a strict conventional hygienic system at an ambient temperature of 22±3 °C and a relative humidity of 50-60% with 12-hour light-dark cycles. They were allowed to access diet and water *ad libitum*. The study received approval from the Institutional Animal Care and Use Committee, Department of Medical Sciences (Ethical Clearance No. 65-016).

Acute toxicity test

Acute oral toxicity testing was carried out according to the Organization of Economic Cooperation and Development (OECD) guideline no. 423. The animals were acclimatized to the laboratory conditions for 24 hours before the study and fasted for 2 hours with free access to water prior to dosing. Female ICR mice were randomly divided into two groups, each comprising three animals. The first group served as a control and received olive oil, while the second group was considered the tested group that received the extract (EAFCP dissolved in olive oil at a concentration of 250 mg/mL) orally at 5,000 mg/kg body weight. After dose administration, food was withheld for 6 hours. The animals were closely observed for mortality and any toxic effects during the first 30 minutes, periodically during the first 6 hours, and daily thereafter for a total of 14 days. At the end of the study, the animals were euthanized by CO_2 inhalation and subjected to gross necropsy. All gross pathological changes were recorded for each animal.

***In vivo* Efficacy Study**

Cell Culture

Luciferase/GFP dual-labeled MDA-MB-231 cancer cell line was purchased from GeneCopoeia, Inc. (GeneCopoeia™, Rockville, MD, USA) and maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and streptomycin at 37 °C in a 5% CO_2 atmosphere.

Animals

Female BALB/c nude mice (BALB/CAJcl-Nu/Nu) of 4-6 weeks old (18-20 g) were obtained from Nomura Siam International Co. Ltd., based in Bangkok, Thailand, and acclimatized to the laboratory conditions for a week prior to the experimentation. The animals were housed in individual ventilation cages in an Animal Biosafety Level 1 (ABSL-1) facility at an ambient temperature of 23±1 °C and a relative humidity of 60±20% with 12-hour light-dark cycles. They were allowed to access diet and water *ad libitum*. The study received approval from the Institutional Animal Care and Use Committee, Department of Medical Sciences (Ethical Clearance No. 63-027).

Xenograft model of MDA-MB-231 cells

For tumor induction, cell suspensions of 5×10^6 cells per 100 µL of FBS-free DMEM or FBS-free DMEM only were injected into the mammary fat pad of mice. After cell implantation for 10–14 days, the tumor was measured for length (L), width (W), and height (H) using a caliper, and the tumor volume was calculated using the formula $0.5236 \times L \times W \times H$. The animals were then randomly divided into four groups (6–9 mice per group). Group I served as a normal control group that was injected with FBS-free DMEM (no cell implantation) and received orally 1% Tragacanth daily. Group II was a xenografting-vehicle control group that received 1% Tragacanth daily. Group III and IV were xenografting groups that received the extract (EAFCP dissolved in 1% Tragacanth) at 10 and 100 mg/kg/day, respectively. The animals were observed for morbidity and mortality at least once per day. The body weights were recorded daily throughout the study. Tumor volumes were monitored weekly using a caliper for a period of study. At the end of the study, the animals were euthanized by isoflurane overexposure and subjected to hematological analysis and gross necropsy. The vital organs were removed and weighted. Metastatic tumor cells were detected using *in vivo* fluorescence imaging (VISQUE® InVivo Smart-LF; Poznan, Poland). The tumors and organs were examined with immunohistochemistry (H&E and Ki67 staining).

Statistical analysis

The experimental data were analyzed using descriptive statistics with the software IBM SPSS Statistics. Student t-test, ANOVA, and Chi-Square tests were used to evaluate statistical significance. The criteria for significance were $p < 0.05$.

III. Results

Extraction, Isolation, and Identification of Active Substances

The dried arial parts of *C. punctatum* were extracted with ethanol and partitioned into a water-ethyl acetate mixture to obtain the ethyl acetate fraction of the ethanolic extract from *C. punctatum* (EAFCP; 6.0% yield). The extract was used for the isolation of bioactive substances as well as *in vivo* safety and efficacy evaluations. Guided by the scratch assay, six active substances were isolated from the EAFCP and tested for their biological activities on the MDA-MB-231 cell line. The chemical structures of isolated substances were identified by comprehensive analysis of ^{1}H, ^{13}C, DEPT135, HMQC, HMBC, COSY, and NOESY NMR spectra. Based on the structure elucidation using NMR data and comparison of their spectral data with the reported data [13-17], six isolated substances were identified as centratherin (**1**), 3-*O*-methyl-kaempferol (**2**), apigenin (**3**), kaempferol (**4**), quercetin (**5**), and luteolin (**6**).

***In vitro* Biological Activities Studies**

To determine the antiproliferative activity of EAFCP and six active substances, the MDA-MB-231 breast cancer cell line was used for cell viability testing by MTT assay. The antiproliferative activity of EAFCP and compounds (**1**-**6**) is presented in **Table 1**. EAFCP and centratherin (**1**) were found to possess strong antiproliferative effects against the MDA-MB-231 cell line, with IC_{50} values of less than 3 µg/mL. 3-*O*-Methyl-kaempferol (**2**), kaempferol (**4**), and luteolin (**6**) exhibited moderate anti-proliferative effects with IC_{50} values ranging from 23.81±1.85 to 36.40± 4.09 µg/mL while apigenin (**3**) and quercetin (**5**) were found to have IC_{50} values of more than 100 µg/mL.

The effects of EAFCP and six active substances (**1**-**6**) on cell motility were examined using the modified scratch assay. The cells were treated with the various concentrations of test samples for 48 hours. The wound areas were determined, and the percentage of wound closure was calculated. After treatment with EAFCP and six active substances (**1**-**6**), migrations of the MDA-MB-231 cell line were significantly inhibited, with the percentage of wound closure reduced to less than 50% at 10-100 µg/mL of test samples. The cell migration of the treated cell line decreased in a dose-dependent manner. (**Figure 1**)

Table 1: Antiproliferative activity of the fractionated extract (EAFCP) and active substances of *C. punctatum* against the MDA-MB-231 breast cancer cell line (IC_{50} values are presented as mean ± standard error).

Test samples	IC_{50} (µg/mL)
EAFCP	0.88 ± 0.06
centratherin (**1**)	1.12 ± 0.20
3-*O*-methyl kaempferol (**2**)	32.58 ± 0.55
apigenin (**3**)	> 100.00
kaempferol (**4**)	23.81 ± 1.07
quercetin (**5**)	>100.00
luteolin (**6**)	36.40 ± 2.36
gefitinib	14.61 ± 1.62

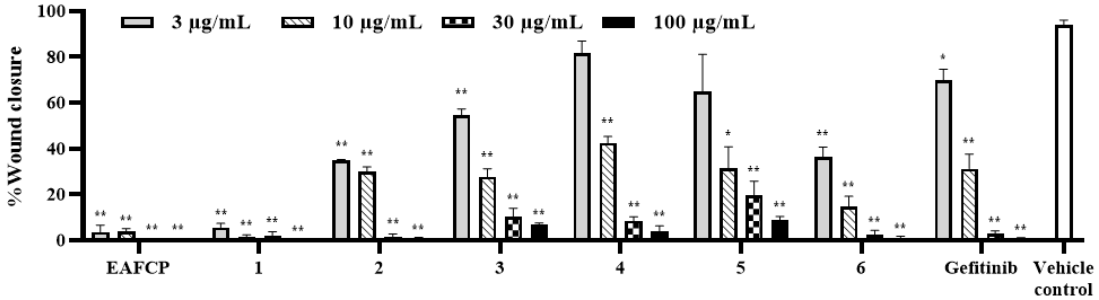

Figure 1: Effects of EAFCP and six active substances (**1**-**6**) on the cell migration of MDA-MB-231. The cell migration inhibition is presented as wound closure (%) after 48 hours of treatment. Results are represented as mean ± standard error. *P<0.05, **P<0.01 as compared with the vehicle (DMSO).

Acute Oral Toxicity Evaluation of EAFCP

The acute toxicity study of EAFCP was carried out following OECD Guideline 423. Female ICR mice were administered orally with 5,000 mg/kg bw of EAFCP and observed for clinical signs, behavioral changes, and mortality for 14 days. It was found that there was no death in any of the animals, and there were no significant differences in clinical signs or body weight changes. In addition, there were no gross pathological changes observed in the vital organs of the treated mice.

***In vivo* Antitumor Activity of EAFCP**

The efficacy study was focused on the antitumor activity of EAFCP in a xenograft model of breast cancer cells. Female BALB/c nude mice were implanted with luciferase/GFP dual-labeled MDA-MB-231 cells into the mammary fat pads. After tumor growth, the animals were treated orally with vehicle or 10 and 100 mg/kg bw of EAFCP daily and observed for food/water intake, body weights, morbidity, and mortality. Tumor volumes were monitored weekly throughout the study. Treatment continued for over 2 months, and the mice were euthanized in week 9 because the animals were considered to have met humane endpoint criteria (the tumors grew to 20 mm, which is the maximum growth allowable by IACUC). During the study period, there were no significant differences in food/water intake or body weight changes between each group. Mortality and morbidity rates in mice are reported in Table 2. It was found that there were no statistically significant differences in mortality and morbidity between groups. In addition, tumor progression in the vehicle control and ETFCP treatment groups was monitored weekly to evaluate the treatment outcomes. As shown in Figure 2, treatment with 100 mg/kg bw of ETFCP yielded a decrease in tumor size. This group showed a significant decrease in tumor growth rate as compared with the vehicle control group in weeks 7, 8, and 9 (p-values = 0.014, <0.001, and < 0.001, respectively). However, there was no significant difference in tumor growth rate between the 10 mg/kg bw ETFCP-treated group and the vehicle control group.

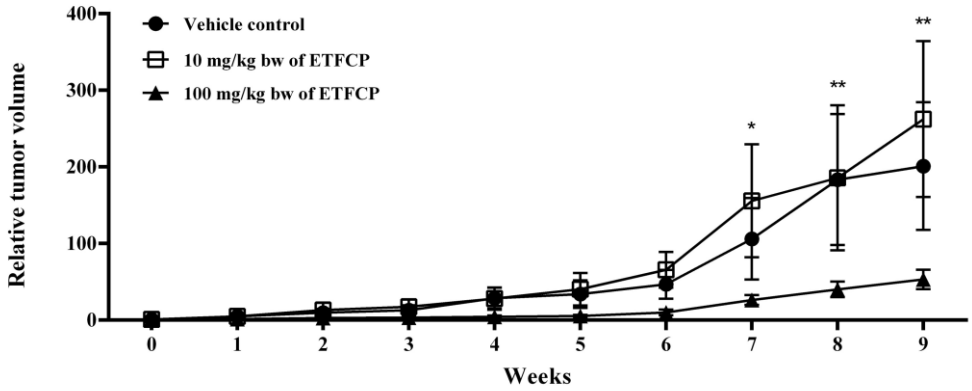

Figure 2: Relative tumor volume of the xenografted tumor after oral administration of ETFCP at 10 and 100 mg/kg bw once daily for 9 weeks. Results are represented as mean ± standard error. *P<0.05, **P<0.01 as compared with the vehicle control group (1% Tragacanth).

Table 2: Clinical observation during the study and hemogram at the end of treatment (All values are presented as mean ± standard error.)

Observation	Groups			
		Xenografting of breast cancer cells		
	I: normal control	II: vehicle control	III: 10 mg/kg bw ETFCP treatment	IV: 100 mg/kg bw ETFCP treatment
n	5	8	9	9
Mortality (until week 9)	1/5 (20.0%)	2/8 (25.0%)	4/9 (44.4%)	3/9 (33.3%)
Morbidity				
Depression/Weakness	0/5 (0.0%)	3/8 (33.3%)	6/9 (66.7%)	3/9 (33.3%)
Nasal discharge	1/5 (20.0%)	0/8 (0.0%)	3/9 (33.3%)	0/9 (0.0%)
Weight loss	0/5 (0.0%)	3/8 (37.5%)	6/9 (66.7%)	3/9 (33.3%)
Jaundice	0/5 (0.0%)	1/8 (12.5%)	0/9 (0.0%)	0/9 (0.0%)
Ascites	0/5 (0.0%)	0/8 (0.0%)	0/9 (0.0%)	1/9 (11.1%)
Tumor volumes (mm^3)				
Week 0	-	0.54 ± 0.12	0.77 ± 0.66	1.16 ± 0.32
Week 9	-	50.00 ± 7.79	48.28 ± 3.18	42.78 ± 17.44
Hematological analysis				
RBC ($\times 10^6/\mu L$)	8.40 ± 0.00	7.63 ± 0.22	7.80 ± 0.12	8.60 ± 0.19
Hemoglobin (g/dL)	14.10 ± 0.00	12.31 ± 0.13	12.47 ± 0.13	12.62 ± 0.47
Hematocrit (%)	39.50 ± 0.35	36.00 ± 0.33	37.00 ± 0.88	39.50 ± 1.42

WBC (× 10^3/μL)	4.45 ± 0.25	19.83 ± 1.53	7.56 ± 1.20	9.80 ± 1.15
Neutrophil (%)	66.00 ± 2.83	72.33 ± 2.59	65.66 ± 3.40	55.25 ± 4.58
Basophil (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.50 ± 0.03
Eosinophil (%)	0.00 ± 0.00	0.33 ± 0.19	0.67 ± 0.19	0.50 ± 0.15
Lymphocyte (%)	32.50 ± 3.18	26.00 ± 2.40	32.67 ± 3.29	42.25 ± 4.49
Monocyte (%)	1.50 ± 0.35	1.33 ± 0.19	1.00 ± 0.00	1.50 ± 0.15
MCV (fL)	47.50 ± 0.35	46.00 ± 0.00	47.50 ± 0.70	46.33 ± 0.58
MCH (pg)	16.80 ± 0.00	16.13 ± 0.35	14.54 ± 0.35	14.55 ± 0.41
MCHC (g/dL)	35.45 ± 0.25	33.85 ± 0.31	29.90 ± 0.56	30.80 ± 0.39
RDW (%)	20.65 ± 1.10	16.50 ± 0.99	15.10 ± 0.24	14.15 ± 0.51
Platelets (× 10^3/μL)	833.00 ± 27.58	854.66 ± 39.05	917.00 ± 86.60	880.00 ± 56.67

At the end of treatment, the hemograms of mice were analyzed to evaluate the possible hemotoxicity of ETFCP. Hematological analysis of xenografted mice treated with either vehicle or EAFCP showed no significant abnormalities in any of the parameters studied when compared to the normal control group. (**Table 2**) These results indicated that there is no hematological toxicity in EAFCP-treated mice. Comparison of organ weights between groups demonstrated an increase in the relative weights of the liver and spleen in all xenografting groups as compared with the normal control group. Specifically, both the xenografting-vehicle control group and the 100 mg/kg bw ETFCP-treated group exhibited significantly higher relative weights of the liver (p-values = 0.026 and 0.028, respectively) and spleen (p-values = 0.021 and 0.011, respectively) compared to the normal control group. However, the relative weights of other organs remained unchanged across all groups. (**Table 3**)

The investigation into the dissemination of tumor cells to distant organs employed fluorescence imaging in conjunction with histological and immunohistochemical staining techniques. Histopathological analysis of xenografted mice revealed the presence of GFP-expressing cancer cells in various organs, including the lung, liver, ovary, and uterus. However, metastases were observed in only certain mice. The application of fluorescence imaging still has limitations in detecting secondary tumors, particularly within organs containing a sparse population of cancer cells. The results of metastatic organs and secondary tumors detected through immunohistochemistry are reported in **Table 3**. Analysis of H&E and Ki67 staining disclosed the metastasis of cancer cells in the lung, liver, stomach, ovary, and uterus. Xenografted mice, administered 1% tragacanth or the ETFCP at daily doses of 10 and 100 mg/kg bw, exhibited secondary tumors at rates of 75.0%, 33.3%, and 44.4%, respectively.

Table 3. Relative organ weights and histopathological examination at the end of the study (All values are presented as mean ± standard error. *P<0.05 as compared with the normal control group.)

Examination	**Groups**			
		Xenografting of breast cancer cells		
	I: normal control	**II: vehicle control**	**III: 10 mg/kg bw ETFCP treatment**	**IV: 100 mg/kg bw ETFCP treatment**
Relative organ weights (g%)				
Brain	1.89 ± 0.42	1.86 ± 0.04	2.01 ± 0.03	1.92 ± 0.04
Heart	0.73 ± 0.01	0.65 ± 0.02	0.68 ± 0.02	0.64 ± 0.01
Liver	5.92 ± 0.11	6.55 ± 0.06*	6.15 ± 0.04	6.52 ± 0.08*
Spleen	0.51 ± 0.02	1.28 ± 0.09*	1.12 ± 0.07	1.34 ± 0.11*
Left kidney	0.80 ± 0.01	0.89 ± 0.04	0.81 ± 0.02	0.80 ± 0.01
Right kidney	0.78 ± 0.00	0.87 ± 0.04	0.81 ± 0.02	0.80 ± 0.01
Left ovary	0.06 ± 0.00	0.05 ± 0.00	0.11 ± 0.02	0.03 ± 0.00
Right ovary	0.05 ± 0.00	0.05 ± 0.00	0.11 ± 0.02	0.07 ± 0.01
Uterus	0.39 ± 0.01	0.26 ± 0.02	0.42 ± 0.03	0.25 ± 0.02
Histopathological examination				
Pneumonitis	0/5	1/8 (12.5%)	1/9 (11.1%)	3/9 (33.3%)
Focal hepatic necrosis	0/5	2/8 (25.0%)	1/9 (11.1%)	0/9 (0.0%)
Nephritis	0/5	0/8 (0.0%)	2/9 (22.2%)	0/9 (0.0%)
Lung metastasis	0/5	2/8 (25.0%)	1/9 (11.1%)	1/9 (11.1%)
Liver metastasis	0/5	1/8 (12.5%)	0/9 (0.0%)	2/9 (22.2%)
Gastric metastasis	0/5	1/8 (12.5%)	0/9 (0.0%)	0/9 (0.0%)
Ovarian metastasis	0/5	2/8 (25.0%)	0/9 (0.0%)	2/9 (22.2%)
Uterine metastasis	0/5	4/8 (50.0%)	3/9 (33.3%)	3/9 (33.3%)
Secondary tumors	0/5	6/8 (75.0%)	3/9 (33.3%)	4/9 (44.4%)

IV. Discussion

Generally, *C. punctatum* is extensively cultivated in various tropical and subtropical areas around the world. The plant features beautiful leaves and bright purple flowers with a distinctive aroma. It has been introduced as an ornamental plant; however, there is a high probability that it has been unintentionally introduced as a contaminant. This is because it produces small seeds that are easily dispersed by the wind, leading to its

growth as a weed in disturbed areas [6]. Phytochemical screening of the aerial parts of *C. punctatum* revealed the presence of terpenes, flavonoids, steroids, protein, sugar, quinone, phenol, and tannin [19]. Additionally, the essential oil compositions of *C. punctatum* have been reported to mainly consist of sesquiterpenes, with β-caryophyllene identified as a major compound, varying from 16.6% to 28.3% of the compositions [20-22]. This compound is also a major constituent in the essential oils of cloves, hops, rosemary, and cannabis, displaying a wide range of biological activities, such as antibacterial, antioxidant, anti-inflammatory, and analgesic properties [23]. Furthermore, *C. punctatum* has been previously reported to comprise sesquiterpene lactones including centratherin, isocentratherin, goyazensolide, isogoyazensolide, and lychnophorolide B [13, 24]. In this study, the sesquiterpene lactone centratherin (**1**) was isolated from the aerial parts of *C. punctatum* and demonstrated a potent antiproliferative effect against the MDA-MB-231 breast cancer cell line. This result is in agreement with previous studies that described centratherin isolated from *Camchaya calcarean* and *Eremanthus crotonoides* as possessing strong antiproliferative effects against small cell lung cancer cell line (NCI-H187) [25] and glioma cancer cell lines (U87-MG and U251) [26], respectively. In order to study the active substances from the aerial parts of *C. punctatum*, not only centratherin (**1**) was isolated, but five flavonoids were also found to possess anti-migration activity against the MDA-MB-231 breast cancer cell line. To date, no other studies have reported the presence of these flavonoids in *C. punctatum*. This study is the first to report the presence of 3-*O*-methyl-kaempferol (**2**), apigenin (**3**), kaempferol (**4**), quercetin (**5**), and luteolin (**6**) in the aerial parts of *C. punctatum*.

While the isolation of *C. punctatum* revealed several active fractions, the identification of active substances in this study only focused on certain fractions. Consequently, it is plausible that the extract of *C. punctatum* may also comprise additional active substances, such as isocentratherin and goyazensolide. Previously, the sesquiterpene lactone goyazensolide was identified as a potent NF-κB inhibitor that inhibits TNFα-mediated NF-κB activation. It increased procaspase-3 expression, leading to apoptosis and cell cycle arrest in G_1-phase. Additionally, goyazensolide significantly suppressed tumor cell growth *in vivo* using the hollow fiber assay [27]. Moreover, the previous investigations of centratherin and goyazensolide on NF-κB DNA binding using an electrophoretic mobility shift assay (EMSA) indicated that both compounds exhibited potent NF-κB inhibiting properties [28-29]. Thus, it is conceivable that the sesquiterpene lactones in *C. punctatum* may serve as potent NF-κB inhibitors, resulting in antiproliferative, anti-migration, and antitumor effects.

Flavonoids are natural polyphenolic compounds widely found in plants, exerting various health benefits. Flavonoids also showed anticancer effects mediated via regulation of key signaling pathways involved in apoptosis, autophagy, cancer cell proliferation, and metastatic progression [30-31]. The flavonoids apigenin, kaempferol, quercetin, and luteolin have been documented to exhibit *in vitro* anti-invasive and *in vivo* anti-metastatic activities [31-32]. In this study, both sesquiterpene lactones and flavonoids were identified as active constituents in *C. punctatum*. The ethyl acetate fraction derived from the ethanolic extract of the aerial parts of *C. punctatum* exhibited strong inhibition of breast cancer cell proliferation and migration. Furthermore, this fractionated extract demonstrated significant *in vivo* antitumor activity with low acute toxicity. Therefore, the extract derived from the aerial part of *C. punctatum*, containing both sesquiterpene lactones and flavonoids, holds potential for the development of an herbal remedy for cancer treatment.

V. Conclusion

In the present study, six active substances were isolated and identified from the arial parts of *C. punctatum*. These substances, including centratherin (**1**), 3-O-methyl-kaempferol (**2**), apigenin (**3**), kaempferol (**4**), quercetin (**5**), and luteolin (**6**), exhibited strong anti-migration activity against the MDA-MB-231 breast cancer cell line. The fractionated extract of *C. punctatum* (ETFCP) and centratherin (**1**), in particular, demonstrated notably inhibitory effects on cell growth and migration, indicating their potential as anticancer agents. Furthermore, the study revealed that the fractionated extract had low acute toxicity, with no mortality or toxic effects observed at the tested dose of 5,000 mg/kg bw. In a xenograft model, oral administration of the fractionated extract significantly reduced the rate of tumor growth, highlighting its promising antitumor activity. These findings suggest that the fractionated extract from *C. punctatum* could be a valuable candidate for the development of herbal remedies for cancer therapy. However, additional studies, including clinical trials, are needed to fully assess the safety and efficacy of the extract in human cancer treatment.

Acknowledgements

The authors are thankful to the Department of Medical Sciences for financial support to this research. We are grateful to the Forest Herbarium Division, Forest and Plant Conservation Research Office, Department of National Park, Wildlife and Plant Conservation for plant authentication and voucher specimen deposition.

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