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The Potential Application of *Clitoria ternatea* for Cancer Treatment

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ABSTRACT

The *Clitoria ternatea* flower, known as *bunga telang* in Indonesia, is commonly mixed with food and beverages to provide a natural blue colour. Aside from its popular culinary use, it is a traditional medicine in Indonesia for diseases in the eyes, urinary tract and skin, as well as functioning as an anti-toxin. Furthermore, recent advances in science and technology have revealed that the *C. ternatea* flower contains a high level of polyphenol compounds that possess anticancer activity, including saponins, tannins, steroids, triterpenoids, kaempferol, and quercetin. This review aims to identify and analyse recent articles regarding the phytochemical activities of *C. ternatea* flower extract as an anticancer agent. The literature on main databases from 2011 to 2021 was searched systematically using the keywords "Anticancer activity of *Clitorea ternatea*" and "Phytochemical activities of *Clitorea ternatea* flower extract against cancer cells". The various extracts of *C. ternatea* flower display a moderate cytotoxic, IC_{50} = 21 µg/mL - 200 µg/mL, for many cancer cell lines, such as MCF-7, MDA-MB-231, CaoV-3, HEp-G2 in aquadest extract and the DLA cell line in petroleum ether extract. The bioactive compounds responsible for the anticancer effect include ternatins, delphinidin, kaempferol, quercetin, sitosterol, and tocopherols. In addition, there have been no reports of any toxic effect on normal cells (Hs27) and oral consumption in mice. According to many studies, the extract is active on multi-molecular targets, with the most conclusive effect on polymerase enzymes, whose inhibition can be an important therapeutic strategy to treat hyperproliferation in cell cancer. Therefore, the findings suggest a potential application of *C. ternatea* for cancer treatment.

Keywords: *Clitoria ternatea flower; phytochemical activity; anticancer activity; multi-molecular targets*

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INTRODUCTION

Cancer-related diseases result in a significant mortality rate globally. According to the American Cancer Society, cancer deaths are increasing gradually by 2-3% yearly (Islami et al., 2018). The Global Burden of Cancer (GLOBOCAN) predicts 20 million new cases globally by 2025, a forecast supported by the World Health Organization (WHO), which has stated that the highest number of cases will be in Asia (Rumgay et al., 2022). In Indonesia, cancer cases rose from 348,809 in 2019 to 396,914 in 2020 (GLOBOCAN, 2020). The highest incidence was breast cancer in women, constituting 30.8%, and lung cancer in men, accounting for 14.1%, followed by cervix uteri, colorectal and liver cancers, at 9.2%, 8.6% and 5.4%, respectively, with similar mortality rates in both sexes. However, cases are projected to rise by 50% and 80% by 2040 for breast and lung cancer respectively (WHO, 2020).

Although cancer is the leading cause of morbidity in the world, considerable progress and research efforts have been made to handle cases, reduce premature deaths, and increase patients' lifespan. First, conventional methods, such as chemotherapy, can eliminate and inhibit the proliferation of cancer cells. The side effects of the cytotoxic drug include its impact on the rapidly-divided healthy cells in the patient's body and weakening of the immune system, hence increasing cancer cell metastasis (Islami et al., 2018). Recently, newer anticancer agents have been developed for drug targeted-therapy, known as cytostatic drugs, which are used during chemotherapy based on the epigenetic characteristics of patients. However, drug resistance often occurs in both therapies due to the mutation of target cells (Gore et al., 2013; Ladislau et al., 2013).

Appropriate treatment must be curative, palliative and preventive (Hasanah et al., 2016). Interestingly, the discovery of herbal agents has suggested an alternative solution, as they produce fewer side effects and show similar effectiveness to synthetic medicines (Irawan et al., 2017). According to WHO, 80% of the world's population use traditional medicine, including for cancer treatment. Several natural compounds isolated from traditional medicinal plants have anticancer effects, such as cytotoxic, proliferation and invasive inhibition, angiogenesis suppression, and chemotherapy enhancement (Jacob & Latha, 2013; Salleh et al., 2013).

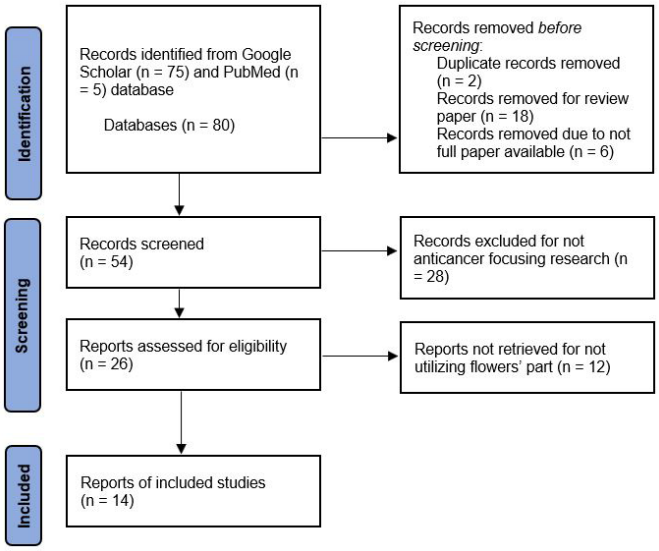


Figure 1. PRISMA flow chart for study selection

Clitoria ternatea flowers are widely recognised as displaying anticancer activity. The vine, known as *bunga telang*, is a member of the Fabaceae family and is widespread in several countries due to its high resistance to various environmental conditions (Purba, 2020). The plant has distinctive flowers with light blue, dark blue, or white petals, and is 4 cm in length and 3 cm wide (Lakshan et al., 2019; Ezzudin & Rabeta, 2018). The brightly coloured flower is also used as a colouring agent in food and beverages. In West Kalimantan, Bali and Central Sulawesi, the primary function of the flower is for decoration and traditional ceremonial usage for the people of Kapuas, Central Kalimantan (Defiani & Kriswiyanti, 2019; Haryanti & Diba, 2015; Sapti, 2019).

In addition, the *C. ternatea* flower has been used as a traditional medicine in various tribes and countries as an anti-inflammatory, analgesic, and antidiabetic agent (Das et al., 2020). Hence, due to its habitual consumption, people accept it as an anticancer supplement or food substitute.

Several recent studies have reported on the phytochemical properties (Ponnusamy et al., 2015; Shen et al., 2016) and activities of *C. ternatea* flower extract (Iamsaard et al., 2014; Lakshan et al., 2019), especially as an anticancer agent, based on in vitro and in vivo studies. However, an in-depth comprehensive systematic review has not been conducted to examine the use of *C. ternatea* flower extract in cancer treatment. This study aims to fill the gaps in the recent literature and contribute further advanced drug development research. Therefore, the study will review the evidence base of specific phytochemical properties of *C. ternatea* flower extract and their anticancer activities in relation to types of extraction methods that produce the prominent cytotoxic effect and toxicity levels.

METHODS

Literature Search Strategy

The research used primary data sources from related articles compiled through the online databases of Google Scholar and PubMed, which were published in English from 2011 to 2021, and using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. Related articles were collected and reviewed using the keywords "Anticancer activity of *Clitorea ternatea*" and "Phytochemical activities of *Clitorea ternatea* flower extract against cancer cells". The search led to the identification of 75 articles from Google Scholar and five from PubMed, of which 26 were removed overall due to duplication (two articles), being review papers (18 articles), and full text not available (six papers). Titles and abstracts were screened from the 49 selected articles to generate a reference list, with 28 studies excluded for not focusing on anticancer research. After screening the 26 articles for eligibility, only 14 were deemed relevant and included in the study. Figure 1 shows the literature collection results and the inclusion and exclusion criteria in a systematic review chart.

RESULTS AND DISCUSSION

Anticancer Agents Derived from Bioactive Compounds of *C. ternatea* Flower Extract

C. ternatea flower extract (Figure 2) contains saponins, tannins, alkaloids, glycosides, and phytosterols (Gollen et al., 2018). Several studies have reported that the active compounds in the extract that inhibit cancer cells consist of anthocyanin, quercetin, flavonols, flavones, and vitamins (Table 1) (Dave et al., 2020; Jeyaraj et al., 2020; Ravishankar et al., 2013; Rizeq et al., 2020). Hence, we highlight the major compounds responsible for the anticancer effect.

(a) (b)

Figure 2. *Clitoria ternatea* (a) blue and white flowers, (b) Flower extract (Gollen et al., 2018; Rana et al., 2020)

Table 1. Active anticancer compounds in *C. ternatea* flower extract

No	Active Compound	Concentration
	Anthocyanin (Shen et al., 2016)	
1	Delphinidin derivative	0.28 ± 0.01 mg/g FW
2	Ternatin A1	0.51 ±0.03 mg/g FW
3	Ternatin B3	0.50 ± 0.03 mg/g FW
4	Ternatin D3	0.54 ± 0.01 mg/g FW
5	Ternatin B2	0.32 ± 0.01 mg/g FW
6	Ternatin C2	1.81 ± 0.09 mg/g FW
7	Ternatin D2	1.45 ± 0.07 mg/g FW
	Flavanol glycosides (Shen et al., 2016)	
8	Kaempferol	1.76 ± 0.05 mg/g FW
	Flavonoid (Shen et al., 2016)	
9	Quercetin	0.37 ± 0.01 mg/g FW
	Phytosterol (Shen et al., 2016)	
	Campesterol	1.24 ± 0.02 mg/100 g FW
	Stigmasterol	6.70 ± 0.83 mg/100 g FW
	β – sitosterol	6.77 ± 0.19 mg/100 g FW
	Tocols (Shen et al., 2016)	
	α – tocopherol	0.20 ± 0.01 mg/100 g FW
	γ – tocopherol	0.24 ± 0.02 mg/100 g FW
	Vitamins (Salleh et al., 2013)	
10	Inositol	38.7%
11	Pentanal	14.3%

Data were measured as milligrams per 100 grams of fresh weight (mg/100 g FW) sample

Ternatin

Several types of ternatins have been detected in blooming flowers, including A1-A3, B1-B4, C1-C5, and D1-D3, consisting of 3,3',5'-triglucoside delphinidin, which binds to malonic acid, glucose, p-coumaric acid, or caffeic acid. This compound is responsible for the bright blue colour (Shen et al., 2016), so is only found in blue and purple flowers, as seen in Figure 2 (Al-Snafi, 2016), including *Delphinium hybridum, Eustoma grandiflora, Gentiana trifloral* (Okitsu et al., 2018), *Rosa,* and *Chrysanthemum* (Noda et al., 2017). According to Shen et al., the concentration of ternatins in *C. ternatea* flower extract is 0.51 ±0.03, 0.50 ± 0.03, 0.54 ± 0.01, 0.32 ± 0.01, 1.81 ± 0.09, 1.45 ± 0.07, and 0.28 ± 0.01 mg/g FW in relation to ternatin A1, B3, D3, B2, C2, D2, and other delphinidin derivatives, respectively.

Ternatin is produced in various forms through biosynthesis, with UDP-glucose and anthocyanin 3'5'-O-glucosyltransferase as the main enzyme (Figure 3). The enzyme binds to delphinidin 3-O-(6''-O-malonyl)- β-glucoside and converts it to delphinidin 3-O-(6''-O-malonyl)-β-glucoside-3'-O-β-glucoside or C5, which is the simplest ternatin. Furthermore, the binding between the enzyme and substrate results in the variation in ternatin structures, as the B-ring of anthocyanin rotates around C2 – C1 as the bifunctional enzyme. Finally, p-coumaroyl and glucosyl groups are added in the 3' and 5' side chain of ternatin to produce a different structure that distinguishes various types (Noda et al., 2017; Vidana Gamage et., 2021). The addition of different amounts and positions of *p*-coumaroyl (C) and glucosyl (G) at the 3' and 5' sidechains, marked by blue and orange respectively, as illustrated in Figure 4, results in distinct types of ternatin. The final compound of the biosynthesis is ternatin A1 with the addition of 4C and 4G, while the others are intermediate products (Oguis et al., 2019).

Ternatin is a distinct flavonoid compound in *C. ternatea* flowers with anticancer properties due to its anthocyanin derivatives. In addition, it has a similar antioxidant effect as flavonoids, which donate hydrogen to radicals and halt chain reactions (Marpaung, 2020). A previous study showed that ternatin and cyanidin glycosides reduce cell viability in the laryngeal cancer cell (Hep-2) by inhibiting adipogenesis and promoting the anti-proliferation effect (Shen et al., 2016). Ternatin binds with eukaryotic translation elongation factors 1 alpha (eEF1A) in melanoma cell lines and suppresses cell proliferation by trapping eEF1A in the ribosome (Carelli et al., 2015; Sanchez-Murcia et al., 2017). Furthermore, eEF1A is translation factors that are highly expressed in human tumors and cause the cancer cell to grow, including in breast, ovarian, and lung cancers (Abbas et al., 2015). Based on the docking analysis by Fan and Sharp (2021), ternatin obtained from *C. ternatea* has a significant antiproliferative potency.

Kaempferol

Kaempferol is an active flavonoid compound widely investigated in many medicinal herb and natural plant diet programs. Additionally, it is soluble in hot ethanol and has hydrophobic properties due to its diphenyl propane structure. Figure 5 shows that kaempferol synthesises under chalcone synthase to produce naringenin chalcone. Furthermore, naringenin is converted into dihydrokaempferol by the catalyst flavanone 3β-hydroxylase (F3H) and then transformed into kaempferol by flavonol synthase (FLS) (Markovic et al., 2014). Shen et al. (2016) identified the concentration of kaempferol in *C. ternatea* flower extract to be 1.76 ± 0.05 mg/g FW.

Kaempferol has been reported to have a strong antioxidant and anti-inflammatory effect on cancer cells, inducing apoptosis (Chen & Chen 2013), including

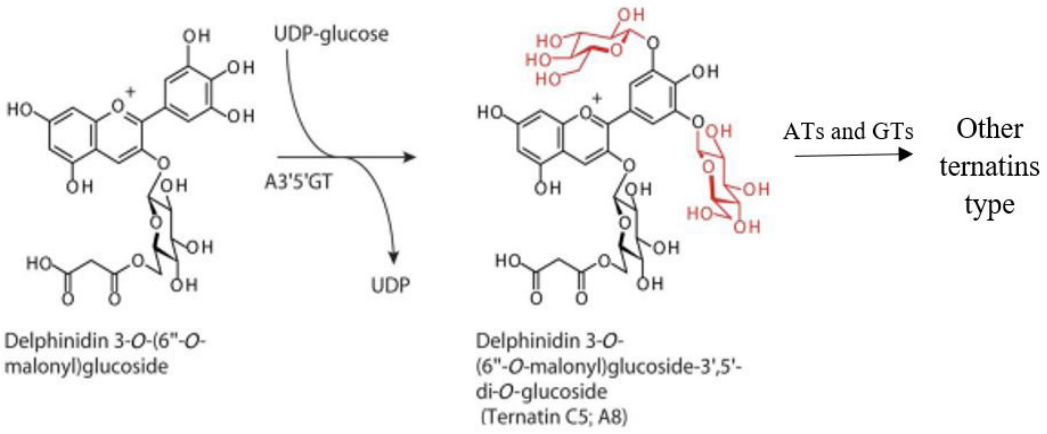


Figure 3. The enzyme binds with substrate converted to ternatin C5 or other types of ternatin (Noda et al., 2017)

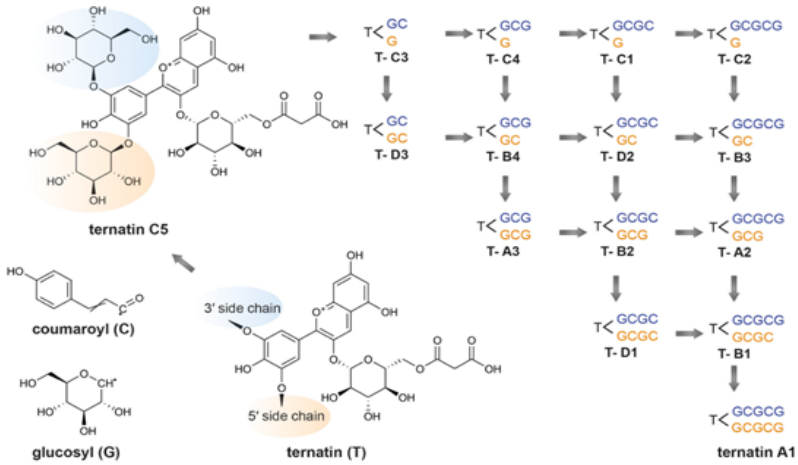


Figure 4. The differences of ternatin type biosynthetic pathways (Oguis et al., 2019)

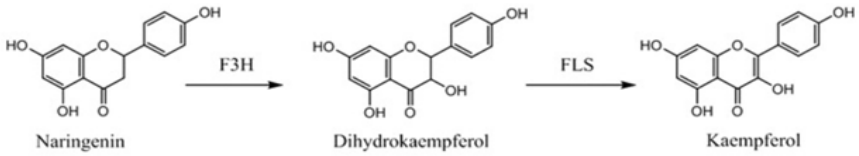


Figure 5. Biosynthetic of kaempferol (Duan et al., 2017)

in esophageal, breast, cervix, ovary, lung, leukemia, pancreas, bladder, bone, brain, colon, kidney, liver, prostate, and osteosarcoma cancer cells. This compound exerts its anticancer activity by enhancing the host's immunity, preventing drug resistance and angiogenesis, and the anti-proliferation effect (Duan et al., 2017; Gao et al., 2018; Imran et al., 2019; Wang et al., 2019).

Quercetin

Chromatographic layer analysis has shown that *C. ternatea* flower extract has a similar Rf value of 0.57 to the quercetin standard (Asysyifa et al., 2020), with the concentration in the extract being 0.37 ± 0.01 mg/g FW (Shen et al., 2016). Furthermore, the phenolic hydroxyl group with double bonds and the keto carbonyl group are the active groups of quercetin, which give it strong antioxidant and anti-inflammatory properties which can help prevent cancer development.

Compared to other flavonoids, several enzymatic complexes on cytosolic that are present on endoplasmic reticulum membranes synthesize quercetin, as shown in Figure 6. Several heterocycles C were first produced, with naringenin as a general product and primary precursor for producing all flavanols, including quercetin (Chouhan et al., 2017; Marín et al., 2018). However, the complex pathways of quercetin synthesis produce kaempferol and dihydrokaempferol as the substrate for flavonoid 3'-hydroxylase (F3'H). The kaempferol is converted into myricetin, then the dihydrokaempferol into dihydroquercetin (taxifolin), before it is finally transformed into quercetin (Crozier et al., 2009).

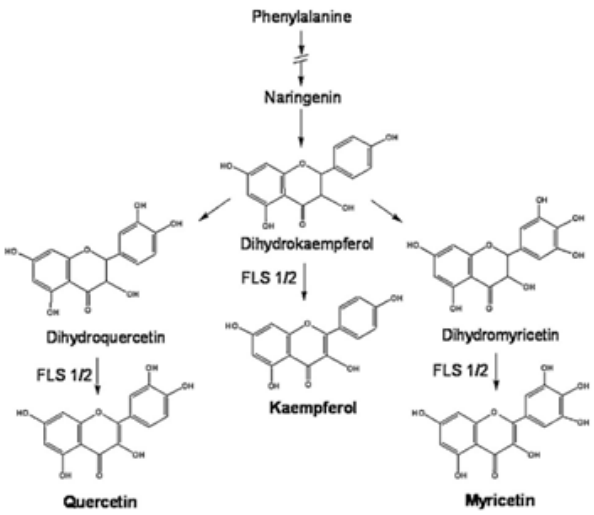


Figure 6. Biosynthetic of quercetin (Li et al., 2013)

Quercetin is widely used for preventing breast cancer metastases by suppressing nuclear factor kappa B (NF-kB). This transcription factor is vital for the metastasis of Human Epidermal Growth Factor Receptor 2 (HER2-positive) in breast cancer cells. Although the transcriptional pathway of the metastatic inhibitory activity of the extract has not been clearly understood, MTT assay has been used in recent studies to discover its prominent anti-proliferation effect on cancer cells, such

as the hormone-dependent breast cancer cell line (MCF-7), non-hormone-dependent breast cancer cell line (MDA-MB-231), human ovarian cancer cell line (Caov-3), human cervical cancer cell line (Hela), human liver cancer cell line (HepG2) and human foreskin fibroblast cell line (Hs27) (Shyam Kumar & Bhat, 2011; Srinivasa Balaji & Shivaprakash, 2016).

Phytosterol

Campesterol, β-sitosterol and stigmasterol are polyester derivatives that prevent the production of carcinogenic compounds from cancer cells by inhibiting the cell metabolism system. These compounds are classified as the main phytosterol, unsaturated molecules containing a core cholesterol skeleton with different side chains. *C. ternatea* flower extract contains campesterol, stigmasterol and β-sitosterol at levels of 1.24 ± 0.02 mg/100 g FW, 6.70 ± 0.83 mg/100 g FW, and 6.77 ± 0.19 mg/100 g FW, respectively (Shen et al., 2016). Beta-sitosterol has an ethyl group at carbons24 on R; campesterol is equipped with a carbons24-methyl group bound with R; and stigmasterol has an additional of an ethyl group at carbons24 positions with unsaturation between carbons22 and 23 (Foley et al., 2011), as illustrated in Figure 7. Generally, phytosterols are not synthesised by humans. Therefore, a diet of plants or vegetables should be followed because they contain this compound (Salehi et al., 2021).

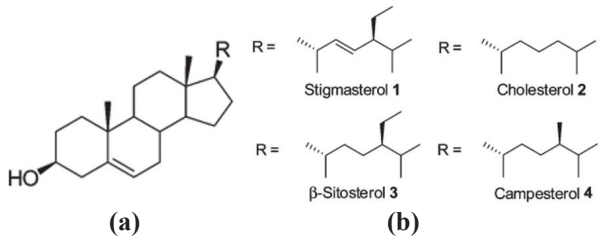


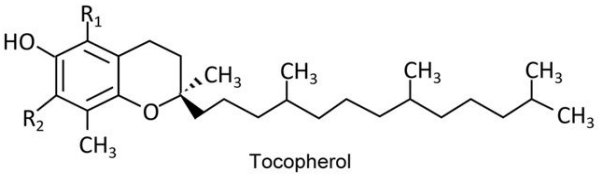
Figure 7. Structure of phytosterol (a) Parent structure as cholesterol skeleton; (b) Differences in the R-side with different derivates of phytosterol (Foley et al., 2011)

Based on in vivo study, campesterol promotes apoptosis in the human breast cancer cell line MDA-MB-231 and human monocyte cell growth (Shahzad et al., 2017). Likewise, clinical tests for the campesterol anticancer effect on esophageal cancer have also shown the same inhibition effect. Therefore, campesterol, with other phytosterols, inhibits cell growth and negates the overexpression of the cancer cell factor gene, which has an impact on the treatment of breast cancer, prostate cancer, and erythroleukemia (Llaverias et al., 2013; O'Callaghan et al., 2014; Shahzad et al., 2017).

According to evidence from vivo research, beta-sterol and stigmasterol as phytosterol display anticancer activities. Beta-sterol has been found to inhibit human tumor cell lines, such as the colon cancer cell line (HT116); human lung cancer cell line (A549); human hepatic cancer cell line (HepG2); and human prostate and human breast cancer cell lines MDA-MB-231 and MCF-7 (Kim et al., 2014). The anticancer activities have also shown downregulation of cell cycle progression, invasion and migration (Jiang et al., 2019). In addition, stigmasterol has also shown anticancer effects on various cancers, such as hepatoma, cholangiocarcinoma, gall bladder carcinoma, endometrial adenocarcinoma, and skin, gastric, breast, prostate and cervical cancer. The main role of stigmasterol anticancer activity has been shown by inhibited cell proliferation and ROS production (Mojarad et al., 2022). Moreover, in vivo studies have reported that cell migration and angiogenesis genes such as VEGFA, PLAU, MMP2, MMP9 and MMP14 expression were suppressed in human ovarian cancer cells (Bae et al., 2020), and proliferation genes, such as Akt/mTOR, were also impeded in the gastric cancer cell line (Zhao et al., 2021).

Tocopherol

Tocopherol is a vitamin E plant derivative containing a lipid-double substance that is differentiated into four types, α-, β-, γ- and δ-forms, based on the number and position of methyl groups on its chromanol ring (Figure 8). This substance has multiple functions, including antioxidant activity, enzyme regulation for proliferation and gene expression related to anticancer activity, prevention of neurological disorders, and induction of immune responses (Kannappan et al., 2012).



	α	β	γ	δ
R_1	CH_3	CH_3	H	H
R_2	CH_3	H	CH_3	H

Figure 8. Tocopherol structure and type (Zulkapli et al., 2017)

The forms of tocopherol in *C. ternatea* flower extract are α-tocopherol and γ-tocopherol, with concentrations of 0.20 ± 0.01 mg/100 g FW and 0.24 ± 0.02 mg/100 g FW respectively. α-tocopherol performs its anticancer activity by scavenging free radicals and inhibiting tumor angiogenesis (Abraham et al., 2019). In addition, γ- and δ tocopherol show antioxidant properties through the NF-kB pathway and enzyme reductase CoA, as well as by inducing an antiangiogenic effect (Abraham et al., 2019; Zulkapli et al., 2017).

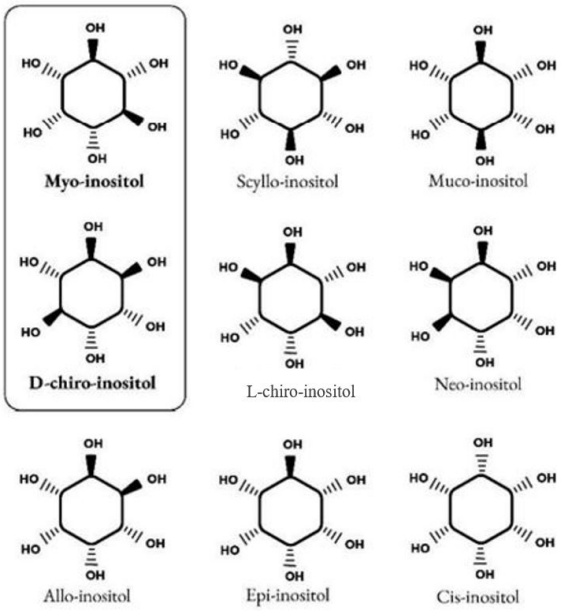


Figure 9. Structure of inositol and the isomers (Dinicola et al., 2021)

Vitamins

Inositol, as illustrated in Figure 9, is a major vitamin in *C. ternatea* flowers (38.7%), with a fairly effective inhibitory effect for cancer cells, especially when combined with phytic acid (Shen et al., 2016). The study of Shen et al. (2016) evaluated that inositol hexakisphosphate and myo-Ins enhance natural killer (NK) cell activity in mice treated with 1,2- dimethylhydrazine (DMH), as a model for colon carcinogen (Salleh et al., 2013; Woyengo et al., 2009). Another study by Tantivejkul et al. (2013) has also demonstrated that inositol hexaphosphate (IP6) significantly inhibits cell proliferation in breast cancer cell lines, such as an estrogen receptor (ER) α-positive (MCF-7), ERα-negative (MDA- MB 231) and adriamycin-resistant MCF-7 (MCF-7/ Adr), using a 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay. Although pentanal found in *C. ternatea* flower extract (14.3%) does not directly play a role in cancer or tumor cells, it has been identified at a high level in lung cancer patients. This shows that it has the potential as a biomarker of lung cancer (Müller-Wirtz et al., 2021), as well as breast and gastrointestinal cancers in humans (Kumar et al., 2015).

Others Compounds

Other phytochemicals identified in *C. ternatea* flowers with anticancer activity include scutellarin, apigenin, baicalein and luteolin, at concentrations of 36.9%, 6.3%, 12.6% and 9.3%, respectively. These compounds have been demonstrated to be able to inhibit the apoptotic signalling of leukemia cells (Liu et al., 2015). Several studies have shown that flavonoids and luteolin prevent the regulation of HIF-1α and molecular VEGF secretion (Samec et al., 2021).

Crude fibre and fat content in *C. ternatea* flowers have also been established as displaying anticancer activity. The results of proximate analysis on the extract show that the levels of crude fibre and fat content that effectively reduced breast cancer activity were 2.1 ± 0.2 mg/100 g FW and 2.5 ± 0.1 mg/100 g FW respectively (Salleh et al., 2013).

Anticancer Activities of *C. ternatea* Flower Extract

C. ternatea flower extract has been evaluated for anticancer activity on several types of cancers, including breast (MCF-7 and MDA-MB-231), ovary (CaoV- 3), cervix (Hela), liver (HEp-G2), skin (Hs27), lung (A549 and sub-row A549/paclitaxel), and on carcinoma cell lines, such as HEp-2, EAC, and DLA cell (Table 2). The anticancer activities of the extract include the induction of apoptosis factors, modulation of cell cycle regulation, inhibition of invasive cells, suppression of angiogenesis, and enhancement of chemotherapy.

Apoptosis-inducing factor

Extract of *C. ternatea* flowers has been identified to possess anthocyanin compounds, especially ternatin anthocyanins, which display anticancer activity. Ternatin in black raspberries has been reported to disrupt cyclooxygenase-2 (COX-2) in tumorigenesis (Oh et al., 2015). This enzyme is responsible for the first step of prostanoid synthesis, which involves inflammation and carcinogenesis. COX-2 promotes tissue invasion of cancer cells or tumors and apoptosis resistance (Liu et al., 2015). Related to ternatin in black raspberries, *C. ternatea* flower extract might display similar anticancer activity, since it contains various ternatin types (Dave et al., 2020).

Cell cycle regulation and metabolic reprogramming of cancer cells

C. ternatea flower with ethanolic extract has shown downregulation of the cell cycle at the pre-G0, G1, and S phases associated with apoptotic induction (Alshamrani et al., 2022). The cell cycle arrest by phenolic compounds that act individually or synergistically affects cell cycle regulation by deactivating key metabolism enzymes, such as pyruvate kinase isoenzyme M2 (PKM2), as the main control point enzyme in metabolic cells. In addition, phenolic compounds interfere with glucose transporters (GLUT), a regulatory enzyme in cancer cell proliferation (Aslan et al., 2016). These enzymes provide rapid ATP production and macromolecular synthesis in the glycolytic pathway, resulting in cancer cells growing more quickly and inducing apoptosis and metastasis in the neighbouring normal cells, in which enzyme inhibition will reduce cancer viability, depending on the metabolic reprogramming system (Suh et al., 2013). Quercetin and kaempferol have been reported to promote cancer cell reprogramming through

Table 2. Research on the anticancer activity of *C. ternatea* flower extract

Study Type	Extraction Method	Results	Reference
In vitro Human carcinoma (HEp-2) cell line	Hydrophilic extraction (methanol) and lipophilic extraction (ethyl acetate and hexane) (50:50; v:v)	Increased concentration of the methanol extract from 0.25 to 0.50 mg/mL showed a 17.2% reduction in cell viability, while the highest concentration (1.0 mg/mL) reduced the viability of HEp-2 cells by 95%. The results of lipophilic extraction did not show a significant difference, even by increasing the concentration to 1.5 mg/mL.	Shen et al. (2016)
In vitro Dalton's Lymphoma ascites cells	Ether and ethanol	Ethanol extract induced higher cytotoxic activity (IC_{50}) at 57 µg/ml than ether extraction at 36 µg/ml.	Shyam kumar & Bhat. (2011)
In vitro Hormone-Dependent Breast Cancer Cell Line (MCF-7), Non-Hormone-Dependent Breast Cancer Cell Line (MDA-MB-231), Human Ovary Cancer Cell Line (Caov-3), Human Cervical Cancer Cell Line (Hela), Human Liver Cancer Cell Line (Hep-G2), Human Foreskin Fibroblast Cell Line (Hs27).	Aquadest and methanol	Cancer cell inhibitions, except in Hela cells, increased dramatically by aquadest extract with any concentration and incubation time, in which the inhibition effect was mos t significant ($p < 0.05$) at 72 hours of incubation, except in Hela cells. The IC50 values in MCF7, Caov3, HepG2, and MDA-MB-231 cells were 175.3 µg/mL, 224.5 µg/mL, 236.3 µg/mL, and 304.7 µg/mL respectively, at 72 hours incubation. In Hela cells, the inhibition effect worked on methanol extract. The IC50 values of MCF-7 and MDA-MB-231 in methanol extract were 536.01 µg/mL and 561.3 µg/mL at 72 hours ($p < 0.05$), respectively. The methanol extract did not act on Caov-3, Hela or HepG2 cells. The aquadest extract had a stronger inhibition effect on MCF-7, MDA-MB-231, CaoV-3, and HEp-G2 compared to the methanol extract ($p < 0.05$).	Salleh et al. (2013)

Table 2. continued

Study Type	Extraction Method	Results	Reference
In vitro Human Mammary Cancer (MCF-7 HER2-Positive)	Ethanol	The IC_{50} value was 862 µg/m. The MTT assay revealed that 50% of cell migration was inhibited by flower extract at 24-hour intervals at a dose of 380 mg/mL. However, a dose of 500 mg/mL for 48 hours had the most significant effect ($p<0.05$).	Asysyifa et al. (2020)
In vivo and molecular analysis Transcription factor of VEGF (HIF-1α protein) from Ehrlich ascites carcinoma (EAC) cells of treated Swiss albino mice (Mus musculus) by MECT	Methanol	Extraction of *C. ternatea* flower significantly affected EAC cell division, which was observed based on cell weight. Control cells (without treatment) showed a 97% increase in weight, while the treated cells showed only a 42% increase. The results of ELISA analysis on the VEGF gene showed a decrease in the expression of HIF-1α protein in the treated cells in the cytosolic fraction and the extract cell nucleus, namely Mean ± SE for optical density at 58.76 ± 1.77% and 278 ± 4.61%. The control cells were 303 ± 4.98% and 77 ± 1.79%.	Srinivasa Balaji & Shivaprakash. (2016)
In vitro Cyclotides protein purification from C. ternatea flower extract in lung cancer cells A549 and sub-row A549/ paclitaxel resistance	20% Ethanol	Extract cyclotides had strong cytotoxicity in A549 and A549/paclitaxel cells (IC_{50}< 20 µg/mL).	Zhang et al. (2013)

C. ternatea flower extract has a moderate cytotoxic effect (IC_{50} = 21 - 200 µg/mL), while a sole active compound of purified cyclotides has a strong cytotoxic effect (IC_{50} < 20 µg/mL).

Nrf2 gene knockdown (Hussain et al., 2022). Hussain et al. revealed that Nrf2 modulation caused inhibition of pro-metastatic transcription in the lung cancer and breast cancer cell lines. Moreover, Shen et al. (2016) revealed that other phytosterol compounds, such as β-sterol and campesterol, have also been known to disrupt cancer cell metabolism.

Inhibiting the invasiveness of cancer cells

The activity of tocotrienol compound, particularly γ-tocopherol, has been reported to reduce cancer cell invasion and the metastasis rate by suppressing death receptor 5 (DR5R) and stoppinng NF-kB activation (Kannappan et al., 2012). Kannappan et al. also found that tacols components in *C. ternatea* extract reduced HEp-2 cell growth. Similarly, γ-tocopherol and ternatin, anthocyanin derivatives, have been reported to block cell proliferation and inhibit cell metastases. Interestingly, although there are various mechanisms of action within the different types of ternatin, A3 has been found to effectively impede cell proliferation by binding with eEF1A as a fundamental elongation cellular process of translation cells (Carelli et al., 2015).

Angiogenesis suppression

An in vivo study by Srinivasa Balaji and Shivaprakash (2016) using Swiss albino mice in Ehrlich ascites carcinoma (EAC) cells treated with methanol extract of *C. ternatea* flower showed a 42% reduction in tumor cell mass compared to the level in control mice, which increased by 97%. This percentage indicated an effective antitumor agent because it had an ILS value > 25%. Microscopic analysis of tumor cells stained with trypan blue dye has revealed that the treated tumor cells experienced nuclear condensation and residual apoptosis. Conversely, the control cells that induced EAC cells in mice were found to have newly formed microvessels and prominent blood vessel growth, indicating the peritoneal angiogenesis properties of the tumor (Srinivasa Balaji & Shivaprakash, 2016).

The anticancer activity of *C. ternatea* flower extract has been analysed further using vascular endothelial growth factor (VEGF), which is responsible for angiogenesis for cancer progression and metastasis. The anti-VEGF 165 antibodies in the ascetic fluid of treated mice after 7, 9 and 11 days of treatment were much lower (below 250 g/mL) than in the control mice (up to 2000 g/mL) when measured using ELISA. This was also followed by a decrease in HIF-1α expression in the cytosol and cell nucleus of treated mice when identified through western blot (Srinivasa Balaji and Shivaprakash 2016; Lakshan et al. 2019). HIF-1α is responsible for VEGF regulation, yet both genes could be significantly impeded by the apigenin and luteolin activity contained in *C.ternatea* flower extract (Cui et al., 2017; Pratheeshkumara et al., 2014).

Chemotherapy enhancement

Besides work on bioactive compounds, research has also been conducted by extracting cyclotides or small circular proteins through RP-HPLC from *C. ternatea*. There are seven purified cyclotide types: CT2, CT4, CT7, CT10, CT12, CT19 and CT20. These purified cyclotides have been used to evaluate cytotoxicity and chemosensitization in lung cancer cell line A549 and sub-line A549/paclitaxel (cancer cells resistant to paclitaxel) using MTT assay. Interestingly, this research by Zhang et al. (2013) reported that certain cyclotides, especially positively charged ones, such as CT2, CT4, CT7, CT10 and CT12, had significant cytotoxic (IC_{50} < 10 μM) and chemosensitization abilities (Zhang et al. 2013).

The Effect of the *C. ternatea* Flower Extraction Method on Anticancer Activities

The anticancer effect of herbal compounds can be affected by the extraction method, including the solvent used (Table 3). *C. ternatea* flower extract with a hydrophilic solution, especially aquadest or methanol, is more effective at inhibiting the viability of cancer cells compared to a lipophilic solution, such as ether, ethyl acetate or hexane. Hence, it may increase chemical extraction or actively regulate phenolic compounds in the hydrophilic solution (Pardo-Botello et al., 2022; Pertuzatti et al., 2014). Other extractions that use petroleum ether rather than ethanol have shown different results based on the dosage. Petroleum ether extract with a concentration of 10 g/mL has shown a decrease of 8% in DLA cells, while 500 g/mL showed a 100% decrease. In addition, ethanol extraction decreased by 1.33% with a 10 g/mL solution, while an 80% decrease in the number of DLA cells was observed at 500 μg/mL (Shyam Kumar & Bhat, 2011). These results indicate that petroleum ether extract is more effective for cytotoxic activity. Additionally, petroleum ether had a smaller IC_{50} value of 36 μg/ml compared to ethanol extract with 57 μg/ml, indicating that it is more effective in reducing DLA cell proliferation.

According to the National Cancer Institute and Geran protocol criteria, the IC_{50} value demonstrates that *C. ternatea* flower extract has moderate anticancer activity. Extracts with IC_{50} ≤ 20 μg/mL are highly cytotoxic; ones with IC_{50} ranging between 21 and 200 μg/mL are moderately cytotoxic; between 201 and 500 μg/mL indicates weakly cytotoxic, while > 501 μg/mL shows no cytotoxicity (Nguyen et al., 2020). Moderate anticancer activity has been shown in MCF- 7, MDA-MB-231, CaoV-3 and HEp-G2 using water extraction, and in DLA cells using petroleum ether and ethanol extraction. Meanwhile, methanol and ethanol extraction has been reported to have non-cytotoxic effects (Salleh et al., 2013). The active compound isolated from *C. ternatea* flower extract has displayed strong anticancer activity. Consequently, a hydrophilic solution would be beneficial to enhance this effect. Isolating each active compound in the flowers is more beneficial for cancer treatment. However, optimisation of the extraction method is necessary in different cancer cell lines before its use in clinical tests.

Toxicity

C. ternatea flowers have not shown toxicity on normal cells with distilled water extraction (Salleh et al., 2013). Furthermore, toxicity tests have been conducted on Wistar rats using ethanol extract and aquadest orally at a 2000 mg/kg dose. The results show that the flower extract caused no mortality nor abnormal activity in rat hematology (Srichaikul, 2017). There have been no signs of poisoning, such as restlessness, respiratory problems, seizures, aggressive activity, coma or death after more than 14 days of treatment (Yee & Than, 2020). In addition, sperm production, serum testosterone and histological and testicular morphology in mice have not been affected (Iamsaard et al., 2014; Mukherjee et al., 2008).

Table 3. Differences in *C. ternatea* flower extraction methods

Extraction Method	Cell Lines	IC_{50}	Reference
Methanol	Human carcinoma (HEp-2)	Data not provided	Shen et al. (2016)
	hormone-dependent breast cancer cell line (MCF-7)	536.01 g/mL	Salleh et al. (2013)
	Non-hormone-dependent breast cancer cell line (MDA-MB-231)	561.3 g/mL	Salleh et al. (2013)
	Human ovary cancer cell line (Caov-3)	*No action*	Salleh et al. (2013)
	Human cervical cancer cell line (Hela)	*No action*	Salleh et al. (2013)
	Human liver cancer cell line (Hepg2)	*No action*	Salleh et al. (2013)
	Human foreskin fibroblast cell line (Hs27)	Data not provided	Salleh et al. (2013)
Ethyl Acetate and Hexane (50:50)	Human carcinoma (hep-2) cell line	Data not provided for IC_{50}	Shen et al. (2016)
Ether	Dalton's lymphoma ascites cells	36 µg/ml	Shyam Kumar and Bhat (2011)
Ethanol	Dalton's lymphoma ascites cells	57 µg/ml	Shyam Kumar and Bhat (2011)
	Human mammary cancer (MCF-7 HER2-Positive)	862 g/mL	Asysyifa et al. (2020)
Aquadest	Hormone-dependent breast cancer cell line (MCF-7)	175.3 g/mL	Salleh et al. (2013)
	Non-hormone-dependent breast cancer cell line (MDA-MB-231)	304.7 g/mL	Salleh et al. (2013)
	Human ovary cancer cell line (Caov-3)	224.5 g/mL	Salleh et al. (2013)
	Human cervical cancer cell line (Hela)	*No action*	Salleh et al. (2013)
	Human liver cancer cell line (HepG2)	236.3 g/mL	Salleh et al. (2013)
	Human foreskin fibroblast cell line (Hs27)	Data not provide	Salleh et al. (2013)

The extraction methods determine the cytotoxic rate. Aquadest extract has shown a stronger effect. Hence, petroleum ether solvent might have the most potential for further analysis

Heavy metal content analysis has been evaluated to by Kushi et al. (2019) to determine safety consumption. Spectrophotometer analysis has shown that *C. ternatea* extract had a low heavy metal content of <0.001, 0.002333±0.0002, and 0.001267±0.0001 in cadmium and arsenic, tin, and nickel, respectively. In addition, the mineral content revealed high concentrations of calcium and magnesium, at 3.09 mg/g and 2.23 mg/g respectively. High concentrations of potassium, zinc, sodium, and iron were also identified at levels of 1.25, 0.59, 0.14 and 0.14 mg/g, respectively, with a significant difference ($p < 0.05$) in the analytical parameters (< 0.01 mg/g). These minerals are necessary for the body's normal cell-building and healing processes (Salleh et al., 2013). Therefore, the results demonstrate that *C. ternatea* flowers are safe for consumption.

CONCLUSION

Several phytochemicals with anticancer activity have been discovered from *C. ternatea* flower extraction, such as the flavonoid group including

ternatins, delphinidin, kaempferol, quercetin, sitosterol and vitamins (tocopherols, inositol and pentanal). The phytochemicals have been measured and used in multi-molecular targets to inhibit the proliferation of tumor or cancer cells, prevent angiogenesis, increase apoptosis cells, and enhance chemotherapy treatments. Furthermore, the safe consumption of the extract is indicated by the high mineral content and low level of heavy metals. Toxicity analysis has shown no lethal effect on healthy cells or test animals. Therefore, the extract can be used as a food substitute, supplement, or in combination with commercial drugs for cancer treatment.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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