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Research Article

Chemical Composition and Potential Cytotoxic Mechanisms of *Camellia flava* (Pitard) Sealy Leaves

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ABSTRACT

Golden camellia is a group of herbal materials belonging to the *Camellia* genus which was utilized as a refreshing and heat-clearing beverage, for treating diarrhea, and as a supportive treatment for cancer. In this current study, we aimed to analyze the chemical composition of *Camellia flava* (Pitard) Sealy and evaluate its cytotoxic effects. *Camellia flava* leaf was extracted exhaustedly with 70% ethanol. The total ethanolic extract was then partitioned with ethyl acetate to obtain ethyl acetate extract (CFLEA). The chemical composition of CFLEA were analyzed using GC-MS method. Next, the network pharmacological studies were conducted to investigate the anti-cancer mechanisms. Finally, cytotoxic activity was evaluated using the MTT staining method. A total of 16 phytochemical compounds in CFLEA were found by GC-MS analysis. The molecular docking results indicated that PRKCB and MAPK9 were the two proteins with the strongest binding affinity to the compounds 3,4-divanillyltetrahydrofuran (-7.7 and -8.2 kcal/mol) and (*E*)-3,3'-dimethoxy-4,4'-dihydroxystilbene (-7.5 and -7.5 kcal/mol). The primary anti-cancer pathways of CFLEA may be the JNK and p38 MAP kinase pathway, the classical MAP kinase pathway, and the calcium signaling pathway. Finally, CFLEA exhibited cytotoxicity on breast and liver cancer cells in a dose-dependent manner, with IC_{50} values of 318.92 ± 12.27 and 291.69 ± 19.97 µg/mL, respectively. A total of 16 plant-derived compounds in CFLEA were identified and their structures were determined using GC-MS. The JNK and p38 MAP kinase pathway, classical MAP kinase pathway, and calcium signaling pathway could be the main pathways through which CFLEA exerted its inhibitory effects on cancer cell growth. Lastly, CFLEA demonstrated significant cytotoxicity and exhibited potent inhibitory effects.

Keywords:
Camellia flava (Pitard) Sealy; chemical composition; cytotoxic effect; GC-MS; network pharmacology

1. INTRODUCTION

Cancer has been becoming a pressing global issue, attracting the attention of the international community and scientists in particular, as it is the second leading cause of death worldwide, following cardiovascular diseases, and is going to become the leading cause of death by 2060 according to the World Health Organization (WHO) and the American Cancer Society (ACS)[1]. Despite the availability of numerous cancer chemotherapy treatments approved for clinical use, drug resistance in cancer cells remains a major cause of treatment failure. Drug resistance mechanisms have been identified, including drug degradation, alteration of drug targets' expression or function, limited drug transport across cell membranes, and ineffective interactions between drugs and their intended targets[2]. Therefore, researching the inhibitory effects of natural substances and compounds on cancer cells not only supports the treatment process but also holds potential for addressing drug resistance.

Golden camellia is a group of herbal materials belonging to the *Camellia* genus, commonly distributed in several Asian countries such as India, Southern China,

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and notably Vietnam, where over 40 species have been discovered and reported[3]. Folk experience has utilized Golden camellia species as a refreshing and heat-clearing beverage, for treating diarrhea, and as a supportive treatment for cancer. Numerous studies on the chemical composition and biological effects of Golden camellia species (predominantly *Camellia nitidissima* Chi) have been conducted and reported. However, many other species of Golden camellia have not received much research attention, particularly *Camellia flava* (Pitard) Sealy, one of the unique species found in Vietnam. The high concentration of polyphenols, total flavonoids, and significant antioxidant capacity of *Camellia flava* leaves were highlighted in our earlier work, which offered early information on the plant's composition and biological effects[4]. In this current study, we aimed to analyze the chemical composition of ethyl acetate extract from *Camellia flava* leaves (CFLEA, flavonoid-rich extract) and evaluate its cytotoxic effects to supplement the chemical profile and provide evidence of the pharmacological effects of this herbal material.

2. MATERIALS AND METHODS

2.1. Materials

The leaves of *Camellia flava* (Pitard) Sealy were harvested in Nghia Hung commune, Chu Pah district, Gia Lai province, Vietnam, in January 2022. The plant material was identified by the Department of Botany, Faculty of Pharmacy, University of Medicine and Pharmacy at Ho Chi Minh City (UMP), Vietnam. The voucher speciment (L5.100222) was deposited at the Department of Analytical Chemistry – Drug Quality Control, Faculty of Pharmacy, UMP, Vietnam. The herbal material was processed by thorough washing, air-drying, and grinding into coarse powder.

DMEM (Dulbecco's Modified Eagle Medium), MEME (Minimum Essential Medium with Eagle salt), L-glutamine, sodium pyruvate, sodium bicarbonate, penicillin G, FBS 10% (Fetal Bovine Serum), Trypsin-EDTA 0.05%, DMSO (dimethyl sulfoxide), TCA (trichloroacetic acid), Tris base, PBS (phosphate buffered saline), Ellipticine (Sigma, USA), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium), acetic acid, and ethanol.

The human breast cancer cell line (MDA-MB-231) and human liver cancer cell line (HepG2) were provided by Prof. JM Pezzuto (Long Island University, USA) and Prof. Jeanette Maier (University of Milan, Italy).

2.2. Methods

2.2.1. Preparation of ethyl acetate leaf extract

One kilogram of powdered *Camellia flava* leaf was soaked with 70% ethanol solvent and extracted exhaustedly over 24 hours (10:1 solvent to herbal material ratio). After that, the extract was collected at a rate of 2-3 mL/minute, and the solvent was recovered under low pressure to yield an ethanol leaf extract. The total ethanolic extract was then evaporated to obtain a concentrated form before being partitioned to liquid-liquid distribution with ethyl acetate solvent. The ethyl acetate fraction (CFLEA) was evaporated and used for the analysis of phytochemical composition by gas chromatography-mass spectrometry (GC-MS) and cytotoxicity assays.

2.2.2. GC-MS analysis

The phytochemical compositions of CFLEA were analyzed using the gas chromatography system (TRACE 1310) coupled with a mass spectrometry detector (ISQ 7000, Thermo Scientific) with the following conditions: an Agilent DB-5MS column (30 m × 0.25 mm × 0.25 µm), Helium as a carrier gas, and a flow rate of 1.5 mL/min. The column temperature program started at 70 ^{o}C (held for 1 minute), following the increase at a rate of 15 ^{o}C/min and linearly ramped to 300 ^{o}C (held for 15 minutes). The injector and MS transfer line temperatures were set at 250 and 280 ^{o}C, respectively. The MS source was set at 260 ^{o}C and the mass range was *m/z* 29-650 amu. One µL of sample was prepared by dissolving 20 mg of CFLEA with 1.0 mL ethanol and was injected at a split ratio of 1:25. The chemical components were identified based on a comparison of their mass spectra values with those in the NIST17 database.

2.2.3. Network pharmacology of CFLEA in treatment of cancer

2.2.3.1. Screening of active ingredients-related target of CFLEA

All phytochemical compounds of CFLEA (analyzed by the GC-MS technique) were imported into Excel 2016 software and their InChI and SMILES names were determined through the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Next, the results were imported into SwissADME (http://www.swissadme.ch/) to evaluate the pharmaceutical characteristics of the compounds. SwissADME is a prediction tool that allows researchers to compute physicochemical

descriptors and predict ADME parameters, pharmacokinetic properties, drug-likeness, and medicinal chemistry friendliness of small molecules[5]. The selection criteria for compounds were high oral absorption and drug-like properties (at least 2 out of 5 "yes" results in the "drug-likeness" section)[6]. Finally, the compounds that passed the screening process using SwissADME were imported into the BATMAN-TCM database (http://bionet.ncpsb.org.cn/batman-tcm/) for the search of potential target interactions, with the screening conditions set at a score cutoff of 20 and $p < 0.05$[7,8]. BATMAN-TCM is a bioinformatics analysis tool for the molecular mechanism of traditional medicine formulations, providing information on target-disease relationships and chemical composition in herbal medicine, and integrating target analysis, allowing researchers to comprehensively and systematically understand the components and targets of traditional medicine formulations[9].

2.2.3.2. Screening of target genes involved in CFLEA against cancer

All human genes related to cancer were searched and screened from 3 databases included GeneCards® (https://www.genecards.org/), Online Mendelian Inheritance in Man® (OMIM®, https://omim.org/) và Terapeutic Target Database (TTD, http://db.idrblab.net/ttd/) with the following keywords "carcinoma", "cancer" and "tumor"[10-12]. The search results were imported into Excel 2016 software and excluded duplicate values.

2.2.3.3. Protein-Protein Interactions (PPI) Network Analysis

Protein-protein interactions were analyzed by importing the overlapping values between the potential target effects of phytochemical compounds in CFLEA and the potential target effects of cancer into STRING version 12.0 (https://string-db.org/). The analysis was performed with the following settings: "*Homo sapiens*" as the organism and an interaction score threshold more than 0.4. STRING is a database of protein-protein interactions that have been studied through computational predictions or synthesized from other reputable databases[13]. The resulting Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway results from the PPI network analysis was extracted into Cytoscape software version 3.10.1.

2.2.3.4. Ingredient-Target-Pathway (ITP) Network Analysis

The network of relationships between chemical ingredients in CFLEA, shared target effects related to cancer, and the associated pathways were analyzed using Cytoscape software version 3.10.1. The structural properties of this complex network were determined using the "Network Analyzer" tool within the software[14].

2.2.3.5. Molecular docking analysis

The process of molecular docking between chemical compounds and target molecules, most relevant to cancer, was performed using ReverseDock (https://reversedock.biologie.uni-freiburg.de/). Reverse-Dock is a tool designed to support researchers in conducting blind high-throughput docking experiments with AutoDock Vina for selected ligands and proteins. The 3D structures of compounds with high "Degree" parameters after ITP analysis were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and saved in *mol2 format. The crystal structures of protein macromolecules were obtained from the RCSB PDB database (https://www.rcsb.org/) and saved in *pdb format. Finally, the molecular docking process was carried out, and the "binding energy" value between the ligand and protein was recorded[15].

2.2.4. In vitro cytotoxicity experiment

The cytotoxic effects of CFLEA was evaluated using the method described by Mosmann T (1983)[16]. The cancer/normal cells were stained with the MTT reagent, and the total cellular protein content was determined by measuring the optical density (OD) at a wavelength of 540 nm. The stock solution was prepared by diluting CFLE in DMSO to a concentration of 20 mg/mL. The solution was diluted in cell culture medium without FBS at concentrations of 500, 100, 20.0, and 4.0 µg/mL. A mixture of 10 µL of the test sample and 190 µL of cells was incubated for 48 hours in a cell incubator. After 48 hours, the mixture was stained with MTT for 4 hours. The medium was then removed, and the formazan crystals were dissolved in 50 µL of DMSO. The OD value at 540 nm was determined using an ELISA Plate Reader (BioTek). A blank well was prepared similarly with 10 µL of 1% DMSO and 190 µL of cells. Ellipticine at concentrations of 10, 2, 0.4, and 0.08 µg/mL was used as a positive control.

The inhibition rate of cancer cells was calculated by the following formula:

$$I(\%) = 1 - \frac{OD_{sp} - OD_0}{OD_{48h} - OD_0}$$

Where: I: the inhibition rate; OD_{sp}: average optical density value of testing sample after incubating 48 hours; OD_0: average optical density value of DMSO at the beginning (0 hour); OD_{48h}: average optical density value of DMSO after 48 hours.

Table 1. Phytochemical constituents of CFLEA

Nº	RT (min)	Compound	MF	MW	Content (%)
1	4.53	Unknown	-	-	0.83
2	4.64	Monomethyl succinate	$C_5H_8O_4$	132.11	4.17
3	5.32	Unknown	-	-	2.47
4	5.73	Methyl salicylate	$C_8H_8O_3$	152.15	3.92
5	5.93	4-Vinylphenol	C_8H_8O	120.15	2.20
6	6.38	4-Hydroxy-4-methyl-cyclohexanone	$C_7H_{12}O_2$	128.17	1.83
7	7.43	Pyrogallol	$C_6H_6O_3$	126.11	55.89
8	7.59	1,2,4-Benzenetriol	$C_6H_6O_3$	126.11	0.78
9	8.22	Unknown	-	-	0.96
10	8.54	*p*-Hydroxybenzoic acid	$C_7H_6O_3$	138.12	1.60
11	9.21	Butyrovanillone	$C_{11}H_{14}O_3$	194.23	1.62
12	10.75	*p*-Coumaric acid	$C_9H_8O_3$	164.16	10.46
13	11.91	Palmitic acid	$C_{16}H_{32}O_6$	256.42	3.79
14	13.07	*trans*-13-Octadecenoic acid	$C_{18}H_{34}O_2$	282.50	2.92
15	13.21	Stearic acid	$C_{18}H_{36}O_2$	284.50	1.84
16	13.95	9-Hexadecenoic acid	$C_{16}H_{30}O_2$	254.41	0.37
17	15.35	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390.60	0.60
18	16.05	(*E*)-3,3'-Dimethoxy-4,4'-dihydroxystilbene	$C_{16}H_{16}O_4$	272.29	0.50
19	17.42	3,4-Divanillyltetrahydrofuran	$C_{20}H_{24}O_5$	344.40	3.23

Note: MW: Molecular weight (g/mol), MF: Molecular Formula

The IC_{50} value (the concentration that inhibits 50% of growth) was determined using the TableCurve 2Dv4 computer software.

2.3. Statistical analysis

The data was processed using Excel 2016 software and Cytoscape version 3.10.1. The results of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were presented using statistical analysis software R. Adjusted p-value (Benjamini–Hochberg) less than 0.05 was considered significantly different. The cytotoxicity experiments were conducted triplicate, and the results were presented as the mean value ± standard deviation (SD).

3. RESULTS AND DISCUSSION

3.1. Ethyl acetate leaf extract preparation

From 1.0 kg of *Camellia flava* leaves, 100.93 g of total ethanolic extract was obtained through exhaustive extraction with 70% ethanol (yield of 18.19%). The extract was then partitioned with ethyl acetate to obtain 1.09 g of CFLEA (yield of 1.08%).

3.2. Phytochemical constituents of CFLEA

The GC-MS analysis results of CFLEA were presented in Table 1. Nineteen phytochemical compounds were found in CFLEA, with 16 compounds identified in decreasing order of content: pyrogallol

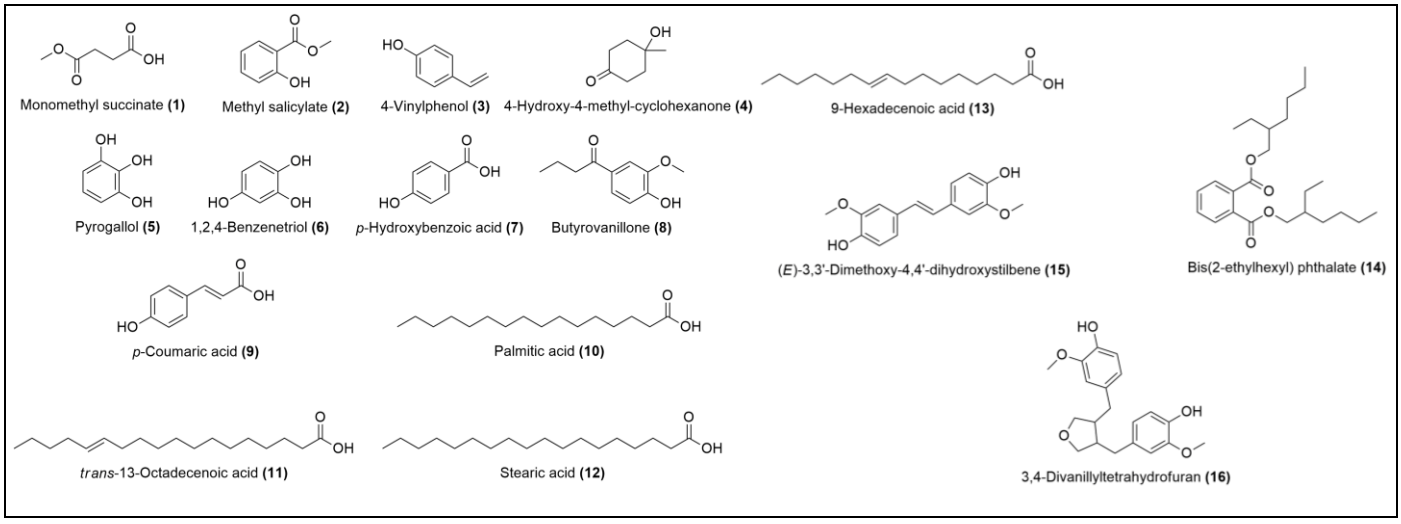

Figure 1. Chemical formula of 16 compounds in CFLEA

Table 2. Pharmaceutical activities of active compounds in CFLE

ID	Compound	Absorption	Drug likeness (n ≥ 2)					
			Lipinski	Ghose	Veber	Egan	Muegge	Total "Yes"
CFLEA01	Monomethyl succinate	High	Yes	No	Yes	Yes	No	3
CFLEA02	Methyl salicylate	High	Yes	No	Yes	Yes	No	3
CFLEA03	4-Vinylphenol	High	Yes	No	Yes	Yes	No	3
CFLEA04	4-Hydroxy-4-methyl-cyclohexanone	High	Yes	No	Yes	Yes	No	3
CFLEA05	Pyrogallol	High	Yes	No	Yes	Yes	No	3
CFLEA06	1,2,4-Benzenetriol	High	Yes	No	Yes	Yes	No	3
CFLEA07	*p*-Hydroxybenzoic acid	High	Yes	No	Yes	Yes	No	3
CFLEA08	Butyrovanillone	High	Yes	Yes	Yes	Yes	No	4
CFLEA09	*p*-Coumaric acid	High	Yes	Yes	Yes	Yes	No	4
CFLEA10	Palmitic acid	High	Yes	Yes	No	Yes	No	3
CFLEA11	*trans*-13-Octadecenoic acid	High	Yes	No	No	No	No	1
CFLEA12	Stearic acid	High	Yes	No	No	No	No	1
CFLEA13	9-Hexadecenoic acid	High	Yes	Yes	No	Yes	No	3
CFLEA14	Bis(2-ethylhexyl) phthalate	High	Yes	No	No	No	No	1
CFLEA15	(*E*)-3,3'-Dimethoxy-4,4'-dihydroxystilbene	High	Yes	Yes	Yes	Yes	Yes	5
CFLEA16	3,4-Divanillyltetrahydrofuran	High	Yes	Yes	Yes	Yes	Yes	5

(55.89%), *p*-coumaric acid (10.46%), monomethyl succinate (4.17%), methyl salicylate (3.92%), palmitic acid (3.79%), 3,4-divanillyltetrahydrofuran (3.23%), *trans*-13-octadecenoic acid (2.92%), 4-vinylphenol (2.20%), stearic acid (1.84%), 4-hydroxy-4-methylcyclohexanone (1.83%), butyrovanillin (1.62%), *p*-hydroxybenzoic acid (1.60%), 1,2,4-benzenetriol (0.78%), bis(2-ethylhexyl) phthalate (0.60%), (*E*)-3,3'-dimethoxy-4,4'-dihydroxystilbene (0.50%), and 9-hexadecenoic acid (0.37%). The chemical formulas of these 16 active compounds in CFLE were presented in Figure 1.

3.3. Potential mechanism of CFLEA on cancer

3.3.1. Screening of Active Compounds in CFLEA

The 16 phytochemical compounds found in CFLEA were evaluated for their pharmaceutical features using SwissADME and the results were presented in Table 2. All compounds showed good absorption through the gastrointestinal tract. Regarding drug-likeness, three compounds did not meet the selection criteria (less than 2 parameters with a "Yes" value), including *trans*-13-octadecenoic acid, stearic acid, and bis(2-ethylhexyl) phthalate. The remaining 13 compounds were further screened for their predicted cancer inhibition pathways.

3.3.2. Screening of Target Genes

When searching the GeneCards, OMIM, and TTD databases using the keywords "Carcinoma", "Cancer", and "Tumor", a total of 98,236 results were found. After removing duplicate values, 47,256 genes were selected for further analysis. Next, these genes were compared to the target impact results of the active compounds in CFLEA proposed by BATMAN-TCM, which revealed 723 related targets associated with the active components and cancer.

3.3.3. PPI Network Analysis

The PPI (Protein-Protein Interaction) network of the cross-targets between CFLEA and cancer was

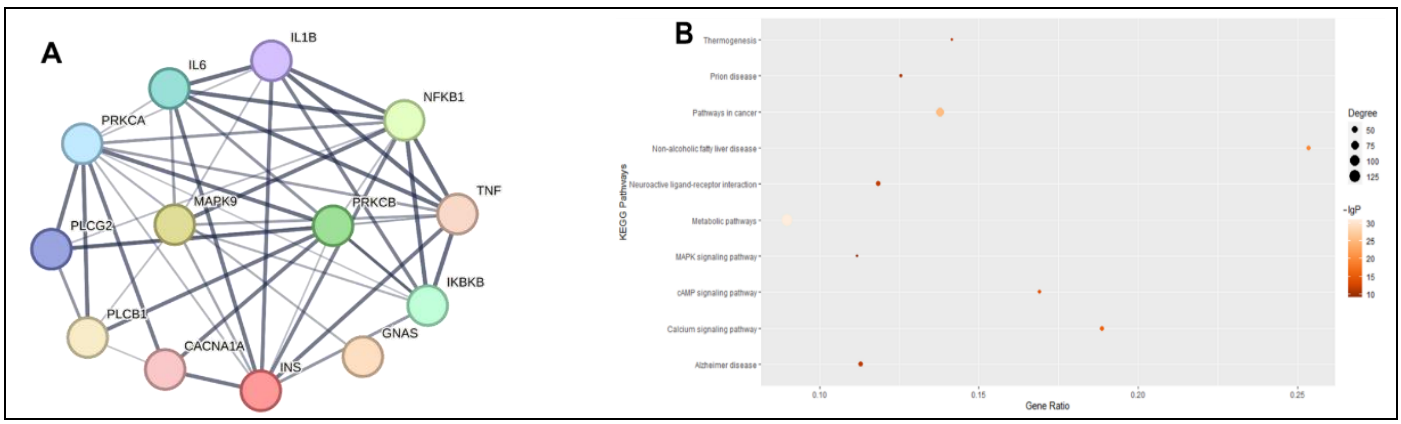

Figure 2. The main therapeutic target interactions of the active compounds in CFLEA for the treatment of cancer (A); Bubble diagram of KEGG enrichment analysis (B)

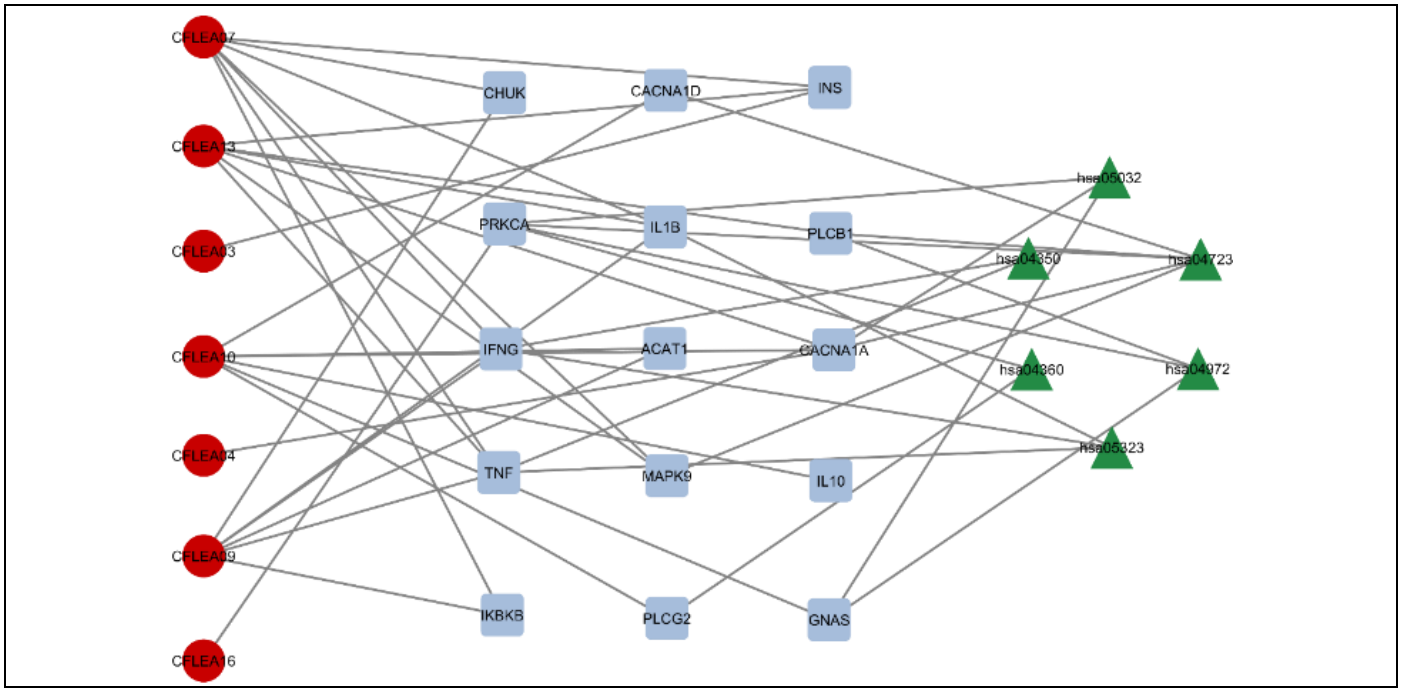

Figure 3. Ingredients – Targets – Pathways network for the treatment of cancer

analyzed using Cytoscape software version 3.10.1. The core targets were identified based on their high degree of interaction and the genes with high degree values, which may play an important role in pharmacological effects. The interaction among the cancer therapeutic targets was analyzed using the STRING tool and is described in Figure 2A. The enrichment of KEGG pathways was presented in Figure 2B. The value "-logP", with P is p-value corrected for multiple testing within each category using the Benjamini–Hochberg procedure describes how significant the enrichment is. "Gene ratio" is the ratio between (i) the number of proteins in the network that are annotated with a term and (ii) the number of proteins in total (in the network and the background) have this term assigned. Finally, "Degree" is the number of gene count in each term.

3.3.4. ITP Network Analysis

The ingredient-target-pathway network of the phytochemical compounds in CFLEA for cancer treatment was analyzed using Cytoscape 3.10.1 software, which consisted of 518 nodes and 713 edges. The ITP network of the main active compounds in CFLEA was shown in Figure 3, where red circles represented the active compounds, blue squares represented the target interactions, and green triangles represented the pathways.

3.3.5. Molecular docking

The molecular docking model was performed to determine the binding energies between the chemical components in CFLEA and the target genes predicted for cancer treatment. The results of the binding energies (kcal/mol) for the ligand-protein interactions were presented in Figure 4.

3.4. Cytotoxicity of CFLEA

The results of the cell viability assessment of CFLEA on breast cancer cells (MDA-MB-231) and liver cancer cells (HepG2) were presented in Table 3 and Figure 5. CFLEA exhibited dose-dependent inhibition of cancer cells, with liver cancer cells (HepG2) being more strongly inhibited compared t breast cancer cells (MDA-MB-231).

4. DISCUSSION

Cancer is a challenging disease in the discipline of medicine worldwide. According to the National Cancer Institute (NCI), cancer is defined as a pathological condition in which some cells in the body develop uncontrollably and spread to other parts of the body[17]. Nowadays, with numerous published studies on the pathophysiology of cancer, the definition of cancer has evolved to be an uncontrolled proliferation of cells resulting from the evolutionary transformation of naturally selected cells[18]. In 2020, statistical data showed that there were over 19 million cancer patients and nearly 10 million cancer-related deaths, with the most common types of cancer being breast, liver, lung, colorectal, prostate, and stomach cancer[19]. Current clinical approaches to cancer treatment include surgery, chemotherapy, radiation therapy, hormone therapy, and

Figure 4. The heatmap illustrating the binding energy values (kcal/mol) between phytochemical compounds in CFLEA and proteins (A); The interactions between CFLEA16 and MAPK9 (B) and PRKCB (C)

immunotherapy[20]. Many cancer treatment drugs have been developed and approved for use, such as alkylating agents (cyclophosphamide, cisplatin, mitomycin C), metabolic inhibitors (methotrexate, fluorouracil, gemcitabine), topoisomerase inhibitors (doxorubicin, topotecan, etoposide), and others[21]. However, drug resistance is one of the major causes of treatment failure in cancer therapy[2]. Therefore, alongside the research and development of new generations of drugs, the use of medicinal herbs and natural compounds to support cancer treatment is currently a trend. Some medicinal herbs that have been studied in preclinical and clinical settings for their anti-cancer effects included Huang Ky (*Astragalus membranaceus*), Ginseng (*Panax ginseng*), Garlic (*Allium sativum*), and Tumeric (*Curcuma longa*)[22].

Out of the 19 compounds analyzed in CFLEA using the GC-MS method, the structures of 16 compounds have been identified. Several compounds made up a significant proportion, including pyrogallol, *p*-coumaric acid, monomethyl succinate, methyl salicylate, palmitic acid, and 3,4-divanillyl-tetrahydrofuran. Pyrogallol is a phytochemical compound belonging to the phenolic acid group, presented in many medicinal herbs, and known for its various pharmacological effects, such as antibacterial, antifungal, antiviral, antioxidant, and cardiovascular protective properties. Notably, pyrogallol exhibited significant inhibitory effects on cancer cells[23].

Besides pyrogallol, *p*-coumaric acid is also a common phenolic acid compound found in various fruits, vegetables, grains, and medicinal herbs. This phenolic compound has been recognized for its multiple biological activities, including anti-tumor, antibacterial, anti-aging, and antioxidant effects[24]. Numerous publications have shown that *p*-coumaric acid exerted strong proliferation-inhibitory effects on colon cancer cells by regulating the downregulation of Grp78, a key protein involved in activating the unfolded protein response, along with two other mechanisms: stimulation of apoptosis and cell cycle arrest[25,26]. Additionally, *p*-coumaric acid has been applied as an effective self-anticancer drug carrier in biologically targeted therapy through polymerization reactions[27].

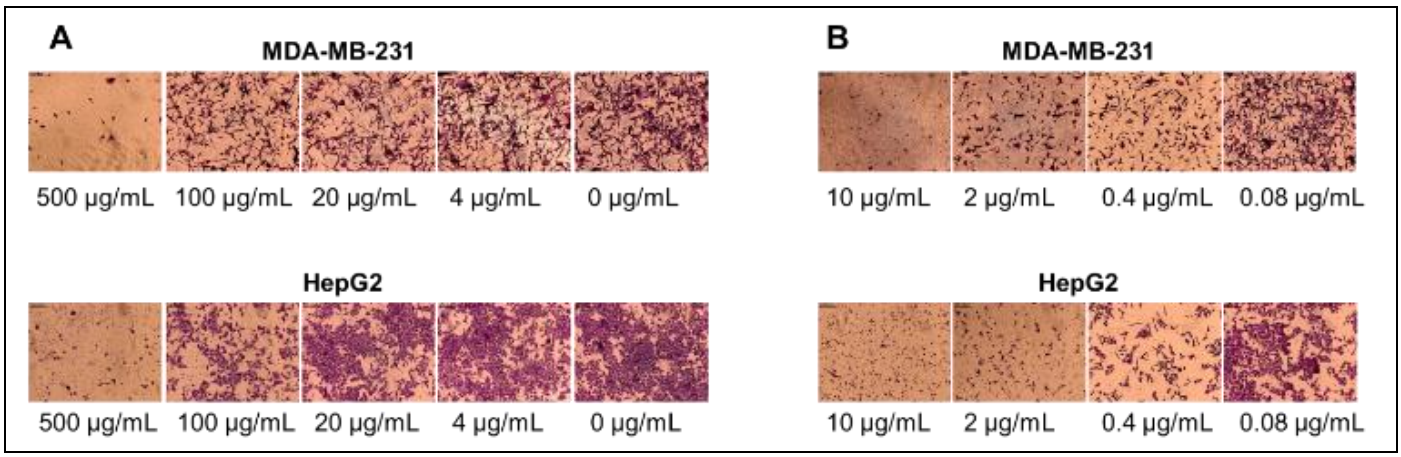

Figure 5. The cells growth when treated with (A) CFLEA or (B) Ellipticine

Table 3. Cytotoxic potential of CFLEA and Ellipticine

%Inhibition of cell growth of leaf extract (n=3, Mean ± SD)		
Concentration (µg/mL)	**MDA-MB-231**	**HepG2**
500	89.44 ± 2.09	61.91 ± 1.20
100	13.92 ± 1.12	32.02 ± 1.35
20	6.37 ± 0.58	22.33 ± 1.17
4	5.03 ± 0.17	11.48 ± 0.87
IC_{50}	318.92 ± 12.27	291.69 ± 19.97
Ellipticine (IC_{50})	0.39 ± 0.44	0.32 ± 0.02

Finally, 3,4-divanillyltetrahydrofuran is a major natural compound found in species belonging to the *Urtica* genus, such as *Urtica dioica* or *Urtica fissa*. This lignan compound has been reported to exhibit several biological activities, including reducing testicular damage and reproductive dysfunction caused by diabetes (through the activation of Nur77 expression and related proteins involved in testosterone synthesis) and inhibiting prostate cancer cells (dependent on the sensitivity of the cells to androgens)[28,29].

The phytochemical compounds in CFLEA are evaluated for their pharmaceutical characteristics using the SwissADME software. The oral absorption characteristic is an important pharmacokinetic property in the process of exploring small molecule compounds. The absorption process provides information on how compounds move from the administration site to the circulatory system. Currently, small molecule therapeutic approaches tend to be designed for oral administration, making a compound with good oral absorption potential desirable for drug development[30].

Protein kinase C (PKC) is a family of protein kinase enzymes that are involved in controlling the function of other proteins through the process of phosphorylation of hydroxyl groups on serine and threonine amino acids[31]. In terms of physiological function, PKC enzymes play various roles related to receptor desensitization, regulation of membrane structural events, transcriptional regulation, modulation of immune reactions, regulation of cell development, as well as learning and memory processes[32]. There are 15 isozymes of protein kinase C, with PRKCB being a commonly studied isozyme that is activated by Ca^{2+} ions and diacylglycerol. Several studies have reported that the methylation process of PRKCB was one of the mechanisms involved in cancer development[33,34]. Therefore, inhibiting the activity of protein kinase C, particularly PRKCB, could be a potential therapeutic approach for cancer treatment. Some pharmaceutical compounds that have been studied and documented to inhibit protein kinase C included midostaurin and enzastaurin[35].

Mitogen-activated protein kinase 9 (MAPK9), also known as c-Jun N-terminal kinase 2 (JNK2), belongs to the MAPK enzyme family. MAPK9 is involved in various cellular regulatory mechanisms such as stress response, apoptosis, proliferation, and differentiation, and has been found to promote tumor formation[36]. Studies on the promotion of tumor formation proposed several mechanisms, such as MAPK9 significantly enhancing proliferation and migration of primary neural crest cells through the Wnt/β-catenin-regulated EMT pathway, or the activation of MAPK9 exacerbating the condition of non-small cell lung cancer[37,38]. Therefore, inhibiting the MAPK9 signaling pathway has the potential to be one of the therapeutic strategies for cancer treatment[39].

The nine selected target proteins with the greatest potential for anticancer effects of CFLEA for molecular docking experiments included TNF, PRKCB, PRCKA, PLCG2, NFKB1, MAPK9, IL1B, IKBKB, and CACNA1A. Among these proteins, PRKCB and MAPK9 exhibited the strongest binding affinity with the compounds 3,4-divanillyltetrahydrofuran (-7.7 and -8.2 kcal/mol) and (*E*)-3,3'-dimethoxy-4,4'-dihydroxystilbene (-7.5 and -7.5 kcal/mol) in the molecular docking experiment. Pyrogallol, which was the compound with the highest content in CFLEA and had potential in cancer treatment, showed the best binding affinity with PRKCB and MAPK9 among the 9 target proteins. However, the binding energy of pyrogallol with these two proteins was relatively low (-5.6 and -5.1 kcal/mol), indicating that pyrogallol may exert its anticancer effects by binding to other proteins rather than PRKCB and MAPK9.

Interestingly, the two proteins, MAPK9 and PRKCB, play important roles in two major cancer pathways, the MAPK signaling pathway and Pathways in cancer (according to KEGG database https://www.kegg.jp/). Specifically, PRKCB is primarily responsible for the JNK and p38 MAP kinase pathway, while MAPK9 is involved in the classical MAP kinase pathway and calcium signaling pathway. Therefore, the JNK and p38 MAP kinase pathway, classical MAP kinase pathway, and calcium signaling pathway could be the main pathways through which CFLEA exerted its inhibitory effects on cancer cell growth.

Finally, cytotoxicity evaluation of CFLEA was conducted on two cancer cell lines, MDA-MB-231 (breast cancer) and HepG2 (liver cancer). In a study by *Lin et al.* in 2013 on the effects of six species of golden

flower leaves on MDA-MB-231 cells, the extract of *Camellia murauchii* exhibited the best activity, with an inhibition rate of approximately 80% at a concentration of 800 μg/mL[40]. Comparing these results, CFLEA showed significantly better cytotoxic effects on MDA-MB-231 cells.

5. CONCLUSION

The 16 phytochemical compounds in CFLEA were identified and their structures were determined using the GC-MS technique. The main active compounds in CFLEA that exhibited anticancer effects were 3,4-divanillyltetrahydrofuran and (*E*)-3,3'-dimethoxy-4,4'-dihydroxystilbene, which were found to bind to the proteins PRKCB and MAPK9 through network pharmacology and molecular docking experiments. The primary anti-cancer pathways of CFLEA may be the JNK and p38 MAP kinase pathway, the classical MAP kinase pathway, and the calcium signaling pathway. Finally, CFLEA was evaluated for its cytotoxic effects and showed good inhibition. Our study contributes to the chemical profile of *Camellia flava* and provides evidence for the anticancer effects of this golden camellia species. However, the study has some limitations, including the limited number of identified phytochemical compounds and the lack of evaluation of the cytotoxicity mechanism of CFLEA at the molecular level. In future studies, we will continue to analyze the chemical composition of CFLEA using modern techniques such as LC-MS and perform molecular biological analyses to further elucidate the anticancer mechanisms of *Camellia flava* leaves.

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Conflict of interest

The authors declare that they have no conflict of interest.

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