RESEARCH ARTICLE

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Evaluation of *In Vitro* Cytotoxic Property Against Cholangiocarcinoma Cell Line and GC/MS Analysis from Leaf of *Erythrophleum succirubrum* Gagnep

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Abstract

Objective: Plants are valuable sources of new pharmaceuticals. Secondary metabolites of the genus *Erythrophleum* exhibit cytotoxicity and may have therapeutic value. The cytotoxic activity of ethanolic leaf extract of *Erythrophleum succirubrum* Gagnep. against a human cholangiocarcinoma cell line was assessed. **Methods:** Crude extract of *E. succirubrum* was prepared by ethanol extraction. The ethanolic leaf extract of *E. succirubrum* was evaluated for cytotoxicity against the human cholangiocarcinoma cell line KKU-M213 using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assays. The chemical composition of *E. succirubrum* leaf extract was analyzed using GC/MS. **Result:** The ethanolic leaf extract of *E. succirubrum* reduced the viability of KKU-M213 cells in a dose- and time-dependent manner. It showed high cytotoxicity, with IC_{50} values of 65.22 ± 1.18 µg/mL and 1.19 ± 1.38 µg/mL at exposure times of 24 and 96 h, respectively. GC/MS analysis of the ethanolic leaf extract of *E. succirubrum* identified 22 components. The main constituents identified were Cyclohexanone, 2-[2-nitro-1-(2-naphthyl)ethyl]-(14.79%) followed by allomycin (14.65%), mome inositol (14.30%), campesterol (11.80%) and ethyl linolenate (10.83%), respectively. **Conclusion:** Five major groups of compounds were found, with lipids dominating, followed by carbohydrates, benzenoids, phenylpropanoids, polyketides and organoheterocyclic compounds. Many of the bioactive components discovered in the ethanolic leaf extract of *E. succirubrum* might be responsible for its cytotoxic properties.

Keywords: Cholangiocarcinoma- cytotoxic activity- *Erythrophleum succirubrum* Gagnep.-GC/MS analysis- gemcitabine

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Introduction

Cancer is defined as uncontrolled cell development, followed by an invasion of cells into surrounding tissue and eventually metastasis to other areas of the body. It is a disorder in which abnormal cells proliferate in the body and can lead to death. Cholangiocarcinoma (CCA) is a cancer of the bile duct epithelium. CCA is an uncommon form of liver cancer but is a serious public health concern in the north-eastern region of Thailand (Sripa and Pairojkul, 2008). Cancer therapies include surgery, radiation, and chemotherapy, but each of these approaches has downsides, such as damage to normal cells and suppression of the functions of bone marrow (Barreto et al., 2014; Atun and Arianingrum, 2015). Natural products derived from plant species, including vincristine, irinotecan, and paclitaxel, are recognized as sources of novel candidate drugs (Da Rocha et al., 2001). Several studies have found that secondary metabolites such as phenolic compounds, flavonoids, terpenes and alkaloids have anticancer and antimutagenic activities (Sanseera et al., 2016). Several phytochemicals with diverse biological characteristics are promising agents for cancer prevention and treatment (Barnes, 2001; Surh, 2003).

Erythrophleum succirubrum Gagnep. belongs to the family Fabaceae. Members of this genus are tall, deciduous tropical trees, occurring in Africa (*E. ivorense, E. suaveolens, E. africanum*), North-East Asia (*E. fordii, E. succirubrum, E. densiflorum*), Australia (*E. chlorostachys*) and Madagascar (*E. couminga*) (Jerome et al., 2013). Several diterpenoids, triterpenoids and alkaloids have been isolated from the genus *Erythrophleum*, and many studies have been published on the biological effects of the alkaloids found in this genus (Cronlund, 1973; Verotta et al., 1995; Qu et al., 2006). Previous studies have revealed that various *Erythrophleum* species, including *E. guineensis*, *E. ivorense*, *E. lasicanthum*, *E. chlorostachys* and

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E. africanum are harmful to cattle and humans and exhibit antitermitic, antibacterial and antifungal properties (Irvine, 1961; Watt and Bayer-Brnadwyle, 1962; Adeoye et al., 2004; Antwi-Boasiako and Damoah, 2010). In this regard, cassaine diterpenoid amines, amides and oleanane-type triterpene saponins have been identified as major compounds produced by the genus *Erythrophleum* and are cytotoxic to many tumor cell lines (Griffin et al., 1971; Cronlund and Sandberg, 1976; Qu et al., 2006; Du et al., 2011). In Thai traditional medicines *E. succirubrum* (local name: Phan-Saat) has been used to relieve fever and skin diseases (Echeverria et al., 1986; Weerapreeyakul et al., 2016).

To the best of our knowledge, there is no information available on the chemical composition and cytotoxic properties of *E. succirubrum* leaf extract. In This study, we investigated the potential cytotoxic effects of *E. succirubrum* leaf extract on a human cholangiocarcinoma cell line and analyzed the chemical components of *E. succirubrum* ethanolic leaf extract using gas chromatography coupled with mass spectrophotometer (GC/MS).

Materials and Methods

Plant Material

E. succirubrum was collected from Chai Nat Province, Thailand, in September 2017 and was identified and authenticated by botanists from the Queen Sirikit Botanic Garden. A voucher specimen registered with the number WP7683 was deposited at the Queen Sirikit Botanic Garden Herbarium, Ministry of Natural Resource and Environment, Bangkok, Thailand.

Preparation of crude extract

The dried leaves of *E. succirubrum* (1,000 g) were ground into powder and then extracted for three days at room temperature with 95% ethanol (12 L). The solvent was evaporated under reduced pressure at 40 – 50 ^{o}C to produce dry crude extract. The yield was calculated according to a method published by Maizura, et al. (2011). The crude ethanolic extract was weighed and refrigerated at 4^{o}C until chemical analysis was performed and the cytotoxic activity was determined.

Cell viability assay

The human cholangiocarcinoma cell line KKU-M213 (JCRB 1557) was obtained from The Japanese Collection of Research Bioresources Cell Bank (JCRB; Osaka, Japan). The KKU-M213 cell line was cultured in Ham/F12 cell culture medium containing 10% Fetal Bovine Serum (FBS), 15 mmol/L HEPES, 100 U/mL Penicillin G Sodium, 100 µg/mL streptomycin sulfate and 0.25 µg/mL amphotericin B. The cell culture was maintained at 37°C under a humid atmosphere with 50 mL/L CO_2.

The ethanolic leaf extract of *E. succirubrum* was evaluated for cytotoxicity against the KKU-M213 cell line using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. KKU-M213 cells were plated at a concentration of 7000 cells per well on a 96-well culture plate. Cell cultures were then incubated at 37°C for 24 hours in 5% CO_2. Following the incubation period, the growth medium was aspirated. The stock solution of *E. succirubrum* crude extract with DMSO was dropped in a growth medium to generate working concentrations of 0, 10, 50, 100 and 200 µg/ml respectively. Gemcitabine (Gemita®, Fresenius Kabi Oncology Ltd., India) was continuously supplied in parallel with *E. succirubrum* crude extract. The cultures were incubated for a further 24 and 96 h. The medium was then replaced with a growth medium containing 0.5 mg/ml MTT and cultured for an additional 4 h. Viable cell converted MTT to formazan crystals, which were then dissolved in DMSO. The quantity of formazan was measured spectrophotometrically at 540 nm using a Tecan Sunrise absorbance reader equipped with the Magellan data software (Tecan Austria GmbH: Austria). The cell viability was calculated using equation:

$$\% \text{ Viability} = [A_{treated} / A_{control}] \times 100$$

where $A_{treated}$ is the absorbance of cells treated with various concentrations of crude extracts and $A_{control}$ is the absorbance of untreated control cells. All experiments were carried out in triplicated, and the data were analyzed for three independent experiments. The half-maximal concentration (IC_{50}) and the nonlinear regression log [inhibitor] versus response (variable slope) were calculated using the GraphPad Prism version 8.0.1 software.

Gas Chromatography - Mass Spectrometry (GC/MS)

The chemical composition of *E. succirubrum* leaf extract was analyzed using GC/MS at the Central Instrument Facility, Faculty of Science, Mahidol University, using an Agilent7890B GC System equipped with an Agilent HP-5MS UI capillary column (30 m length x 0.25 mm I.D. and 0.25 µm film thickness). The temperature of the column was first set to 80°C for 6 min, increased by 5°C/min to 200°C and then by 20°C/min to 280°C. The isothermal temperature was kept at 280°C for 10 min. Helium was used as the carrier gas at a flow rate of 1 mL/min. An ethanolic crude extract of *E. succirubrum* was prepared at a concentration of 0.1% (w/v). The injection volume was 2 µl, split at a ratio of 250:1, and the injector temperature was 250°C. Mass spectra quantity was operated at 70 eV. A scan period of 0.5 seconds was used, with fragments ranging from 45 to 450 Da. The overall running time for the GC was 46 min.

Identification of composition

The chemical compositions were identified by comparing of their mass spectrum fragmentation patterns with those of the National Institute of Standards and Technology (NIST) and Wiley GC/MS libraries. The PubChem database and Classy Fire batch (Djoumbou et al., 2016) were used to classify the chemical taxonomy of the components. The proportions of the chemicals identified were computed using a total ion chromatogram (Adams, 2007).

Statistical analysis

The experimental data were analyzed using SPSS 17 for Windows software (SPSS Inc; IBM Corp.; Armonk,

NY, USA) using one-way analysis of variance and independent samples t test. The results were presented as means± standard error of the mean and $p < 0.05$ was considered statistically significant.

Results

Preparation of E. succirubrum ethanolic extract

The ethanol-derived crude extract obtained following vacuum evaporation from *E. succirubrum* leaves was sticky, dark green in color and odorless, with a yield of 14.04 % w/w.

Cell viability assay

The cytotoxic effects of *E. succirubrum* leaf extract on CCA cells were assessed using MTT assays. KKU-M213 cells were treated with 0, 10, 50, 100, 200 µg/mL of the extract for 24 or 96 h. The ethanolic leaf extract of *E. succirubrum* significantly reduced the viability of KKU-M213 cells in a dose- and time-dependent manner. *Erythrophleum succirubrum* extract exhibited significantly higher effectiveness than the standard control, gemcitabine, after both 24 and 96 h of exposure, particularly at concentrations of 50, 100 and 200 µg/mL (Tables 1 and 2). The principal reduction of cell viability of both *E. succirubrum* extract and gemcitabine began at 10 µg/mL and persisted at higher concentrations. *Erythrophleum succirubrum* extract, had a more significant effect than gemcitabine (Figures 1 and 2). The IC_{50} value of *E. succirubrum* was determined with respect to its suppression of the viability of KKU-M213 cells. The plant extract had a high level of cytotoxicity. After exposure intervals of 24 and 96 h, the IC_{50} values were 65.22 ± 1.18 µg/mL and 1.19 ± 1.38 µg/mL, respectively. *Erythrophleum succirubrum* extract was shown to be more effective than gemcitabine at inhibiting the growth of KKU-M213 cells.

Chemical composition of E. succirubrum ethanolic extract

The GC/MS analysis of *E. succirubrum* ethanolic extract resulted in the identification of 22 phytochemical compounds (Table 3), and the spectrum is shown in Figure 3. The chemical constituents identified included lipids (42.05%), carbohydrates (28.95%), benzenoids (16.57%), phenylpropanoids and polyketides (5.84%), organoheterocyclic chemicals (3.95%), and other compounds (2.64%). Cyclohexanone, 2-[2-nitro-1-(2-naphthyl)ethyl]- had the highest peak area (14.79%) followed by allomycin (14.65%), mome inositol (14.30%), campesterol (11.80%) and ethyl linolenate (10.83%).

Discussion

Members of the genus *Erythrophleum* produce various classes of substances, including alkaloids, terpenoids, phytosterols, saponins, flavonoids, and their derivative

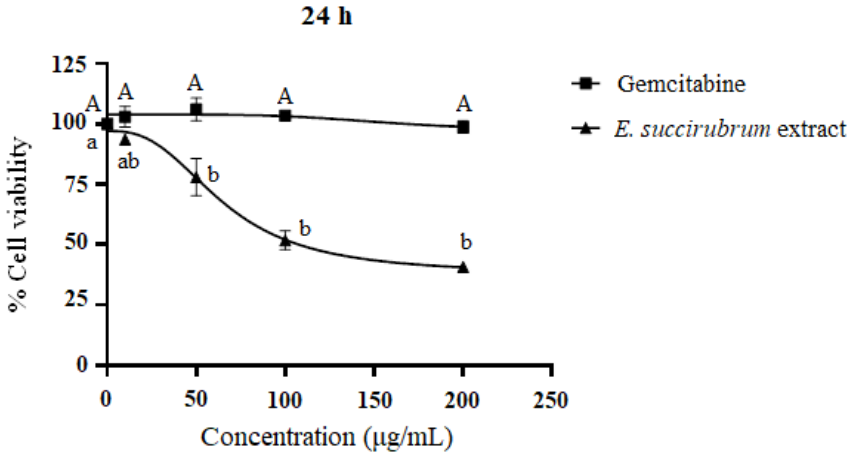

Figure 1. Cytotoxic Effect of Ethanolic Leaf Extract of *E. succirubrum* against Cholangiocarcinoma Cell Line after 24 h Incubation Time. Mean values with different lowercase superscripts within each horizontal row express significant (p<0.05) difference between groups of *E. succirubrum* extract. Mean values with different uppercase superscripts within each horizontal row express significant (p<0.05) difference between groups of gemcitabine.

Table 1. The Percentage of Cell Viability of KKU-M213 Treated with *E. succirubrum* leaf Extract and Gemcitabine at 24 h Incubation Time

Sample	% viability				
	concentration of extract (µg/mL)				
	0	10	50	100	200
E. succirubrum	100 [a]	93.62±2.12 [a]	77.96±9.40 [a]	51.73±4.84 [a]	40.75±2.47 [a]
gemcitabine	100 [a]	100 a	100 [b]	100 [b]	98.76±2.63 [b]

Results are representing the average of three separate experiments. Mean values with different lowercase superscripts within each column express significant (p<0.05) differences between groups.

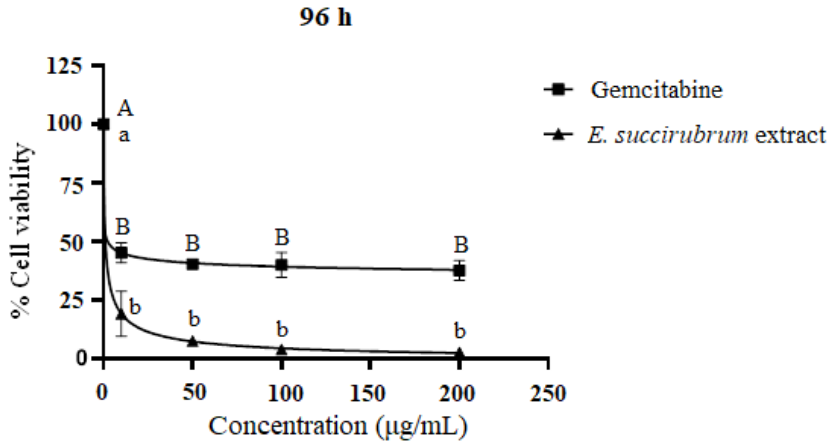

Figure 2. Cytotoxic Effect of Ethanolic Leaf Extract of *E. succirubrum* against Cholangiocarcinoma Cell Line after 96 h Incubation Time. Mean values with different lowercase superscripts within each horizontal row express significant (p<0.05) difference between groups of *E. succirubrum* extract. Mean values with different uppercase superscripts within each horizontal row express significant (p<0.05) difference between groups of gemcitabine.

Table 2. The Percentage of Cell Viability of KKU-M213 Treated with *E. succirubrum* Leaf Extract and Gemcitabine at 96 h Incubation Time

Sample	% viability				
	concentration of extract (µg/mL)				
	0	10	50	100	200
E. succirubrum	100 [a]	19.12±11.81 [a]	7.54±1.52 [a]	4.15±1.45 [a]	3.07±1.25 [a]
gemcitabine	100 [a]	45.14±4.25 [a]	40.30±1.88 [b]	39.90±5.32 [b]	37.53±4.24 [b]

Results are representing the average of three separate experiments. Mean values with different lowercase superscripts within each column express significant (p<0.05) differences between groups.

glycosides. The amount of each compound depends on the collection time, geographic factor, climatic environment, organ, course heredity and other factors. Alkaloids and terpenoids were predominant among the 140 compounds found in the genus *Erythrophleum*. Additionally, *Erythrophleum* species have become more significant in drug development, due to their economic potential (Son, 2019). It is therefore essential to analyze the majority of chemicals identified from *E. succirubrum* leaf extract, as well as their biological activity. Several diterpenoids and sesquiterpenoids classified as lipids were detected among the 22 chemicals in our study. The bark, root, leaves, and seeds of *Erythrophleum* contained alkaloids that produced digitoxin-like poisoning effects (Echeverria et al., 1986). There was a report that it was isolated in *E. succirubrum* bark, which led to the isolation of several alkaloids including Erythrophlesin A, B, C, and D, but there are no reports that it was identified in the leaf (Miyagawa et al., 2009; Du et al., 2011). However, Sattayasia et al., (1983) reported that the toxicity of *E. succirubrum* leaf extract was toxic to experimental animals. Aqueous extracts of the leaves were provided intraperitoneally to mice, causing them to become tremulous, sluggish and had breathing problems. The intravenous injection of a leaf extract to anesthetic rats resulted in an increase in blood pressure and a reduction in heart rate.

The cytotoxic activity of *E. succirubrum* leaf extract against the KKU-M213 cell line was measured using MTT assays. The viability of KKU-M213 cells was inhibited in a dose- and time-dependent manner by the ethanolic leaf extract of *E. succirubrum*. In comparison with gemcitabine, one of the most frequently used medications for the treatment of CCA (Dokduang et al., 2010), we found that *E. succirubrum* leaf extract had a stronger inhibitory effect on CCA. Furthermore, the results indicated potent cytotoxicity, with cell viability of less than 50% after 96 h exposure at a concentration of 1.19 ± 1.38 µg/mL which was comparable to the IC_{50} of gemcitabine of 10.10 ± 0.05 µg/mL (Chaiyong et al., 2021). Piperine-free *Piper nigrum* L. extract, exerted a cytotoxic effect against KKU-M213 cells, with an IC_{50} value of 13.70 ± 1.14 µg/mL (Tedasen et al., 2020). Maungchanburi et al., (2022) reported that fraction DE15 from dichloromethane extract of *Piper cubeba* L. seed had cytotoxic activity against cholangiocarcinoma cell line KKU-M213 with an IC_{50} value of 6.76 ± 0.52 µg/mL. While Janeklang et al., (2014) found that tiliacorinine had an antiproliferative effect on human cholangiocarcinoma cell lines, with IC_{50} values ranging from 2.59 to 7.00 µg/mL. Compared with the results of Janeklang et al., (2014), Tedasen et al., (2020) and Maungchanburi et al., (2022), the *E. succirubrum* leaf extract was effective against CCA. The information provided by the US National Cancer Institute Plant Screening Program is pertinent, as it indicates that a crude

Table 3. Chemical Compositions of *E. succirubrum* Ethanolic Extract

RT (minutes)	Compounds	Peak Area (%)	Classification of compound
14.67	2-(1-hydroxyethyl)-2-methyl-1,3-oxathiolane	1.19	Miscellaneous
23.15	Trisilane	1.45	Miscellaneous
24.22	Mome inositol	14.3	Carbohydrates (Polysaccharides)
24.33	Allomycin	14.65	Carbohydrates (Aminosaccharides)
27.38	1-cyano-7-methoxy-6-methyl-5,8-isoquinolinedione	0.85	Organoheterocyclic compounds (Isoquinoline quinones)
27.44	5,9-diamino-2,4-dimethyl-7H-pyrazolo[3,4-h][1,6]naphthyridine	1.42	Organoheterocyclic compounds (Pyrazolylpyridines)
28.17	Neophytadiene	1.2	Lipids (Sesquiterpenoids)
31.21	Ethyl palmitate	5.75	Lipids (Fatty acid esters)
33.412	Phytol isomer	3.95	Lipids (Acyclic diterpenoids)
34.29	Ethyl linoleate	2.03	Lipids (Lineolic acids and derivatives)
34.41	Ethyl linolenate	10.83	Lipids (Lineolic acids and derivatives)
34.85	Ethyl stearate	2.21	Lipids (Fatty acid esters)
37.04	2-(2-Furyl)-5,6-dimethoxy-3-methylindan-1-one	1.78	Benzenoids (Indanones)
39.9	2-amino-α-[2-chlorophenyl]cinnamic acid	4.69	Phenylpropanoids and polyketides (Stilbenes)
40.32	2-methyl-Z-7,8-epoxyhexadecane	1.02	Organoheterocyclic compounds (Epoxides)
40.9	4-methoxy-2,5-bis(2-phenylethyl)phenol	1.15	Phenylpropanoids and polyketides (Stilbenes)
41.88	5,16-androstadien-3β-ol	1.06	Lipids (Androstane steroids)
41.96	8β,13β-kaur-16-ene	1.78	Lipids (Diterpenoids)
42.97	Cyclohexanone, 2-[2-nitro-1-(2-naphthyl)ethyl]-	14.79	Benzenoids (Naphthalenes)
44.65	2,6,10,14,18-pentamethyl-2,6,10,14,18-eicosapentaene	1.44	Lipids (Sesquiterpenoids)
45.39	Campesterol	11.8	Lipids (Ergostane steroids)
45.65	Azetidin-2-one 3,3-dimethyl-4-(1-aminoethyl)-	0.66	Organoheterocyclic compounds (Beta lactams)
	Benzenoids		16.57
	Carbohydrates		28.95
	Lipids		42.05
	Organoheterocyclic compounds		3.95
	Phenylpropanoids and polyketides		5.84
	Miscellaneous		2.64
	Total		100

RT, Retention time

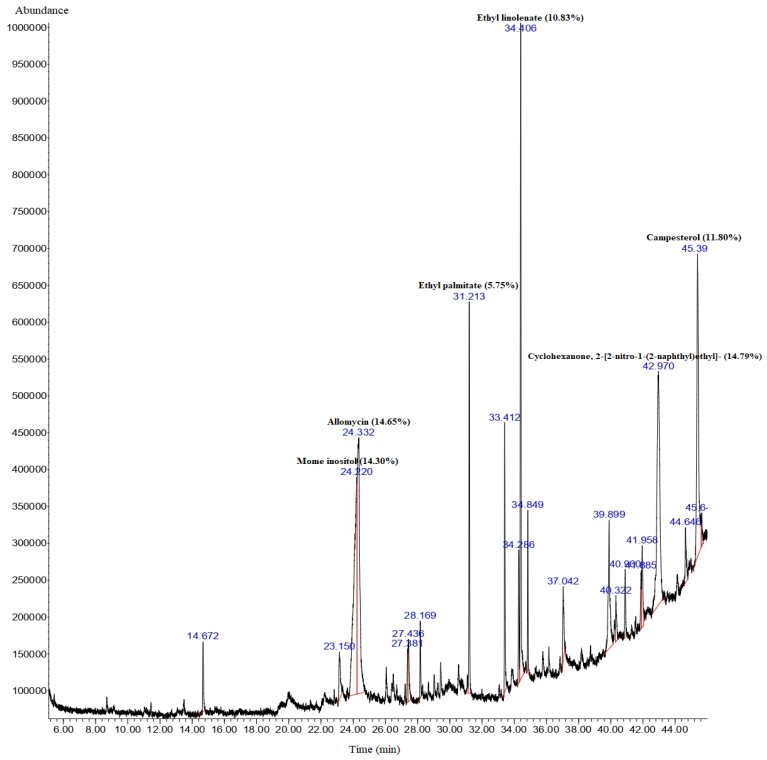

Figure 3. GC/MS Chromatogram of Ethanolic Leaf Extract of *E. succirubrum*

extract is generally examined for in vitro cytotoxic activity if the IC_{50} value in the carcinoma cells 48 – 72 h after incubation is less than 20 µg/mL, and for pure compounds when the IC_{50} is less than 4 µg/mL (Boik, 2001). Even though the exposure time in this experiment was 96 h, our finding might imply that *E. succirubrum* may have strong cytotoxic activity against CCA.

However, the biological activity of some components identified in *E. succirubrum* leaf extract should not be neglected since they may be directly responsible for the plant's properties. Dr. Duke's phytochemical and ethnobotanical databases, for instance, revealed that ethyl linolenate and ethyl linoleate, both fatty acid substances, have anticancer activity (Duke, 2002; Prakash et al., 2020). Mome inositol, a polysaccharide, is antiproliferative in MCF-7, MDA-MB-231, HepG2 and Hs27 cell lines (Mathi et al., 2015). Additionally, Neda et al. (2013) reported the anticancer activity of mome inositol present in *Clitoria ternatea* flowers. Furthermore, the anticancer effect of campesterol, an ergostane steroid, has been frequently reported in recent years. Campesterol may have inhibited the growth of leukemia, hepatocarcinoma, and prostate cancer cells. (Awad et al., 2001; Chuu et al., 2007; O'Callaghan et al., 2013). In similar research, campesterol was found to suppress cell proliferation, cell cycle progression and cell aggregation in ovarian cancer cells (Bae et al., 2021). *E. succirubrum* may be a potentially valuable addition to this wealth of knowledge, which constitute the basis for developing innovative natural medications. This finding could provide a new source for isolating and identifying bioactive molecules for pharmaceutical applications. Therefore, further study into the isolation of bioactive components and their bioactivities is needed.

Author Contribution Statement

S. Chaiyong: contribution to the research as research executive, experimental establishment and in charge of determination of plant specimen for taxonomy and plant preparative for biological and phytochemical study. N. Sutthanont: contribution to the identification of phytochemical constituent of *E. succirubrum*. A. Menakongka: contribution to the investigation of plant effective on human carcinoma cell. All authors reviewed the results and approved the final version of the manuscript.

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Ethical approval

This research was reviewed and approved by the Institutional Review Board of the Faculty of Medicine Vajira Hospital with the International guidelines for human research protection such as Declaration of Helsinki, The Belmont Report, CIOMs Guideline and International Conference on Harmonization in Good Clinical Practice (ICH-GCP) (Approval number: COE_NOT HS 9/2017).

Availability of data

The data that used during the current study are available from corresponding author on reasonable request.

Conflict of interest

The authors declare that there are no conflicts of interest.

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