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Original article

Exploring *in vitro* and *in silico* Biological Activities of *Calligonum Comosum* and *Rumex Vesicarius*: Implications on Anticancer and Antibacterial Therapeutics

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ABSTRACT

Introduction: The adverse effects of clinically used anti-cancer medication and the rise in resistive microorganisms have limited therapeutic options. Multiple anti-cancer drugs are derived from medicinal herbs which also have shown anti-bacterial effects. This study aimed to identify the optimal extraction solvent for detecting the cytotoxic and anti-bacterial effects of *Calligonum comosum* (*C. Comosum*) and *Rumex vesicarius* (*R. Vesicarius*) extracts. Additionally, the study aimed to identify active metabolites and assess their potential as future drug candidates for anti-cancer and anti-bacterial therapeutics.

Methods: Leaves from both plants were extracted using ethanol, ethyl acetate, chloroform, and water. The cytotoxic effects of the extracts were tested on liver, colon, and breast cancer cell lines. Apoptosis was assessed using High Content Imaging (HCI) and the ApoTox triplex Glo assay. The anti-bacterial effects were determined using agar-well diffusion. Liquid chromatography-mass spectrometry (LC-MS) was used to tentatively identify the secondary metabolites. *In silico* computational studies were conducted to determine the metabolites' mode of action, safety, and pharmacokinetic properties.

Results: The ethanolic extract of *C. Comosum* exhibited potent cytotoxicity on breast cancer cell lines, with IC_{50} values of 54.97 μg/mL and 58 μg/mL for KAIMRC2 and MDA-MB-231, respectively. It also induced apoptosis in colon and breast cancer cell lines. All tested extracts of *C. Comosum* and *R. Vesicarius* demonstrated anti-bacterial activity against *Staphylococcus aureus* and *Escherichia coli*. Seven active metabolites were identified, one of which is Kaempferol 3-O-Glucoside-7-O-Rhamnoside, which showed strong (predicted) anti-cancer activity. Kaempferol 3-O-Glucoside-7-O-Rhamnoside and Quercetin-3-O-Glucuronide also exhibited potential anti-bacterial effects on gram-positive and negative bacteria.

Abbreviations: *C. Comosum*, *Calligonum comosum*; *R. Vesicarius*, *Rumex vesicarius*; *S. aureus*, *Staphylococcus aureus*; *E. coli*, *Escherichia coli*; HCT8, Human ileocecal adenocarcinoma cell line; HepG2, Human liver cancer cell line; MDA-MB-231, Triple-negative breast cancer; KAIMRC2, King Abdullah international medical research center 1 cell line; IC_{50}, Half-maximal inhibitory concentration; P_a, Probability of being active; P_i, Probability of being inactive; NA, Not Active; GPCR, G-protein-coupled receptors; MW, Molecular weight; HBA, Hydrogen-bonding-acceptor; HBD, Hydrogen-bonding-donor; Log P, Predictions of The Pharmacokinetics Parameters; Log Kp, skin permeation; BBB, Blood-brain barrier; POLAR, Polarity; INSOLU, Solubility; LIPO, oral bioavailability; FLEX, Flexibility; INSATU, Saturation; SIZE, size; AR, Androgen receptor; ER, Estrogen receptor; PR, Progesterone receptor; GR, Glucocorticoid receptor; MR, Mineralocorticoid receptor; PXR, Pregnane X receptor; PPAR, Peroxisome proliferator-activated receptors; RXR, Retinoid X receptor; RAR, Retinoic acid receptors; TR, Thyroid receptor; FXR, Farnesoid X receptor; LXR, Liver X receptors; AST, Antimicrobial Susceptibility Testing; HCI, High content imagining.

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Conclusion: Ethanol extraction of *C. Comosum* solubilizes active metabolites with potential therapeutic applications in cancer treatment and bacterial infections. Kaempferol 3-O-Glucoside-7-O-Rhamnoside, in particular, shows promise as a dual therapeutic drug candidate for further research and development to improve its efficacy, safety, and pharmacokinetic profile.
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1. Introduction

Cancer is a multifaceted disease characterized by abnormal cell growth and proliferation. With over a hundred different types, each displaying distinct behavior and response to treatment, finding effective therapies can be challenging (Cooper et al., 2000). The current reliance on cytotoxic drugs, like paclitaxel derived from plants, has drawbacks such as adverse effects including neutropenia, nausea, and hair loss (Fridlender et al., 2015; Oun et al., 2018). To overcome these limitations and develop more efficient treatments, it is crucial to explore novel approaches, such as investigating plant-derived metabolites with potential anticancer properties (Balunas et al., 2005). Additionally, infectious diseases further contribute to the complexity of cancer treatment. Infections can impact cancer progression and outcomes, making it essential to investigate plant-derived metabolites that exhibit promising bioactivity as both anticancer and antibacterial agents.

Infectious diseases are a major global health concern, and antimicrobial resistance has been on the rise (Gan et al., 2020; van Boeckel et al., 2014). Antibiotic resistance reduces the efficacy of antibiotics, leading to the need for higher doses that may cause toxicity (Brauner et al., 2016). This resistance is associated with increased treatment failure and relapse, contributing to rising morbidity and mortality rates (Wong, 2011). To combat microbial resistance, it is crucial to explore novel antimicrobials or further develop existing ones. Additionally, chronic bacterial infections have been identified as a risk factor for cancer, with *Helicobacter pylori* (*H. pylori*) being associated with various malignancies (Hu et al., 2021). Bacterial infections can also promote chemoresistance mechanisms, further complicating cancer treatment (Nanayakkara et al., 2021). Moreover, certain chaperone proteins found in bacteria, such as DnaK, have been implicated in improper protein folding and cellular dysregulation, potentially contributing to neoplasia (Zella et al., 2021).

Traditional medicinal herbs and natural compounds have gained significant attention in recent years due to their therapeutic activity against various diseases including cancer. These natural products have been extensively studied for their ability to induce apoptosis, a crucial process in regulating cell proliferation (Wong, 2011). One of these medicinal plants is the *Polygonaceae* family, also known as the buckwheat family, which has attracted increasing interest due to its potential anticancer activity. This family consists of approximately 1200 species of herbs distributed worldwide, including in Saudi Arabia. These herbs have been traditionally used for various medicinal purposes, including the treatment of inflammatory conditions, jaundice, kidney disease, leprosy, and more (Uddin et al., 2014).

Two members of the *Polygonaceae* family, *Calligonum comosum* and *Rumex vesicarius*, have shown promising anticancer properties. *C. Comosum* has been found to induce apoptosis and cell cycle arrest in triple-negative breast cancer cells, making it a potential treatment option for this aggressive form of breast cancer (Lajter et al., 2013). Additionally, the fruit hairs of *C. Comosum* exhibit antioxidant and anti-proliferative effects against hepatocellular carcinoma cells (Alzahrani, 2021). Moreover, *R. Vesicarius*, has demonstrated anticancer activity against breast, colon, and liver carcinoma cells. It contains bioactive compounds that target antiangiogenic proteins involved in tumor growth. Some of the identified compounds include Propanoic acid, 2-[(trimethylsilyl) oxy]-, trimethylsilyl ester, Butane, 1,2,3-tris(trimethylsiloxy), and Butanedioic acid, bis(trimethylsilyl) ester (Farooq et al., 2020). Furthermore, *C. Comosum and R. Vesicarius* have demonstrated significant antibacterial activity. *C. Comosum* ethanolic, methanolic, and acetonic extracts showed antibacterial activity against *Listeria ivanovii*, *Staphylococcus aureus*, including MRSA, and *Escherichia coli* (Riadh et al., 2011; Soliman et al., 2021). *R. Vesicarius* extracts exhibited antibacterial activity against *E. coli*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa* (Panduraju et al., 2009, 2013).

Further research and exploration of the medicinal properties of plants from the *Polygonaceae* family are necessary to better understand their potential as anticancer and anti-bacterial agents. Thus, the biological activity of *C. Comosum* and *R. vesicarius* remains an important topic in cancer and infectious disease research. Herein, we utilized various solvents to prepare extracts of *C. Comosum* and *R. vesicarius*, identifying the optimal extraction conditions that confer cytotoxic effects on multiple cancer cells and bacterial strains. Furthermore, we identified the metabolites involved and explored the mode of anti-cancer and anti-bacterial actions using multiple *in-silico* approaches.

2. Materials and Methods

2.1. Chemicals and Reagents

Dulbecco's Modified Eagle Medium (DMEM) plus GlutaMax-1 (4.5 g/l D-Glucose, 25 mM HEPES, Pyruvate), fetal bovine serum (FBS), TrypLE™ Express, and Dulbecco's phosphate-buffered saline (PBS) were purchased by Gibco® (Waltham, MA). NP-40 cell lysis buffer was purchased from Thermo Fisher Invitrogen (Carlsbad, CA). Ethyl acetate, ethanol, and chloroform were obtained from Honeywell Riedel-de Haen (Seelze, Germany). DMSO was procured from Calbiochem (San Diego, CA). The solvents were chromatography-grade or equivalent. Purified carbon dioxide (CO_2) gas was provided by Saudi Industrial Gas (Dammam, Saudi Arabia). Ultra-pure water was produced using a Millipore (Billerica, MA) system with a resistivity reading of 18.2 MΩ·cm at 25 °C.

2.2. Plant Extract Preparation and Metabolite Identification

2.2.1. Collection and Authentication of C. Comosum and R. Vesicarius

The traditional herbal medicines were wildcrafted around Riyadh city in the Central Region, Kingdom of Saudi Arabia. The herbs were identified, classified, and approved by a Professor of Botany from King Saud University. The plants' parts were rinsed with filtered water and then dried under warm air. Using an electric-motor grinder, *R.Vensicarius* atrial and *C. Comosum* leaves were fine powdered and then placed in the dark at room temperature until extraction. Moreover, voucher samples were deposited at King Saud University (Herbium, Department of Botany and Microbiology) with accession number 24,325 for *R. Vesicarius* and 24,328 for *C. Comosum.*

2.2.2. Extraction of C. Comosum Leaf and R. Vesicarius Arial

Approximately 500.0 mg of each dried *C. Comosum* leaf and *R. Vesicarius* arial was extracted using 10.0 mL of high-purity ethanol,

ethyl acetate, chloroform, and water under high-power sonication using a Sonics (Newton, CT, USA) Vibra-Cell Ultrasonic Liquid Processor (Model GEX-130 probe-sonicator) for 90 minutes. The sonicated extract was filtered using a Sartorius stedim biotech (Göttingen, Germany) quantitative ashless paper filter under gravity flow and dried in an incubator set at 40.0C. The remaining dried pellet residue was weighed and reconstituted with 100.0 to 500.0 µL of DMSO by vortex until completely dissolved. The reconstituted extract was stored at a cool temperature in the dark until use. The averaged extraction yield percentage is presented in Table S1 (Supplementary Table).

2.2.3. Metabolite Identification Using ESI-LC-QTOF

The method of separation and identification of metabolites in *C. Comosum* and *R. Vesicarius* extracts were carried out utilizing an Agilent 1260 Infinity HPLC system connected to an Agilent 6530 quadrupole time-of-flight (Q-TOF). Using mobile-phase A (0.1% formic acid in water) and mobile-phase B (0.1% formic acid in methanol), the separation was conducted by using an Agilent SB-C18 column (4.6 mm 150 mm, 1.8 m) with the following elution gradient: 0–2 minutes, 5% B; 2–17 minutes, 5–100% B; 17–21 minutes, 95% B; 21–25 minutes, 5% B. The injection volume was 10 µL, and the flow rate was set at 250 µL/min. The gas temperature was set at 300 °C, the gas flow was 8 µL/min, the nebulizer pressure was 35 psi, and sheath gas was used. The scanning range was set to 50–800 (*m*/*z*), and was performed utilizing an Agilent 1260 Infinity HPLC system connected to an Agilent 6530 quadrupole time with a gas flow rate of 8 µL/min, a nebulizer pressure of 35 psi, a sheath gas temperature of 350 °C, and a sheath gas flow rate of 11 µL/min. Agilent MassHunter (version B.06.00) qualitative analysis software was used to produce the data.

2.3. Anti-cancer and anti-bacterial activity investigations

2.3.1. MTT Cytotoxic Assay

The MTT assay was utilized to examine *C. Comosum* and *R. Vesicarius* anti-proliferative activity against liver cancer cell line (HepG2), breast cancer cell lines (KAIMRC2 and MDA-MB-231), and colorectal cancer cell lines (HCT8), purchased from ATCC, USA (Ali et al., 2021). All the cells used in the study were between passage numbers 8 and 12. Cells were grown in T75 flasks and upon 90% confluency trypsinized using Triplex reagent for 5 minutes in an incubator. Cells were then centrifuged and counted. 100 µL of growth media with 5×10^3 cells/well were seeded into 96-well plates. Post 24 hours incubation, the cells were treated with varying concentrations of each substance ranging from 0 to 500 µg/ml. Each well received 50 mL of serum-free media and 50 mL of MTT solution after aspirating the media. Following a three-hour incubation period at 37 °C, each well received 150 µL of the MTT solvent. After that, plates were shaken for 15 minutes on an orbital shaker while being covered in foil. At OD590 nm, the absorbance was finally measured after 1 hour. From the dose–response curve, half-maximal inhibitory concentration (IC_{50}) values were obtained for each extract. Experiments were carried out in triplicate.

2.3.2. High Content Imaging (HCI)

KAIMRC2 and HCT8 cells were seeded at 20,000 cells per well in a 96-well plate. For 48 hours, the cells were treated with *C. Comosum* ethanol extract and the positive control mitoxantrone. The treatment consisted of four graduated concentrations: 46.87 µg/mL, 93.75 µg/mL, 187.5 µg/mL, and 375 µg/mL. Following treatment, the cells were stained for 45 minutes at 37 °C and 5% CO_2 with YO-PRO™-1 Iodide (491/509, Cat. no. Y3603, ThermoFisher Scientific) at 2 µg/mL, HOECHST33342 (355/465, Invitrogen™ H3570) at 2.5 µg/mL, and Propidium Iodide (533/617, Invitrogen™ P3566) at 2.5 µg/mL. Plates were scanned on a Molecular Devices ImageXpress® Microsystem, and the collected image data were processed using Molecular Devices' MetaXpress® software, Downingtown, PA, USA. The MetaXpress software's Cell Health module was used to calculate the % viability of live and dead cells. Experiments were carried out in triplicate.

2.3.3. ApoTox Triplex Glo

The Promega ApoTox-Glo® triplex assay was performed following the manufacturer's instructions. The cells were treated for 24 hours with different doses of *C. Comosum* in ethanol extract after being incubated for 48 hours at 37 °C. After that, each well received 100 µL of the Viability and Cytotoxicity Reagent, which was then briefly mixed on an orbital shaker. The plates were incubated at 37 °C for 30 minutes. Using the Envision plate reader, the fluorescence was then measured at the following two-wavelength sets: 400Ex/505Em (Viability) and 485Ex/520 Em (Cytotoxicity) (Perkin Elmer). Following the addition of the Caspase-Glo® 3/7 Reagent (100 µL/well), plates were briefly shaken on an orbital shaker (300 to 500 rpm for 30 seconds), and then incubated for an additional 30 minutes at room temperature. The luminescence associated with caspase 3/7 activation was evaluated with an assay to determine the degree of apoptosis Imagine using a Perkin Elmer plate reader for about 30 seconds, followed by an additional 30 minutes of room temperature incubation.

2.3.4. Antibacterial Screening

The prepared extract was tested for antibacterial susceptibility Test (AST) against Gram-positive bacteria: methicillin-resistant *S. aureus* (MRSA) and Gram-negative *E. coli* using the agar well diffusion method. The Bio-house medical lab in Riyadh, Saudi Arabia, donated the microbes. Pure cultures of each strain were subcultured on nutrient agar medium (Oxoid) and cultivated at 37 °C for 24 hours. For 0.5 McFarland standard bacterial suspensions (1.5×10^8 CFU/mL) prepared in saline, direct colonies from each culture were employed. When test plates were cultivated with tested microorganisms, 40 µl of each extract was placed into each well that was produced in the agar plates independently and kept for one hour under aseptic conditions to allow easy diffusion of the tested substance in the agar. As a negative control, distilled water was used, and Ampicillin was employed as a positive control. According to the Clinical and Laboratory Standards Institute, plates were maintained at 37 °C for 24 hours (Weinstein et al., 2020). The clean area around each well was measured in millimeters and presented as the inhibition zone, demonstrating the efficacy of the tested medicines.

2.4. Statistical Analysis

Based on three different experiments, the data are presented as the mean standard deviation (SD). An unpaired student *t*-test was performed to compare the two groups. The IC_{50} values were calculated using GraphPad Prism 9 (San Diego, California USA), and Microsoft Excel was used for the ApoTox Triplex Glo data. The statistical significance level was set at P 0.05.

2.5. Computational Investigations

2.5.1. Anti-cancer Activity Predictions

The 2D chemical structures of the discovered metabolites were utilized as input for the Prediction of activity spectra for substances (PASS) website (https://way2drug.com/PassOnline/) to predict anti-cancer activity. The predicted outcomes provide an activity score abbreviated as P_a indicating that the metabolite is active if the score is greater than 0.7, moderately active if the score

is between 0.5 and 0.7, and inactive if the score is less than 0.5 (Filimonov et al., 2014).

2.5.2. Molecular Target Predictions

Molinspiration Chemoinformatics tools (https://www.molinspiration.com/cgi-bin/properties) were used to predict the molecular targets for the identified metabolites, which provide an estimated bioactivity score for metabolites against a variety of biological targets such as G protein-coupled receptors (GPCR), ion channels, nuclear receptors, kinases, proteases, and enzymes. The bioactivity score value categorizes substances as active (if the anticipated score is equal to or more than 0.00), moderately active (scoring between 0.50 and 0.00), or inactive (score less than 0.50) against the examined targets.

2.5.3. Evaluation of Pharmacokinetic Parameters

Using the Swiss ADME webserver https://www.swissadme.ch/, multiple pharmacokinetic parameters were predicted for the discovered metabolites in order to analyze and quantify drug-likeness qualities for oral bioavailability Lipinski's rule of five (ROF), central nervous system (CNS) distribution, and projected inhibition of major cytochromes P450 enzymes are among these evaluated parameters (Lipinski et al., 2001).

2.5.4. Toxicity Assessment

Several toxicity endpoints, including hepatotoxicity, cytotoxicity, carcinogenicity, mutagenicity, and immunotoxicity, were assessed for the identified metabolites using the Prediction of Toxicity of Chemicals (ProTox-II) webpage (https://tox-new.charite.de/protoxII/) (Banerjee et al., 2018).

2.5.5. Cardiac Toxicity Prediction

Using the pred-hERG 4.2 website (https://predherg.labmol.com.br/), the identified metabolites were computationally screened against the blocking of the hERG K^+ channels, which have been associated with cardiac damage. The produced output categorizes the compounds as non-blockers, weak/moderate blockers, or strong blockers, using probability maps that reflect the atoms' contributions to hERG K^+ channel blockage. The intensity, contour lines, and color all contribute to the blockage (green), non-blockage (red), or none at all (grey) (Braga et al., 2015).

2.5.6. Endocrine Disruptome Predictions

The metabolites were computationally evaluated and assessed for binding to 14 nuclear receptors, including androgen receptor (AR); estrogen receptors (ER) and; glucocorticoid receptor (GR); liver X receptors (LXR), and; mineralocorticoid receptor (MR); peroxisome proliferator-activated receptors (PPAR), and; progesterone receptor (PR); retinoid X receptor (RXR); and thyroid receptor. The online server uses the docking interface for target systems (DoTS) technique to dock the chemicals into the fourteen receptors, and the findings are classified into four main classifications based on the binding probability to the receptor: red, orange, yellow, and green. The color red denotes a high probability of binding, orange/yellow indicates a medium probability of binding, and green indicates a low probability of binding (Kolšek et al., 2014).

3. Results

3.1. MTT Cytotoxic Assay

The anticancer assay was designed to test the influence of plant extracts on cell proliferation. According to preliminary screening assays conducted by the American National Cancer Institute (NCI), extracts achieving 50% cytotoxic effect at a concentration of 10 μg/ml are considered strongly active, those between 11 and 100 μg/ml are considered moderately active, and those above 100 μg/ml are considered non-active (K. (Ed.) HOSTETTMANN, 1991). The ethanol, ethyl acetate, chloroform, and water extracts of the plants were tested for cytotoxic activity using the MTT assay comparing it to mitoxantrone as a positive control in liver cancer (HepG2) and colorectal cancer cells (HCT8). As shown in Fig. 1, all the extracts had IC_{50} values above 100 μg/ml, which is considered inactive. The half-maximal inhibitory concentration IC_{50} (μg/ml) of each extract was stated by plotting a dose–response curve. The ethanolic and aqueous *C. Comosum* extracts had the lowest IC_{50} values and, as such, were used for further testing the cytotoxic effects in breast cancer cell lines, KAIMRC2 and MDA-MB-231 (Fig. 2). As presented in Table 1, the IC_{50} values of ethanolic *C. Comosum* extract were 54.97 μg/ml and 58.39 μg/ml on KAIMRC2 and MDA-MB-231, respectively, which indicated moderate cytotoxic activity.

3.2. HCI- Apoptosis Assay

Breast cancer KAIMRC2 and colorectal cancer HCT8 cells were treated with increasing concentrations of the ethanolic extract of *C. Comosum* and mitoxantrone was used as a positive control. The cells were stained with HOECHST33342 to visualize the nucleus (blue), Propidium Iodide to visualize dead cells (red), and Yo Pro to visualize apoptotic cells (green). In the KAIMRC2 cells, green apoptotic cells were evident at 187.5 μg/ml and 375 μg/ml concentrations. Whereas very low concentrations of 46.87 μg/ml and 93.75 μg/ml demonstrated no apoptotic effect in cell growth but very few apoptotic cells as compared to DMSO control (Fig. 3). However, in HCT8-treated cells, green apoptotic cells were visualized at lower doses of 93.75 μg/ml (Fig. 4).

3.3. ApoTox Triplex Glo Assay

The ApoTox-Glo® triplex assay was employed to investigate the effect of the ethanolic extract of *C. Comosum* on the viability, cytotoxicity, and apoptosis of KAIMRC2 and HCT8 cancer cells. This assay measures caspase activity, which is utilized as a marker for apoptosis within cells, using a luminogenic peptide substrate for caspase 3/7. Treatment of the ethanolic extract of *C. Comosum* induced apoptosis and cytotoxicity in a dose-dependent manner in both the cell lines and as expected there was a dose-dependent decline in cell viability, as shown in Fig. 5.

3.4. Antibacterial Activity

The study was designed to assess and screen *R. Vesicarius* and *C. Comosum* extracts as antibacterial agents against *E. coli* and *S. aureus,* comparing it to the positive control, ampicillin, and negative control, water. However, our study was limited due to the accidental loss of some extracts, including ethanolic extracts of both plants and the ethyl acetate and chloroform extracts of *C. Comosum*. A concentration of 1 mg/ml was prepared from each extracted plant material in DMSO for the antibacterial assay. As expected, the water showed no activity against the tested bacteria (data not shown). Both plant types extracted with different solvents showed significantly high activity against *S. aureus* and *E. coli* compared to water, and a difference was observed compared to ampicillin ($P < 0.0001$), as shown in Fig. 6. The activity of each extract type was more than 50% of ampicillin activity against *S. aureus*; however, the effect against *E. coli* was varied according to the extraction solvent, where the activity for both plant aqueous extracts were approximately 50% of ampicillin activity but for ethyl acetate and chloroform extracts the activity were lower than 50% of the ampicillin activity.

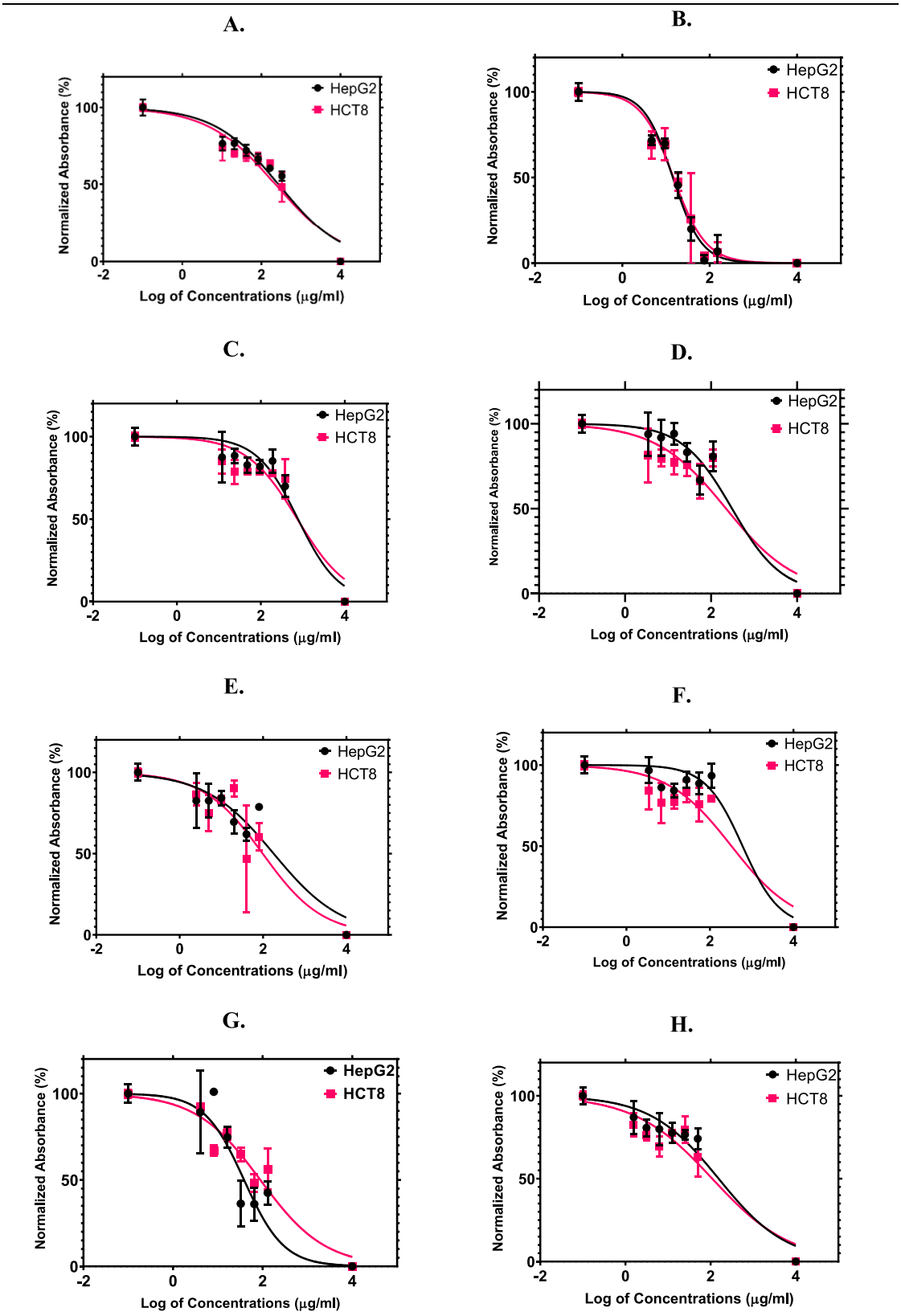


Fig. 1. MTT Assay Against Cancer Cell Lines HepG2 and HCT8 of (A) *R. Vesicarius* in Ethanol, (B) *C. Comosum* in Ethanol, (C) *R. Vesicarius* in Chloroform, (D) *C. Comosum* in Chloroform, (E) *R. Vesicarius* in Ethyl Acetate, (F) *C. Comosum* in Ethyl Acetate, (G) *R. Vesicarius* in Water, (H) *C. Comosum* in Water.

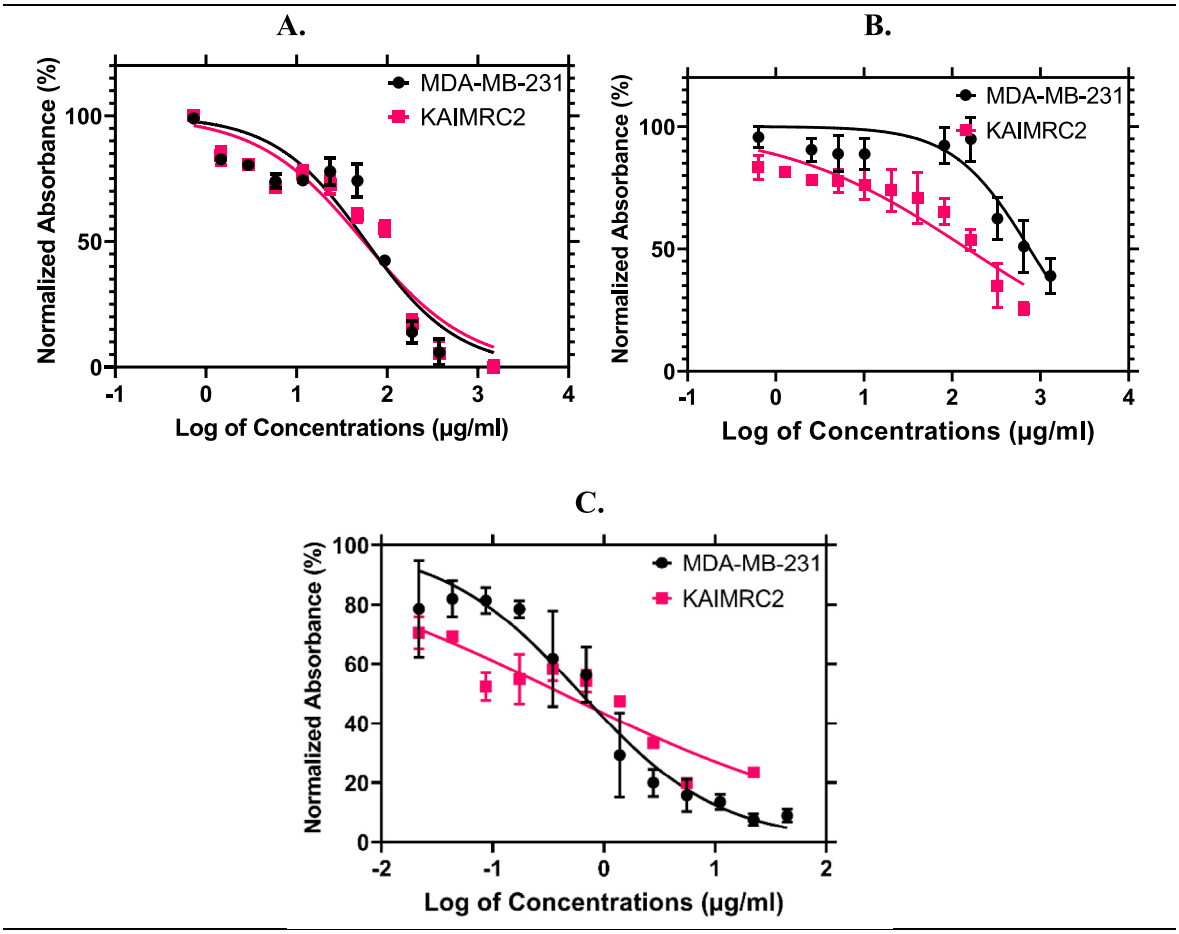


Fig. 2. MTT Assay Against Breast Cancer Cell Lines (KAIMRC2 and MDA-MB-231) of (A) *C. Comosum* in Ethanol (B) *C. Comosum* in Water, (C) Mitoxantrone.

Table 1
The IC_{50} Values of *C. Comosum* and *R. Vesicarius* Extracts Against the Following Cancer Cell Lines.

Extract	Cell Lines			
	KAIMRC2	MDA-MB-231	HCT8	HepG2
	IC_{50} (µg/ml)			
Mitoxantrone	0.4215	0.6228	N/A	N/A
C. Comosum in ethanol	54.97	58.39	147.4	143.1
C. Comosum in Chloroform	N/A	N/A	2227	3064
C. Comosum in Ethyl Acetate	N/A	N/A	1095	1545
C. Comosum in Water	148.9	754.2	888.9	385.3
R. Vesicarius in ethanol	N/A	N/A	2271	2713
R. Vesicarius in Chloroform	N/A	N/A	7416	7680
R. Vesicarius in Ethyl Acetate	N/A	N/A	980.5	1796
R. Vesicarius in Water	N/A	N/A	3290	6063

N/A: not available.

3.5. LC-QTOF-MS

Three replicates of ethanolic extract of the *C. Cosmosum* were subjected to total ion current spectra (TIC), and the qualitative and quantitative analysis software program Mass Hunter (Agilent Technologies) was used for data analysis. After conducting a mass screening on the below spectrum (Fig. 7), the chemical features were extracted from the LC-MS data using the Molecular Features Extraction (MFE) algorithm and the recursive analysis workflow. Features have been extracted by screening the detected nodes at various retention times per minute, with a minimum intensity of 6,000 counts, and aligned with previously detected compounds considering adducts ($[M + H]^+$, and $[M-H]^-$). The tentatively identified compounds are Catechin, (Markham, 1982), Dehydrodicatechin A, (Markham, 1982), Quercetin-3-O-Glucuronide, (Markham, 1982), Tamgermanetin, (Jayaprakasha et al., 2006), Methyl Gallate, (Jayaprakasha et al., 2006), 5-(3-Hydroxyphenyl)Pentanoic Acid, (Jayaprakasha et al., 2006), and Kaempferol 3-O-Glucoside-7-O-Rhamnoside, (el Sayyad and Wagner, 1978), Means *m/z* implies measured *m/z*.

3.6. In silico Computational Investigation

3.6.1. Anti-cancer Activity Prediction

PASS Online webserver was used to predict the anti-cancer activity of the seven tentatively identified metabolites of the ethanolic *C. Cosmosum* extract. The highest P_a values and

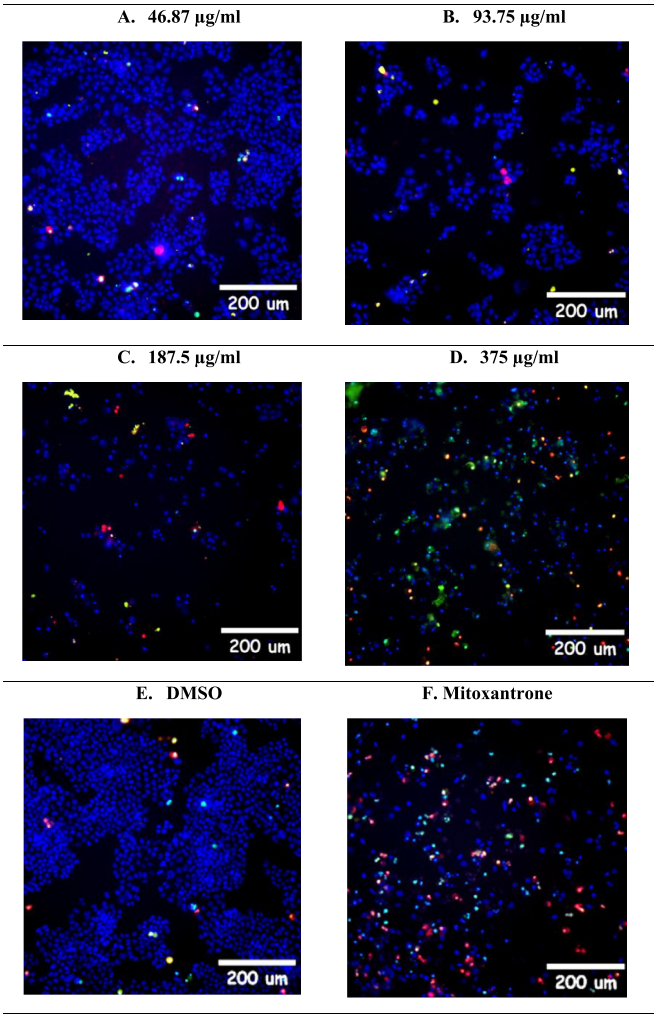


Fig. 3. HCl-based Live/Dead/Apoptosis Assay of *C. Comosum* in Ethanol on KAIMRC2 Cells Compared with the Controls Including DMSO and Mitoxantrone (5.56 µg/ml). HOECHST33342 (blue), and Propidium Iodide (red). Yo Pro (green).

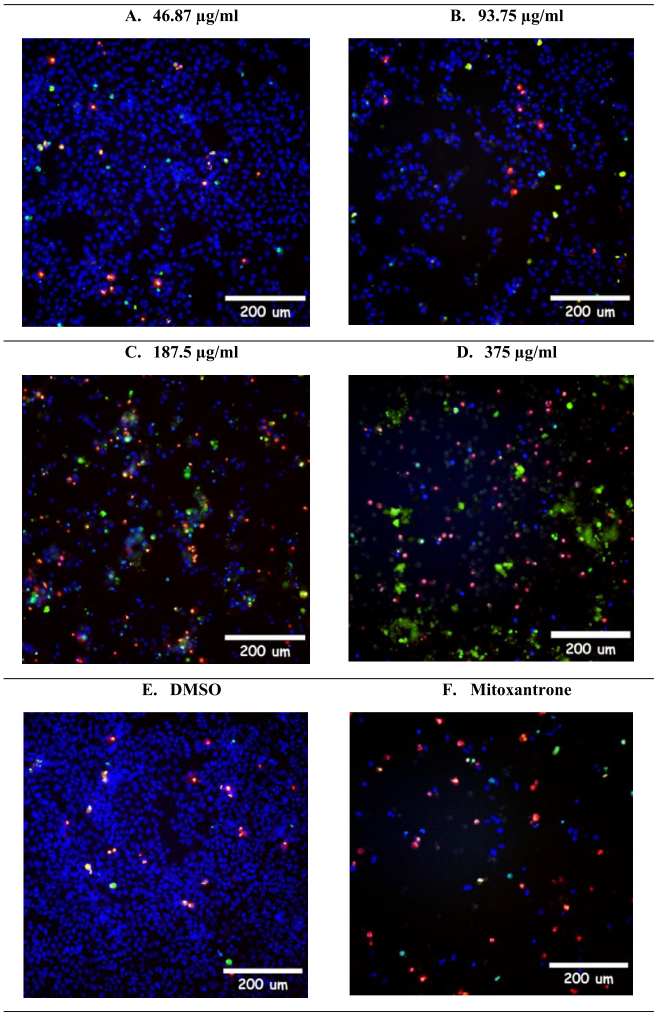


Fig. 4. HCl-based Live/Dead/Apoptosis Assay of *C. Comosum* in Ethanol on HCT8 Cells Compared with the Controls Including DMSO and Mitoxantrone (5.56 µg/ml). HOECHST33342 (blue), and Propidium Iodide (red). Yo Pro (green).

lowest P_i values indicate that the compound could exhibit the highest anti-cancer activity when tested experimentally. As shown in Table 2, Dehydrodicatechin A possessed the highest anti-cancer activity with a P_a value of 0.941 followed by Kaempferol 3-O-Glucoside-7-O-Rhamnoside with a P_a value of 0.842. The remaining metabolites showed lower P_a values which suggests lower anti-cancer activity. Moreover, the anti-bacterial predictions suggest that Quercetin-3-O-Glucuronide and Kaempferol 3-O-Glucoside-7-O-Rhamnoside could exhibit a 60% probability of being active anti-bacterial agents if tested experimentally.

3.6.2. Prediction of the Bioactivity Score

The bioactivity of the metabolites was predicted by using the Molinspiration web server, as represented in Table 3. If the bioactivity score at a specific target is greater than 0.00, then the result suggests that metabolite is considered to be potentially bioactive. Our predictions and results demonstrated that metabolite Catechin showed activity at all targets, with the highest activity as a nuclear receptor ligand. In addition, metabolite Dehydrodicatechin A exhibited potential activity for all targets except as an ion channel modulator and kinase inhibitor. Moreover, metabolites Quercetin-3-O-Glucuronide and Tamgermanetin revealed potential activity as G protein-coupled receptor ligands, nuclear receptor ligands, and enzyme inhibitors. While metabolite Methyl Gallate demonstrated no activity at any target; however, metabolite 5-(3-Hydroxyphenyl) Pentanoic Acid and Kaempferol 3-O-Glucoside-7-O-Rhamnoside possessed potential activity as enzyme inhibitors.

Moreover, the SWISS target prediction webserver was utilized as an additional tool to evaluate the potential molecular targets that could be modulated by the metabolites. The prediction results are summarized as a pie chart shown in Fig. S1. First, Catechin exhibited no similarity in the database, which resulted in no prediction. The pie chart of Dehydrodicatechin A showed a probable protease target of 46.7% and a kinase target of 26.7%. Quercetin-3-O-Glucuronide demonstrated lyase, enzyme targets of 26.7%, and 20% of family A G protein-coupled receptor. Tamgermanetin's pie chart revealed 46.7% kinase, 20% protease, hydrolase, and oxidoreductase targets of 13.3%. Methyl Gallate showed a probable lyase target of 80%, and enzyme, transferase, and secreted protein targets of 6.7%. 5-(3-Hydroxyphenyl) Pentanoic Acid pie chart exposed an enzyme target of 26.7%, kinase 13.3%, and protease 13.3%. Kaempferol 3-O-Glucoside-7-O-Rhamnoside exhibits enzyme and lyase probable targets of 26.7% and 20% of family A G protein-coupled receptors.

3.6.3. Pharmacokinetic Parameters Evaluation

Based on Lipinski's rule of five, a metabolite with poor oral absorption is most likely to possess a molecular weight (MW) of more than 500, number of H-bond acceptors (HBA) of more than

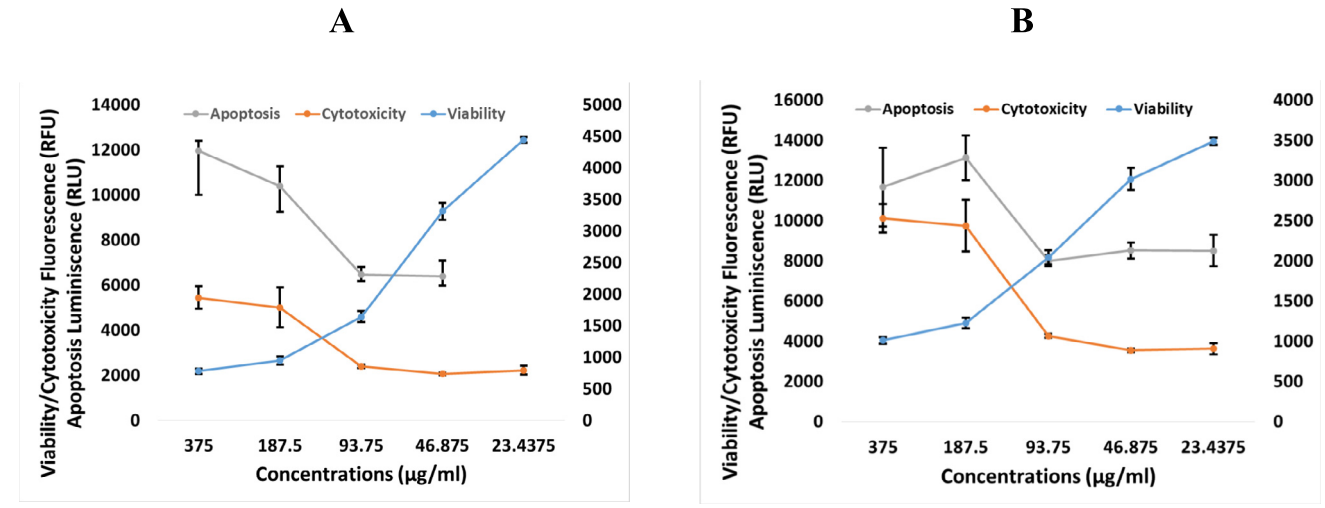


Fig. 5. The Effect of *C. Comosum* in Ethanol on Breast Cancer KAIMRC2 (A) and HCT8 (B) Cell Apoptosis, Cytotoxicity, and Viability.

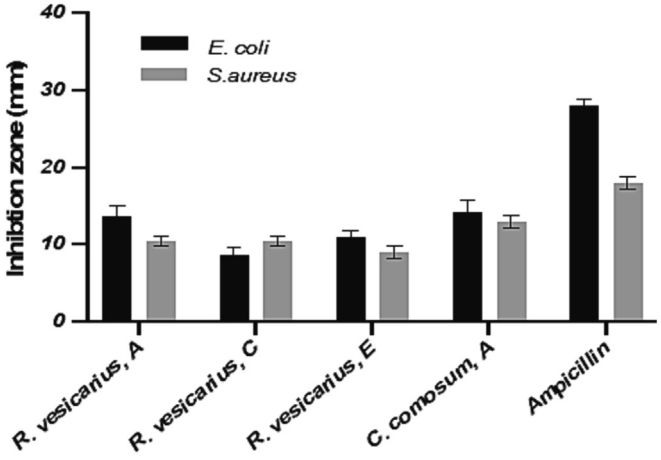


Fig. 6. Inhibition Zone (mm) as Antibacterial Activity of *R. Vesicarius* and *C. Comosum* Extracts Using Water (A), Chloroform (C), and Ethyl Acetate (E) As Well As the Ampicillin.

10, number of H-bond donors (HBD) of more than 5 and lipophilicity MlogP more than 4.15. As illustrated in Table 4, Catechin, Quercetin-3-O-Glucuronide, Tamgermanetin, Methyl Gallate, and 5-(3-Hydroxyphenyl) Pentanoic Acid demonstrated a molecular weight of less than 500 g/mol. Moreover, Catechin, Tamgermanetin, Methyl Gallate, and 5-(3-Hydroxyphenyl) Pentanoic Acid exhibit HBA less than ten, and HBD less than five except Catechin possessed HBD more than five. According to MlogP all the metabolites are within the range of less than 4.15, as shown in Table 4. Moreover, high solubility causes good absorption and oral bioavailability, and based on SwissADME, all the metabolites were within the acceptable range of (−6.5 to 0.5). Skin permeability which is assessed by log K_p is a term that refers to how easily a substance can penetrate the stratum corneum. According to SwissADME, the more negative the log K_p, the less skin permeant the compound. Thus, Dehydrodicatechin A, Quercetin-3-O-Glucuronide, and Kaempferol 3-O-Glucoside-7-O-Rhamnoside possessed skin permeability with log K_p of −8.98, −8.78, and −10.35, respectively. All metabolites did not penetrate the blood-brain barrier (BBB) except 5-(3-Hydroxyphenyl) Pentanoic Acid. As demonstrated in Table 4, all metabolites were not predicted to inhibit CYP enzymes except Dehydrodicatechin A predicted to inhibit CYP2C9, and Tamgermanetin predicted to inhibit CYP2D6 and CYP3A4. Furthermore, based on the Lipinski rule of five, the compounds that violated drug-likeness properties are Catechin, Tamgermanetin, Methyl Gallate, and 5-(3-Hydroxyphenyl) Pentanoic Acid.

The oral bioavailability graphs of the identified metabolites were predicted and summarized in Fig. S2. The expected properties for the evaluated molecules are represented by the red line while the recommended range for the attributes to be orally active is illustrated by the pink-shaded zone. Polarity (POLAR), solubility (INSOLU), lipophilicity (LIPO), flexibility (FLEX), saturation (INSATU), and size (SIZE) are among the parameters. None of the metabolites were predicted to be orally bioavailable except 5-(3-Hydroxyphenyl) Pentanoic Acid.

3.6.4. Toxicity Assessment

The ProTox-II website evaluates chemical toxicity, including oral toxicity, organ toxicity (hepatotoxicity), and toxicological endpoints like mutagenicity, carcinogenicity, and immunotoxicity. As demonstrated in Table 5, all metabolites are considered safe except Quercetin-3-O-Glucuronide, Tamgermanetin, and Kaempferol 3-O-Glucoside-7-O-Rhamnoside which were predicted to be immunotoxin; moreover, Quercetin-3-O-Glucuronide is predicted to be carcinogenic.

3.6.5. Prediction of Cardiac Toxicity

Lethal cardiac arrhythmias are directly related to the blocking of hERG K^+ channels. One of the most significant anti-targets to be considered at the beginning of the drug development process is hERG because of this channel's infamous ligand promiscuity. The pred-hERG 4.2 webserver was used to predict cardiotoxicity, as shown in Table 6**,** Catechin, Dehydrodicatechin A, and Tamgermanetin were predicted to have potential cardiotoxicity with the confidence of 50%, 60%, and 50%, respectively. On the probability map, pink atoms denote contributions to hERG blockage, green atoms denote contributions to hERG blockage's reduction, and gray atoms denote the position of a split between positive (green) and negative (pink) contributions.

3.6.6. Predictions of Endocrine Disruptome

Predicting a metabolite's potential for endocrine disruption is a necessary undertaking task. The following 14 different human

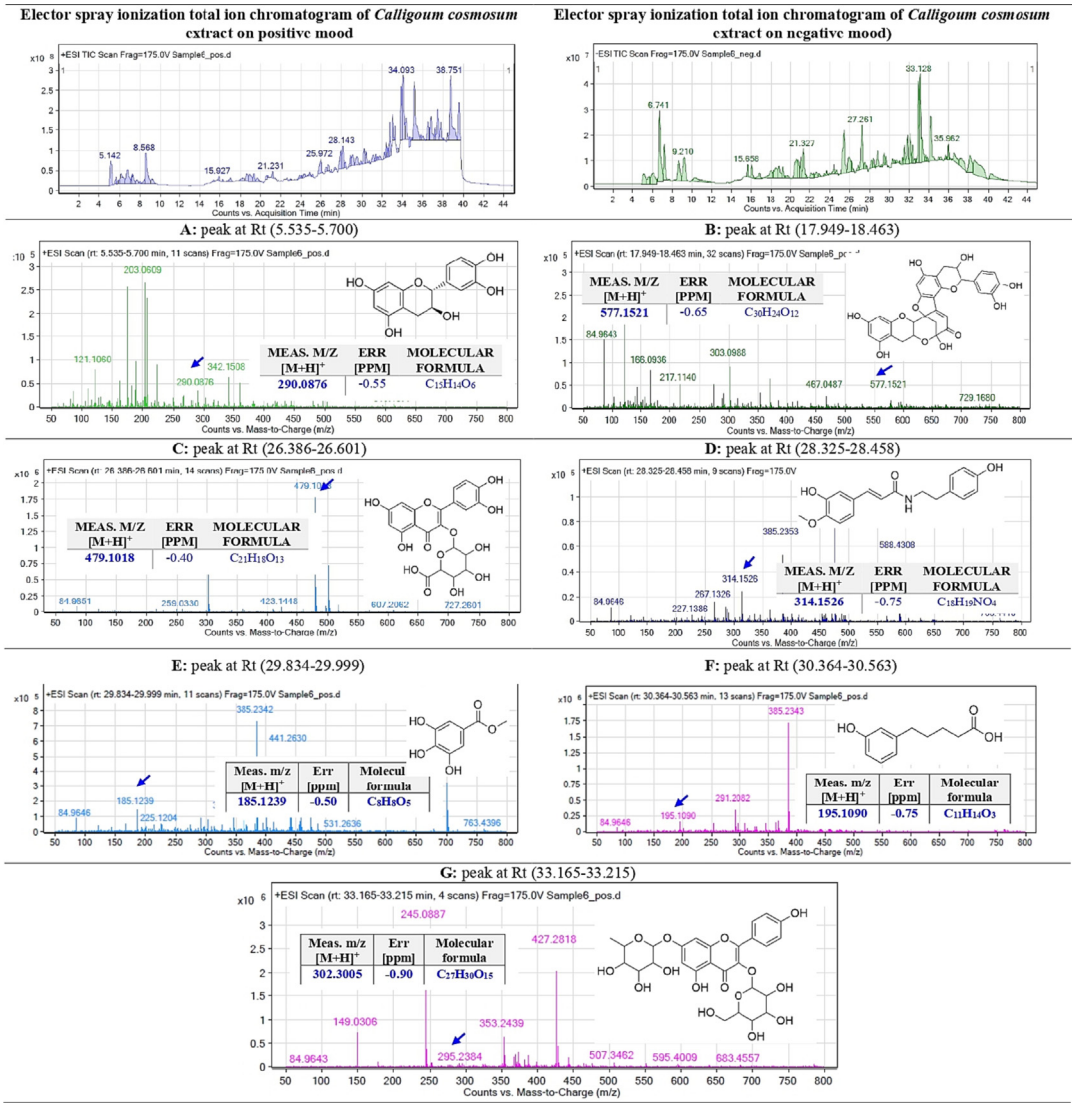


Fig. 7. Base Peak Chromatogram of *C. Comosum* Ethanolic Extract: A Visual Representation of the Chromatographic Profile Highlighting the Dominant Peaks Observed in The Ethanolic Extract of C. *Comosum*. Means *m*/*z* implies measured *m*/*z*.

Table 2
The Prediction of Anti-cancer Activity of Identified Metabolites from Ethanolic *C. Cosmosum* Extract.

Biological Activities for Metabolites	Anti-cancer Activity		Anti-bacterial Activity	
	P_a	P_i	P_a	P_i
Catechin	0.795	0.005	0.320	0.053
Dehydrodicatechin A	0.941	0.004	0.399	0.030
Quercetin-3-O-Glucuronide	0.788	0.013	0.640	0.007
Tamgermanetin	0.453	0.063	0.184	0.133
Methyl Gallate	0.328	0.035	0.349	0.043
5-(3-Hydroxyphenyl) Pentanoic Acid	0.378	0.129	0.210	0.108
Kaempferol 3-O-Glucoside-7-O-Rhamnoside	0.842	0.008	0.645	0.007

P_a: Probability of being active. P_i: Probability of being inactive.

nuclear receptors are docked with compounds including androgen receptor (AR), estrogen receptors α (ER α) and β (ER β), glucocorticoid receptor (GR), liver X receptors α (LXR α) and β (LXR β) mineralocorticoid receptor (MR); peroxisome proliferator-activated receptors α (PPAR α), β/δ (PPAR β), and (PPAR γ), progesterone receptor (PR), retinoid X receptor α (RXR α), and thyroid receptors α (TR α) and β (TR β). The results are divided into four main classes including red, orange, yellow, and green. Red color denotes a high likelihood of binding, orange/yellow color denotes a medium likelihood of binding, and green color denotes a low likelihood of binding. As shown in Fig. S3, all metabolites are predicted to bind to androgen receptors except

Table 3
Molecular Target Prediction of Ethanolic *C. Cosmosum* Identified Metabolites Using Molinspiration Webserver.

Biological Metabolites	GPCR Ligand	Ion Channel Modulator	Kinase Inhibitor	Nuclear Receptor Ligand	Protease Inhibitor	Enzyme Inhibitor
Catechin	0.41	0.14	0.09	0.60	0.26	0.47
Dehydrodicatechin A	0.10	−0.32	−0.22	0.33	0.10	0.15
Quercetin-3-O-Glucuronide	0.08	−0.06	−0.01	0.33	−0.05	0.42
Tamgermanetin	0.10	−0.06	−0.16	0.05	−0.05	0.02
Methyl Gallate	−0.89	−0.36	−0.89	−0.72	−1.03	−0.36
5-(3-Hydroxyphenyl) Pentanoic Acid	−0.13	0.07	−0.68	0.00	−0.32	0.14
Kaempferol 3-O-Glucoside-7-O-Rhamnoside	−0.04	−0.45	−0.10	−0.05	−0.04	0.15

Table 4
Predictions of the Pharmacokinetics Parameters for Identified Metabolites from Ethanolic *C. Cosmosum* Extract Using SwissADME Computational Tool.

Properties	Parameters	Catechin	Dehydrodicatechin A	Quercetin-3-O-Glucuronide	Tamgermanetin	Methyl Gallate	5-(3-Hydroxyphenyl) Pentanoic Acid	Kaempferol 3-O-Glucoside-7-O-Rhamnoside
Physicochemical Properties	MW (g/mol)	290.27	576.50	478.36	313.35	184.15	194.23	594.52
	HBA	6	12	13	4	5	3	15
	HBD	5	7	8	3	3	2	9
Lipophilicity	iLOGP	1.47	2.09	0.75	2.65	0.97	1.53	1.80
Log Po/w	XLOGP3	0.36	1.18	0.61	2.10	0.86	2.26	−0.59
	MLOGP	0.24	−0.32	−2.60	1.89	0.18	1.96	−3.43
Absorption	Water solubility	−2.14 (Soluble)	−4.11(Moderately soluble)	−1.04(Soluble)	−4.78 (Moderately soluble)	−0.75 (Soluble)	−2.82(Soluble)	−0.88(Soluble)
	GI	High	Low	Low	High	High	High	Low
	Log Kp (skin permeation) cm/s	−7.82	−8.98	−8.78	−6.72	−6.81	−5.88	−10.35
Distribution	BBB permeant	No	No	No	No	No	Yes	No
Metabolism	CYP1A2 inhibitor	No	No	No	No	No	No	No
	CYP2C19 inhibitor	No	No	No	No	No	No	No
	CYP2C9 inhibitor	No	Yes	No	No	No	No	No
	CYP2D6 inhibitor	No	No	No	Yes	No	No	No
	CYP3A4 inhibitor	No	No	No	Yes	No	No	No
Drug likeness	Lipinski	Yes; 0 violation	No; 3 violations: MW > 500, NorO > 10, NHorOH > 5	No; 2 violations: NorO > 10, NHorOH > 5	Yes; 0 violation	Yes; 0 violation	Yes; 0 violation	No; 3 violations: MW > 500, NorO > 10, NHorOH > 5

Table 5
Toxicity Profiles of Identified Metabolites from *C. Cosmosum* in Ethanol Extract Using ProTox-II Online Tool.

Metabolite Name	Classification				
	Organ Toxicity (% Probability)	Toxicity Endpoint Probability (% Probability)			
	Hepatotoxicity	Carcinogenicity	Immunotoxicity	Mutagenicity	Cytotoxicity
Catechin	Inactive(0.72)	Inactive(0.51)	Inactive(0.96)	Inactive(0.55)	Inactive(0.84)
Dehydrodicatechin A	Inactive(0.72)	Inactive(0.56)	Inactive(0.67)	Inactive(0.64)	Inactive(0.76)
Quercetin-3-O-Glucuronide	Inactive(0.75)	Active(0.50)	Active(0.58)	Inactive(0.68)	Inactive(0.91)
Tamgermanetin	Inactive(0.77)	Inactive(0.57)	Active(0.97)	Inactive(0.61)	Inactive(0.98)
Methyl Gallate	Inactive(0.62)	Inactive(0.63)	Inactive(0.98)	Inactive(0.91)	Inactive(0.93)
5-(3-Hydroxyphenyl) Pentanoic Acid	Inactive(0.68)	Inactive(0.72)	Inactive(0.99)	Inactive(0.90)	Inactive(0.82)
Kaempferol 3-O-Glucoside-7-O-Rhamnoside	Inactive(0.81)	Inactive(0.90)	Active(0.92)	Inactive(0.73)	Inactive(0.66)

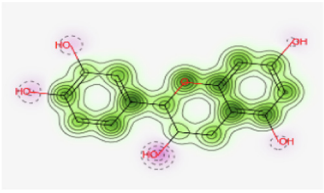
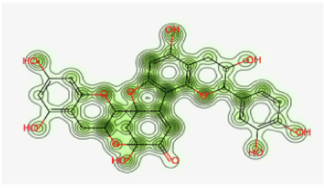
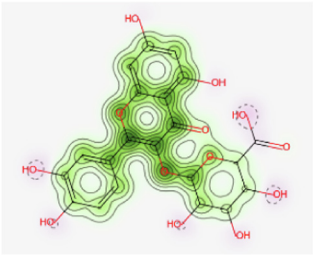
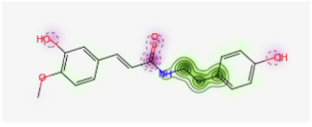
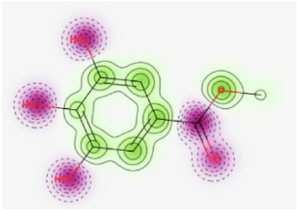
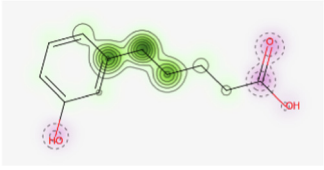
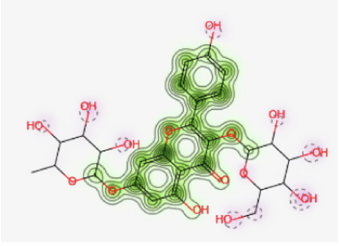
Dehydrodicatechin A and Kaempferol 3-O-Glucoside-7-O-Rhamnoside. Additionally, Dehydrodicatechin A possessed a high probability of binding to the glucocorticoid receptor. Moreover, Catechin, Quercetin-3-O-Glucuronide, and Tamgermanetin revealed a medium likelihood of binding to mineralocorticoid receptors (Kolšek et al., 2014).

4. Discussion

4.1. In vitro Biological Effects

Current drug discovery research from medicinal plants has a diverse approach that combines botanical, phytochemical,

Table 6
Prediction of Cardiac Toxicity of Ethanolic *C. Cosmosum* Extract Identified Metabolites by Using the Pred-hERG 4.2 Webserver.

Metabolite Name	Prediction/Potency	Confidence	Probability Map
Catechin	Potential cardiotoxic (+)	50%	
Dehydrodicatechin A	Potential cardiotoxic (+)	60%	
Quercetin-3-O-Glucuronide	Non-cardiotoxic (−)	50%	
Tamgermanetin	Potential cardiotoxic (+)	50%	
Methyl Gallate	Non-cardiotoxic (−)	80%	
5-(3-Hydroxyphenyl) Pentanoic Acid	Non-cardiotoxic (−)	50%	
Kaempferol 3-O-Glucoside-7-O-Rhamnoside	Non-cardiotoxic (−)	50%	

biological, and molecular techniques. Medicinal plant drug research continues to yield novel and significant leads against a broad range of pharmacological targets that involve multiple diseases (Balunas et al., 2005). Therefore, the cytotoxic potential of *R. Vesicarius* and *C. Comosum* were tested against the MDA-MB-231 (triple-negative, resistant, and highly metastatic) (Simu et al., 2021) and other types of cancer. Our MTT results demonstrated that all the tested extracts were inactive against liver cancer (HepG2) and colorectal cancer cells (HCT8). This finding suggested that the extracts from this particular plant source may not possess strong anticancer properties against these specific cancer types. To further investigate the potential cytotoxic effects of the plant extracts, the ethanolic and aqueous extracts of *C. Comosum* were selected due to their relatively high potency values compared to others. These extracts were then tested on breast cancer cell lines, specifically KAIMRC2 and MDA-MB-231, to evaluate their

effects on cell proliferation. Our findings suggest moderate cytotoxic activity of the ethanolic *C. Comosum* extract against the tested breast cancer cell lines including highly proliferative and spontaneously immortalized breast cancer cell line, KAIMRC2 (Ali et al., 2021). Furthermore, *C. Comosum* in ethanol demonstrated cytotoxicity and induction of apoptosis via activation of caspase 3 on HCT8 and KAIMRC2 cell lines which also indicated the possibility of a wide clinical application. Earlier studies have reported that *C. Comosum* exhibited both cytotoxicity and genotoxicity to human hepatocellular carcinoma (HepG2) cells (Alzahrani, 2021; Shalabi et al., 2015). In another study, *C. Comosum* demonstrated cytotoxicity against human triple-negative MDA-MB231 breast cancer cells via the intrinsic apoptotic pathway (Alehaideb et al., 2020).

The development of microbial resistance to the known antibiotics drives research to explore new antibiotics from natural origin. Current findings demonstrated *R. Vesicarius* and *C. Comosum* as antibacterial agents against *E. coli* and *S. aureus* when plants were extracted using different solvents. A recent study reported substantial antibacterial activity against *Salmonella typhi*, *Bacillus cereus,* and *Staphylococcus aureus* for the *R. Vesicarius* methanolic extract (Salama et al., 2022). Furthermore, aqueous and organic extracts from *C. Comosum* were investigated *in vitro* and showed a high ability to suppress the growth of *Listeria ivanovii* (Riadh et al., 2011).

The current study extracted the medicinal plants in four solvents with different polarities, including water, ethanol, ethyl acetate, and chloroform to isolate various phytochemicals (Truong et al., 2019). Aqueous mixes of ethanol, methanol, acetone, and ethyl acetate polar solvents are commonly used to extract polyphenols from plant matrices (Sultana et al., 2009). Other studies have shown that ethyl acetate may extract phenolic chemicals from onion and orange peel (Abdille et al., 2005; Li et al., 2006; Peschel et al., 2006; Zia-ur-Rehman, 2006). Additionally, optimum phenolic compounds were produced from barley flour using ethanol and acetone combinations (Bonoli et al., 2004). Similarly, aqueous methanol was discovered to be more effective in recovering the maximum amounts of phenolic compounds from rice bran (Chatha et al., 2006). Moreover, methanol was the best solvent for obtaining bioactive compounds from *Severinia buxifolia* since it provided the maximum content of phenolics, alkaloids, flavonoids, and terpenoids (Truong et al., 2019).

In our study, the ethanolic extract of *C. Cosmosum* exhibited the greatest bioactivity in cancer cells, and other solvent extracts from both plants had antibacterial efficacy against the tested gram-positive and gram-negative bacteria. *C. Cosmosum* had several bioactive metabolites including Catechin, Dehydrodicatechin A, Quercetin-3-O-Glucuronide, Tamgermanetin, Methyl Gallate, 5-(3-Hydroxyphenyl) Pentanoic Acid, and Kaempferol 3-O-Glucoside-7-O-Rhamnoside. Some metabolites appear to have limited data available on their antibacterial and anti-cancer activity. However, Catechin is a polyphenol that was reported to inhibit prostate cancer cell PC-3 proliferation by the induction of apoptosis inhibiting of bcl-2 expression, and increasing cytochrome *C* expression for activation of caspase-3, caspase-8, and caspase-9 (Ranjan et al., 2019; Tsai and Chen, 2016). Moreover, Catechin demonstrated an antimicrobial effect against methicillin-resistant *S. aureus* (MRSA) via oxidative stress by increasing ROS production and decreasing antioxidant enzymes (Sinsinwar and Vadivel, 2020). Additionally, one study suggested that Quercetin-3-O-Glucuronide controlling 2-adrenergic signaling may act to suppress breast cancer cell invasion (Yamazaki et al., 2014). Tamgermanetin which is a unique isoferuloyl derivative has exhibited potential cytotoxic activity against the liver (Huh-7), prostate (PC-3), and breast cancer cell line (MCF-7) (Nawwar et al., 2013). Moreover, Methyl Gallate is phenolic gallic acid that exhibited cytotoxic activity against Hepatocellular carcinoma inhibiting cell proliferation by increasing ROS production (Huang et al., 2021). Another study suggested that Methyl Gallate suppresses melanoma tumor development through the induction of apoptosis and blocking tumor angiogenesis and metastasis (Park et al., 2022). Furthermore, Methyl Gallate has antimicrobial activity by disturbing the membrane integrity of *Vibrio cholerae* (Sánchez et al., 2013). Furthermore, one study demonstrated that the combination of Methyl Gallate and Tylosin synergistically decreased the *Salmonella Typhimurium* membrane integrity and intracellular survival. Therefore, the secondary metabolites extracted from *C. Cosmosum* possessed anticancer and antibacterial activities, which confer biological activity to the ethanolic extract of *C. Cosmosum.* Furthermore, the only extract tested for antimicrobial activity of *C. Cosmosum* was the aqueous extract, which seemed to be the most active compared to all the other plant extracts tested. Further experiments testing the antibacterial effect of the ethanolic extract are essential.

The MTT assay, although widely used in research, has several limitations that need to be considered. One limitation is its lack of sensitivity, which may result in the inability to detect subtle changes in cell viability. Additionally, the assay can be subject to chemical interference, leading to inaccurate results. Furthermore, the MTT assay is time-dependent, meaning that the duration of treatment can greatly influence the assay results. Therefore, these limitations should be taken into account when interpreting the results of MTT assays in scientific studies (Ghasemi et al., 2021; Riss, 2017). Thus, in the current study, we verified the cytotoxic data using the ApoTox Triplex Glo assay as well as making a qualitative assessment by performing the HCI apoptosis assay, confirming a dose-dependent effect of inducing apoptosis and cytotoxicity. Furthermore, it is crucial to expand the range of microbes and cancer cell lines, including normal cell lines, for testing the effectiveness of plant extracts in treating various cancers and microbes. Ongoing studies are underway to evaluate the therapeutic potential and safety profile of these extracts, as well as to determine their precise mechanisms of action. By comparing two different plants that share the same solvents, we highlight the diversity observed in plant metabolomics across species and emphasize the need to optimize the extraction procedure based on the solubility of active metabolites for each plant. This study serves as a significant foundation for future research in drug discovery, indicating that ethanol is the optimal solvent for extracting active metabolites from *C. Cosmosum* for further investigation and development of novel anti-cancer and anti-bacterial drugs.

4.2. Purification & Identification of Metabolites

In addition, Liquid chromatography-mass spectrometry (LC-MS) was applied to *C. Comosum* in ethanol to tentatively identify and elucidate the structure and chemical characteristics of the metabolites present in the ethanolic extract. Peak A the appeared *m/z* value at retention time (5.3535–5.700) was correlated with the parent compound Catechin, with *m/z* $[M + H]^+$ 290.0876 Da and a molecular formula of $[C_{15}H_{14}O_6]^+$, $[M + H]^+$ *m/z* 290.14 in positive ion mode, and $[M-H]^-$ with *m/z* 290.06 Da in negative mode, indicating that the compound has a molecular weight of 290.16 g mol^{-1} (Markham, 1982). Moreover, peak B showed *m/z* value at retention time (17.396–18.698) was correlated with the parent compound Dehydrodicatechin A, with *m/z* $[M + H]^+$ 577.1521 Da and a molecular formula of $[C_{30}H_{24}O_{12}]^+$, $[M + H]^+$ *m/z* 576.56 in positive ion mode, and $[M-H]^-$ with *m/z* 575.63 Da in negative mode, indicating that the compound has a molecular weight of 576.56 g mol^{-1}. Also, peak C manifested *m/z* value at retention time (26.396–26.608) were correlated with the parent compound Quercetin-3-O-Glucuronide, with *m/z* $[M + H]^+$ 479.1018 Da and a molecular formula of $[C_{21}H_{18}O_{13}]^+$, $[M + H]^+$ *m/z* 576.56 in positive ion mode, and $[M-H]^-$ with *m/z* 575.63 (Markham, 1982). Furthermore, peak D demonstrated *m/z* value at retention time (28.365–28.4087) was

correlated with the parent compound Tamgermanetin with *m/z* $[M + H]^+$ 314.1526 Da and a molecular formula of $[C_{18}H_{19}NO_4]^+$, $[M + H]^+$ *m/z* 313.35 in positive ion mode, and $[M-H]^-$ with *m/z* 312.67 Da in negative mode, indicating that the compound has a molecular weight of 313.32 g mol^{-1} (Nawwar et al., 2013) Additionally, peak E appeared *m/z* value at retention time (29.865–29.997) were correlated with the parent compound Methyl Gallate, with *m/z* $[M + H]^+$ 185.1239 Da and a molecular formula of $[C_8H_8O_5]^+$, $[M + H]^+$ *m/z* 184.35 in positive ion mode, and $[M-H]^-$ with *m/z* 183.312 Da in negative mode, indicating that the compound has a molecular weight of 184.32 g mol^{-1} (Jayaprakasha et al., 2006). Moreover, peak F showed *m/z* value at retention time (30.365–30.560) was correlated with the parent compound 5-(3-Hydroxyphenyl) Pentanoic Acid, with *m/z* $[M + H]^+$ 195.1090 Da and a molecular formula of $[C_{11}H_{14}O_3]^+$, $[M + H]^+$ *m/z* 194.23 in positive ion mode, and $[M-H]^-$ with *m/z* 193.73 Da in negative mode, indicating that the compound has a molecular weight of 194.23 g mol^{-1} (27). In addition, peak G exhibited *m/z* value at retention time (33.165–33.215) was correlated with the parent compound Kaempferol 3-O-Glucoside-7-O-Rhamnoside, with *m/z* $[M + H]^+$ 595.4009 Da and a molecular formula of $[C_{11}H_{14}O_3]^+$, $[M + H]^+$ *m/z* 594.50 in positive ion mode, and $[M-H]^-$ with *m/z* 593.83 Da in negative mode, indicating that the compound has a molecular weight of 594.36 g mol^{-1} (el Sayyad and Wagner, 1978).

4.3. In silico Investigation

There are several strategies for predicting good bioavailability, and the Lipinski rule of five is among the most well-known rules. The governing principle relates to the physicochemical proprieties crucial to a drug's pharmacokinetics in the human body. The anticancer and antibacterial activity was anticipated utilizing the PASS Online web server and Dehydrodicatechin A exhibited the highest anti-cancer activity. A previous study has reported that Dehydrodicatechin A possessed the highest cytotoxic activity between *C. Comosum* in ethyl acetate isolated compounds in the brine shrimp lethality assay (Badria et al., 2007). Furthermore, Quercetin-3-O-Glucuronide and Kaempferol 3-O-Glucoside-7-O-Rhamnoside could demonstrate a 60% probability of being active as antibacterial agents. In addition, According to Molinspiration Webserver, Catechin is demonstrated to be a nuclear receptor ligand. Various biological processes are regulated by nuclear receptors, which overlap with cancer cell hallmarks. Nuclear receptors are typically hormonally responsive; thus, they would display the most efficacy in malignancies that are hormone-sensitive such as breast, ovarian, and prostate cancers (Yang et al., 2021). Moreover, Based on the SWISS target prediction webserver which was used to predict molecular targets illustrated that Methyl Gallate revealed a probable lyase target. Extra-mitochondrial enzyme ATP citrate lyase (ACL or ACLY) is present in a variety of human tissues. ACL is abnormally expressed in many immortalized cells and malignancies, including breast, colon, liver, lung, and prostate cancers. It possesses a negative prognostic correlation with tumor stage and differentiation. As a result, ACL is a crucial enzyme in cellular lipogenesis and a powerful target for cancer treatment (Zu et al., 2012). Before a biomolecule is transformed into a drug, pharmacokinetics proprieties are essential and *C. Comosum* in ethanol identified metabolites were evaluated. Lipophilicity is a propensity to partition into a nonpolar lipid matrix as opposed to an aqueous matrix. Additionally, it has an impact on physiochemical, metabolic, and toxic properties. It assesses by Log $P_{o/w}$ which was calculated by three methods iLOGP, XLOGP3, and MLOGP. According to MLOGP, all the metabolites are within the range. Moreover, all the metabolites were soluble which indicated good absorption and oral bioavailability.

Additionally, different permeation mechanisms lead to the blood–brain barrier (BBB) being penetrated. Even though BBB penetration has a significant impact on central nervous system (CNS) drug recovery, it is also crucial for peripheral brain exposure, as this might produce CNS adverse reactions (Kerns and Di, 2015). However, all metabolites except 5-(3-Hydroxyphenyl) Pentanoic Acid failed to cross the BBB. Moreover, Cytochrome P450 enzymes are necessary for the metabolism of several drugs. Although this class contains more than 50 enzymes, 90% of medicines are metabolized by CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4. Moreover, drugs can inhibit or activate cytochrome P450 enzymes, causing clinically important drug-drug interactions that may result in unanticipated adverse effects or treatment failures (Lynch and Price, 2007). All metabolites were not predicted to inhibit CYP enzymes except Dehydrodicatechin A predicted to inhibit CYP2C9 and Tamgermanetin predicted to inhibit CYP2D6 and CYP3A4. Therefore, Dehydrodicatechin A and Tamgermanetin may cause drug-drug interactions that may result in unpredictable adverse events or therapy failure (Lynch and Price, 2007). Furthermore, regarding the toxicity of the metabolites, all metabolites are classified as harmless except Quercetin-3-O-Glucuronide, Tamgermanetin, and Kaempferol 3-O-Glucoside-7-O-Rhamnoside were immunotoxin; moreover, Quercetin-3-O-Glucuronide was carcinogenic. In addition, QT prolongation and potentially lethal arrhythmias can be caused by blocking the hERG K^+ channel. Catechin, Dehydrodicatechin A, and Tamgermanetin are considered to be potentially cardiotoxic. Dehydrodicatechin A exhibited a high probability of binding to the glucocorticoid receptor (GR). High expression of GR in triple-negative breast cancer (TNBC) correlates strongly with increased recurrence and chemotherapy resistance. Therefore, the pretreatment of a GR antagonist may be an effective method for inducing tumor cell death in chemotherapy-resistant GR + TNBC (Skor et al., 2013). Our *in silico* study predicts the cytotoxic metabolites' pharmacodynamic and pharmacokinetic activities in addition to predicting the safety profile. Thus, we shed light on the potential of *C. Comosum* metabolites as lead drug candidates for further application into medicinal chemistry, to improve their therapeutic profile, and testing in biological systems.

5. Conclusion

Metabolites from *C. Comosum* ethanolic extract were cytotoxic against liver, colorectal, and breast cancer cell lines. In particular, the ethanolic extract of *C. Comosum* exhibited moderate cytotoxic activity against breast cancer cell lines. Moreover, *R. Vesicarius* extracts and *C. Comosum* aqueous extract conferred significant antibacterial activity against *S. aureus* and *E. coli*. In addition, the *in silico* approach, which harnesses the analytical and predictive power of computation to generate hypotheses, helped verify possible drug candidates. Dehydrodicatechin A and Kaempferol 3-O-Glucoside-7-O-Rhamnoside were predicted to have high anticancer activity, and Quercetin-3-O-Glucuronide and Kaempferol 3-O-Glucoside-7-O-Rhamnoside were predicted to exert strong antibacterial activities. Therefore, Kaempferol 3-O-Glucoside-7-O-Rhamnoside may be suitable for further *in vitro* tests as a dual activity candidate. However, further research and development are required to identify the *in vitro* and *in vivo* potential of herbal metabolites in treating cancer and microbial infections.

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Institutional review board statement

The study was approved by the Institutional Review Board of King Abdullah International Medical Research Center (IRB number is SP21R/463/12).

Data availability statement

The data presented in this study are available on request from the corresponding author.

CRediT authorship contribution statement

Sahar S. Alghamdi: Conceptualization, Funding acquisition, Project administration, Supervision, Visualization, Writing – review & editing, Software, Validation, Resources, Data curation, Writing – original draft. **Raghad A. Alshafi:** Methodology, Visualization, Writing – review & editing, Writing – original draft, Formal analysis, Investigation. **Sarah Huwaizi:** Methodology, Investigation, Data curation. **Rasha S. Suliman:** Conceptualization, Project administration, Validation. **Afrah E. Mohammed:** Methodology, Formal analysis, Investigation. **Zeyad I. Alehaideb:** Conceptualization, Validation, Resources. **Allulu Y. Alturki:** Methodology, Visualization, Formal analysis, Investigation, Data curation. **Sara A. Alghashem:** Methodology, Visualization, Investigation. **Ishrat Rahman:** Data curation, Project administration, Supervision, Writing – review & editing, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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