Current Research in Nutrition and Food Science

Bioactive Potential and *In silico* Insights of Flavonoid-Enriched Femivine V Jelly and *Kaempferia Angustifolia* Extracts in Antioxidant and Anticancer Applications

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Abstract

Kaempferia angustifolia, a notable medicinal herb within the ginger family, comprises around 60 species of rhizomatous plants. Commonly referred to as *temu kunci, kunci pepet,* or *Thao Nhang Haeng* in Southeast Asia, it is renowned for its aromatic rhizomes, traditionally utilized to address ailments like colds, digestive issues, and fevers. Recent research underscores its potential in cancer chemoprevention, alongside antioxidants, antimicrobial, anti-obesity, and anti-allergic properties. This study focuses on four key flavonoids found in the innovative FemiVine V jelly product and the raw extract of the *Kaempferia angustifolia* plant. The jelly exhibited a 60% higher total phenolic content (31.93 ± 0.20 mg/g) compared to the raw extract (12.0 ± 0.23 mg/g, $p<0.05$). Antioxidant assays revealed superior DPPH radical scavenging activity in the jelly (67.6 ± 0.15%) versus the extract (30.54 ± 0.10%, $p<0.05$), supported by FRAP values of 5.08 ± 0.01 mg/100 g (jelly) and 4.81 ± 0.05 mg/100

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ml (extract). Cytotoxic screening against MDA-MB-231 breast cancer cells showed stronger activity in the raw extract (IC$_{50}$: 82 ± 0.06 mg/ml) compared to the jelly (IC$_{50}$: 125 ± 0.20 mg/ml, p<0.05). *In-silico* analysis highlighted vitexin and β-sitosterol as promising candidates for peripheral cancer treatment due to favorable pharmacokinetic properties, including gastrointestinal absorption and lack of brain penetration. Further research is needed to assess their selective cytotoxicity against cancer cells versus normal fibroblasts.

Introduction

Kaempferia angustifolia, commonly known as *temu kunci* or white turmeric, is a medicinal plant in the Zingiberaceae family, which comprises approximately 60 species of small rhizomatous herbs.[1] Native to East Tropical Asia, this genus is characterized by small rhizomatous herbs with short rhizomes and thick, aromatic tuberous roots.[2] While *Kaempferia angustifolia* is less prominent in Malaysia compared to its relatives *K. galanga* and *K. rotunda*,[3] it is recognized in Indonesia by names such as *Kunci pepet, Kunci menir,* and *Kunci kunot,* and in Vietnam as *Thao Nhang Haeng*.[1]

Traditionally, the rhizomes of *K. angustifolia* are prized for their pleasant aroma and are widely used in folk medicine to treat ailments such as colds, stomachaches, coughs, diarrhea, fever, and dysentery.[4] Recent studies have revealed the therapeutic potential of edible Southeast Asian plants, including *K. angustifolia*, for applications such as cancer chemoprevention and antimicrobial activity.[3] Additionally, *K. angustifolia* has demonstrated biological activities, including antioxidant, antimicrobial, anti-obesity, and anti-allergic effects.[4] Despite these promising properties, research on *K. angustifolia* as an anti-obesity agent remains limited. In Indonesia, the rhizomes are traditionally used in slimming remedies, prompting further investigation into their potential to regulate adipocyte differentiation in murine 3T3-L1 cells.[4,5]

The essential oil of *K. angustifolia* has shown moderate antimicrobial activity, particularly against *Pseudomonas aeruginosa* (10.63 ± 0.23 mm and 10.03 ± 0.22 mm inhibition zones), but it exhibits minimal efficacy against pathogens such as *Escherichia coli, Bacillus subtilis, Streptococcus faecalis, Candida albicans,* and *Microsporum gypseum*.[6] Extracts and compounds from *Kaempferia*

species have demonstrated diverse therapeutic applications, including inhibition of *Staphylococcus aureus* and *Pseudomonas spp.*, treatment of knee osteoarthritis, and inhibition of breast cancer resistance protein (BCRP). They also exhibit anti-inflammatory, anti-acne, and anticholinesterase activities, support wound healing, mitigate obesity-induced dermatopathy, and combat drug-resistant strains of *Mycobacterium tuberculosis*. Additionally, Kaempferia species possess neuroprotective and anti-nociceptive properties and have shown activity against HIV-1, in vitro anti-allergenic effects, and larvicidal activity against Aedes aegypti.[7]

Specific compounds from *K. angustifolia*, such as 20-hydroxy-4,4,6-trimethoxychalcone and 25-dien-3-ol, exhibit cytotoxic effects against human cancer cell lines, including HL-60 (promyelocytic leukemia) and MCF-7 (breast cancer).[4] The Zingiberaceae family, known for its antimicrobial properties, produces extracts and essential oils effective against bacteria, fungi, and yeasts. Antioxidant compounds in *K. angustifolia* include boesenboxide, crotepoxide, 2'-hydroxy-4,4',6-trimethoxychalcone, kaempfolienol, and zeylenol, alongside catechin, kaempferol, quercetin, gallic acid, caffeic acid, and α-tocopherol, which are particularly notable for their anticancer and antioxidant properties. This species is a rich source of secondary metabolites, including diterpenoids, steroids, saponins, cyclohexane oxides, flavonoids, and chalcones.[8]

These metabolites have applications in medicine, nutrition, beverages, fragrances, cosmetics, and other industries.[9] Chalcones, characterized by small molecular structures, act as Michael acceptors, enabling covalent interactions with proteins that drive biological activity.[10] The composition and concentration of these metabolites vary with factors such as growth environment, harvest season, plant

part used, and solvent properties, directly influencing their biological activity.[11] Flavonoids in *K. angustifolia* include glycosidic and aglycone types. C-glycosidic flavonoids, with sugar substituents at C(6) and/or C(8) of the aglycone, are of particular interest. Their moderate polarity and thermolability make reversed-phase liquid chromatography (LC) a preferred analysis method, with advanced techniques like LC-MS and LC-MS/MS providing precise identification of flavonoids and their substituent positions.[9]

In silico pharmacology, a growing field leveraging computational models to analyze biological and medical data, facilitates the prediction and discovery of therapeutic compound.[12] Previous studies on *K. angustifolia* have analyzed its chemical composition and biological activities, with metabolomics revealing changes in metabolite profiles under various conditions.[13] Techniques like LC-MS/MS provide the sensitivity and selectivity needed for detailed metabolite profiling.

In overall, the research on Kaempferia angustifolia and FemiVine V Jelly highlights their antioxidant and cytotoxic potential, yet certain gaps remain. While the study demonstrates the superior antioxidant activity of FemiVine V Jelly and the stronger cytotoxic effect of the raw extract on MDA-MB-231 breast cancer cells, further investigation is needed to understand the mechanisms underlying these differences. Additionally, the in-silico analysis suggests promising pharmacokinetic properties for vitexin and β-sitosterol, but their selective cytotoxicity against normal fibroblasts remains unexplored. Future studies should focus on optimizing flavonoid extraction, evaluating potential synergistic effects, and conducting *in vivo* experiments to validate their therapeutic efficacy. Thus, this study aims to evaluate the antioxidant properties, cytotoxic potential, and pharmacokinetic characteristics of flavonoids in *K. angustifolia* extracts, focusing on their therapeutic potential against MDA-MB-231 breast cancer cells. The innovative FemiVine V jelly product and raw extracts are analyzed as part of this investigation.

Materials and Methods
Materials and Chemicals
The following materials were used in this study: Folin-Ciocalteu reagent, sodium nitrite, catechin, gallic acid, Sephadex G-25, and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), acetate buffer (pH 3.6),10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) , hydrochloric acid (HCl), iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) all obtained from Sigma Chemical Co. (St. Louis, MO, USA). LH-20 was procured from Amersham Biosciences (Uppsala, Sweden), and Whatman No. 1 filter paper was purchased from Fisher Scientific (Fair Lawn, NY, USA). High-performance liquid chromatography, HPLC-grade acetonitrile and acetic acid were supplied by Merck (Darmstadt, Germany). Two types of samples were analyzed: the raw extract and the FemiVine V jelly commercial product. The raw extract was in powder form, the process of drying *K. angustifolia* were washed and cut into small slices then put onto the aluminum tray and oven-dried at 50 °C for 24 hrs. The dried samples were then ground to powder form and kept in bottles prior to extraction process. The raw extract and FemiVine V jelly were similarly dissolved at the same concentration. Both samples were centrifuged at 10,000 rpm for 10 minutes, and the resulting supernatant was filtered using a 0.22 μm syringe filter. The filtered solutions were then transferred into 20 μL inserts prior to injection into the Liquid Chromatography/Mass Spectrometry (LC/MS) system.

Phytochemicals Molecular Profiling Analysis
The Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry (LC/MS-QTOF) system employed in this study comprised an Agilent 1200 liquid chromatography system equipped with a binary pump, a vacuum degasser unit, an auto-sampler, and a 6520-quadrupole time-of-flight mass spectrometer with an electrospray ionization (ESI) source. Chromatographic separation was carried out using an Agilent ZORBAX Eclipse Plus C18 Rapid Resolution HT column (2.1 × 100 mm, 1.8 μm particle size) maintained at a temperature of 40 °C. The mobile phase consisted of two components: (A) 0.1% formic acid in deionized water and (B) 0.1% formic acid in acetonitrile. The gradient elution program was as follows: a) 0.00–18.00 minutes: Linear gradient from 5% (B) to 95% (B); 18.00–23.00 minutes: Held at 95% (B) and c) 23.01 minutes: Returned to 5% (B). The total run time was 30 minutes, with the system pre-equilibrated for 2 minutes before each new injection. A sample injection volume of 2 μL was used, and the mobile phase flow rate was maintained at 0.25 mL/min. The mass spectrometer operated in positive ESI mode under the following conditions: a) Gas temperature: 325 °C; b) Gas flow rate: 11 L/min;

c) Nebulizer pressure: 35 psi. Data were analyzed using Agilent Mass Hunter Qualitative Analysis software (version B.05.00, Agilent Technologies, Santa Clara, CA, USA). Chromatographic profiles were assessed based on the accurate mass measurements obtained from the LC/MS system.[14,15]

Determination of Total Phenolic Contents

The total phenolic content of the raw *Kaempferia angustifolia* extract and FemiVine V jelly was quantified using a modified Folin-Ciocalteu method. A 125 µL aliquot of a known dilution of the extract was transferred into a test tube and mixed with 0.5 mL of Folin-Ciocalteu reagent. The mixture was vortexed for 15 seconds and left to stand at room temperature (20 °C) for 6 minutes. Subsequently, 1.25 mL of a 7% sodium carbonate solution was added to the mixture, and the volume was adjusted to 3 mL using distilled, deionized water. The reaction mixture was allowed to develop color over 90 minutes. Absorbance was then measured at 760 nm using a Hewlett-Packard UV-Vis spectrophotometer (Palo Alto, CA, USA). The total phenolic content was calculated by comparing the absorbance values to a standard curve prepared with gallic acid solutions, and the results were expressed as milligrams of gallic acid equivalents (mg GAE). All measurements were conducted in triplicate, and the results are presented as the mean of three determinations (n = 3).[16]

DPPH Radical Scavenging

A 0.1 mL aliquot of the acetone extract prepared earlier was combined with 3.9 mL of an 80% ethanolic 0.6 mM DPPH solution in a test tube. The mixture was vortexed for 15 seconds and allowed to stand for 180 minutes at room temperature.[17] After this incubation period, the absorbance was measured at 515 nm using a Hewlett-Packard UV/Vis spectrophotometer (Palo Alto, CA, USA). Under these conditions, most tested compounds are expected to react completely within 180 minutes, while vitamin C, due to its rapid oxidation, reacts in less than 1 minute. An 80% ethanol solution was used as the blank, while the control consisted of the DPPH solution without any test sample (3.9 mL of DPPH mixed with 0.1 mL of 80% ethanol). All tests were performed in triplicate (n = 3). The antioxidant activity of the samples was evaluated using two parameters: a) Median Effective Concentration (EC_{50}): The concentration of antioxidant (test sample) required to achieve a 50% reduction in the absorbance of DPPH radicals, expressed as total phenolics (mg); b) Scavenging Activity (%): Calculated using the formula:

$$\text{Inhibition (\%) of DPPH absorbance} = (A_{control} - A_{test})/A_{control} \times 100 \qquad ...(1)$$

A dose-response curve (absorbance of DPPH vs. concentration of antioxidants) was plotted to establish standard curves and calculate the EC_{50}. Vitamin C was used as the standard reference, and the results were expressed as vitamin C equivalent antioxidant capacity (VCEAC).

FRAP Assay

The reducing capacity of the sample was evaluated using the FRAP (Ferric Reducing Antioxidant Power) method, as described by Elshamy *et al.* (2019).[7] Briefly, the FRAP reagent was freshly prepared by mixing the following components: a) 300 mM acetate buffer (pH 3.6), b) 10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) dissolved in 40 mM hydrochloric acid (HCl), and c) 20 mM iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$). A 1.0 mL aliquot of the FRAP reagent was combined with 50 µL of the sample or Trolox standard. The mixture was incubated in the dark at room temperature for 30 minutes to allow the reaction to develop. The reducing capacity of the sample was then determined by measuring absorbance at 595 nm using a spectrophotometer (BMG Labtech).[17]

2-D Cell Culture and MTT Viability Assay

The MDA-MB-231 adenocarcinoma cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM), as described by Kim *et al.* (2014).[18] The medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S). The cells were maintained in a humidified incubator at 37°C with 5% CO_2. Adherent BV-2 cells, at ≥70% confluency, were detached from the flask by trypsinization and subcultured every 72 hours. For the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, 180 µL of BV-2 cells at a density of 20,000 cells per well were seeded into a 96-well plate and incubated for 24 hours. Subsequently, the cells were treated with 20 µL of the following: a) Medium alone, b) 0.01% dimethyl sulfoxide (DMSO), c) Donepezil, or d)

labelled dextran (FD) at various concentrations (0.1 µg/mL, 1 µg/mL, 10 µg/mL, and 100 µg/mL). After a further 24-hour incubation period, 50 µL of MTT reagent was added to each well, followed by incubation at 37 °C for 4 hours. Following this, the supernatants were carefully removed, and 100 µL of DMSO was added to each well to dissolve the formazan crystals.[19] Absorbance was measured at 550 nm using a microplate reader (Molecular Devices, USA). The normalized viability (%) of the cells was calculated using SoftMax® Pro Software for accuracy and reliable data acquisition and analysis.

In silico Metabolism, and Absorption, Distribution Excretion (ADME) Prediction and Drug-Likeness Assessment

The QikProp 4.4 software was employed for absorption, distribution, metabolism, and excretion (ADME) predictions. Recognized for its accuracy, simplicity, and efficiency, this program provides key physical descriptors and relevant pharmaceutical properties of active molecules (QikProp 4.4 User Manual, 2015). The "drug-likeness" of the compounds was evaluated based on Lipinski's "Rule of Five." Critical physical properties, including molecular weight, hydrophobicity (logP), the number of hydrogen bond acceptors, and the number of hydrogen bond donors, were analyzed to determine their compliance with the rule. Furthermore, the potential of each compound to cross the blood-brain barrier (BBB) was assessed, as described by Zolkiffly *et al.* (2021).[12]

Statistical Analysis

Results are given as mean values ± standard deviation from measurements of three replicates (n = 3). Statistical analysis data can be obtained from Minitab statistical software (Version 22.0, Minitab Inc., United States). The independent t-test was then performed to compare the mean values obtained at a significant level of 95% ($p < 0.05$).

Results

In the present study, four flavonoid compounds were identified in *K. angustifolia* extracts through Q-TOF LC-MS analysis (Table 1, Figures 1 and 2). These compounds were detected in two samples: FemiVine V jelly and the raw (powdered) species. Identification was achieved using full mass spectra and MS² fragmentation patterns, which were compared with published data and MS² references, such as those from Mass Bank. The base peak chromatograms obtained in positive ionization mode (Figures 1 and 2) revealed distinct metabolite profiles for the two extracts.

The fragmentation patterns of the identified compounds were consistent across samples, especially at low collision energies (<70% eV). For instance, isoorientin (luteolin 6-C-glucose) exhibited the loss of a water molecule, resulting in a fragment at m/z 448 [M–H–18]−. This was observed at retention times of 20.643 minutes in FemiVine V jelly and 23.132 minutes in the raw extract. In contrast, orientin (luteolin 8-C-glucose) did not show this loss of water.
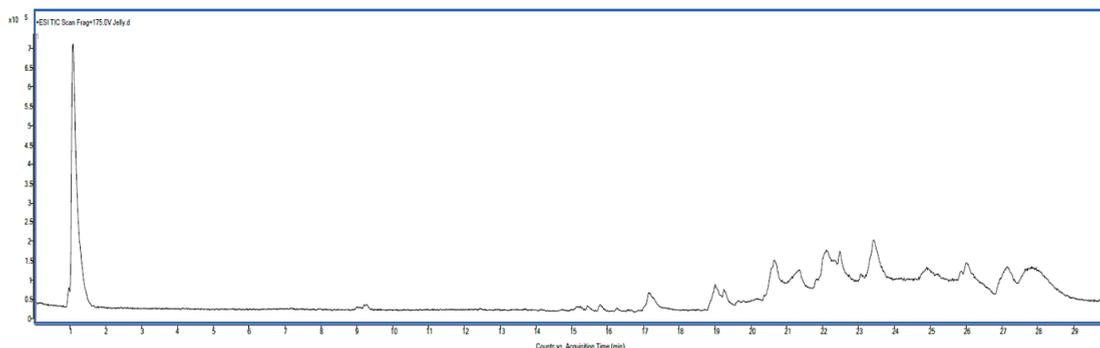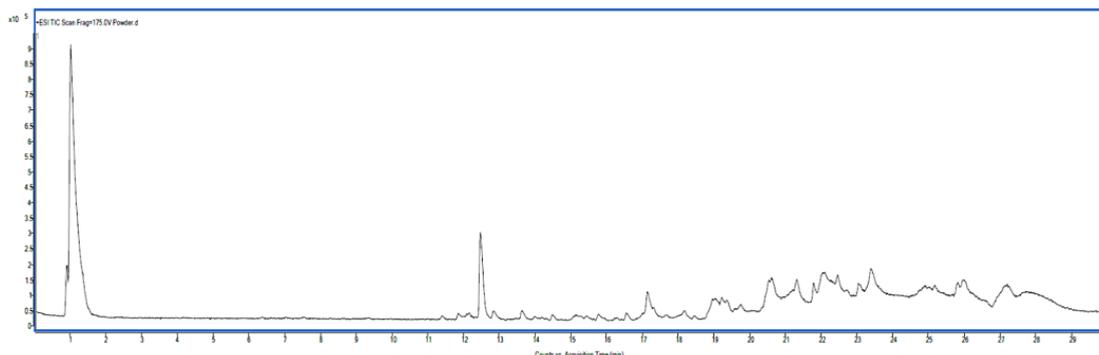

Fig. 1: LC-MS Chromatogram of FemiVine V Jelly commercialized product with the number of molecular features (M/Z) detected as of 208

The antioxidant properties of FemiVine V jelly and its corresponding raw extract were assessed using three methods in Table 2: total polyphenol content (expressed as gallic acid equivalents, GAE), the DPPH assay, and the FRAP assay. This study

represents the first documented evaluation of the antioxidant potential of extracts and phytochemicals derived from *Kaempferia angustifolia*, as summarized in Table 2.

Fig. 2: LC-MS chromatogram of raw extract (powder) with the number of molecular features (M/Z) detected as of 121

Table 1: Selected metabolites profiling of K. Angustifolia extracts FemiVine V jelly and raw extract as identified by LC-MS

	Compound	m/z	Formula	RT (min)	Height
FemiVine V jelly	Orientin	447.69	$C_{21}H_{20}O_{11}$	20.643	1119
	Vitexin	432.24	$C_{21}H_{20}O_{10}$	20.664	2048
	Kaempfolienol	354.5	$C_{20}H_{34}O_5$	1.064	3126
	β- Sitosterol	414.7	$C_{29}H_{50}O$	15.793	3064
Raw extract (powder)	Orientin	447.40	$C_{21}H_{20}O_{11}$	23.132	1004
	Vitexin	435.36	$C_{21}H_{20}O_{10}$	20.619	2322
	Kaempfolienol	289.05	$C_{20}H_{34}O_5$	1.062	1503
	β- Sitosterol	413.32	$C_{29}H_{50}O$	22.113	4695

Table 2: Antioxidant activity for FemiVine V jelly and species raw of *K. angustifolia*

Antioxidant Activity	FemiVine V Jelly	Raw Extract (Powder)
Total Polyphenol (GAE) mg/g	31.93 ± 0.20[a]	12.00 ± 0.23[b]
DPPH (% Inhibition)	67.60 ± 0.15[c]	30.54 ± 0.10[d]
FRAP	5.08 ± 0.01 (mg/100 g ascorbic acid)[e]	4.81 ± 0.05 (mg/100 ml VCEAC)[f]

[a-f]Mean with different alphabet shows significant difference between samples ($p<0.05$)

Table 3: IC_{50} values for both commercialized product and raw extract (powder) of *K. angustifolia* against MDA-MB-231

| Product	Nilai IC_{50} mg/ml
FemiVine V jelly	125 ± 0.20[a]
Raw extract (powder)	82 ± 0.06[b]

[a-b]Mean with different alphabet shows significant difference between samples ($p<0.05$)

The cytotoxic screening results (see Table 3) demonstrate the antagonistic effect of the isolated compounds from *K. angustifolia* against the MDA-MB-231 breast cancer cell line. The tested products were found to be inactive, showing IC_{50} values of 125 mg/mL for FemiVine V jelly and 82 mg/mL for the raw extract. The raw extract exhibited a stronger IC50 value compared to FemiVine V jelly (p<0.05).

Furthermore, Figure 3 presents the cell morphology of MDA-MB-231 cells at both low and high densities from ATCC. Figures 4(a) and (b) display cell morphology for negative and positive controls, along with initial post-treatment observations following the administration of FemiVine V jelly at concentrations of 500 mg/mL (c), 250 mg/mL (d), and 125 mg/mL (e). Figures 4 (f), (g), and (h) show the morphology for the raw extract. Furthermore, as the concentration of the product increases, cell morphology begins to display signs of interaction, with noticeable stress appearing across the cell colonies. At a concentration of 500 mg/mL, cells exhibit shrinkage and asymmetric membrane development (Figures 4(c) and (f)) for both FemiVine V jelly and the raw extract.
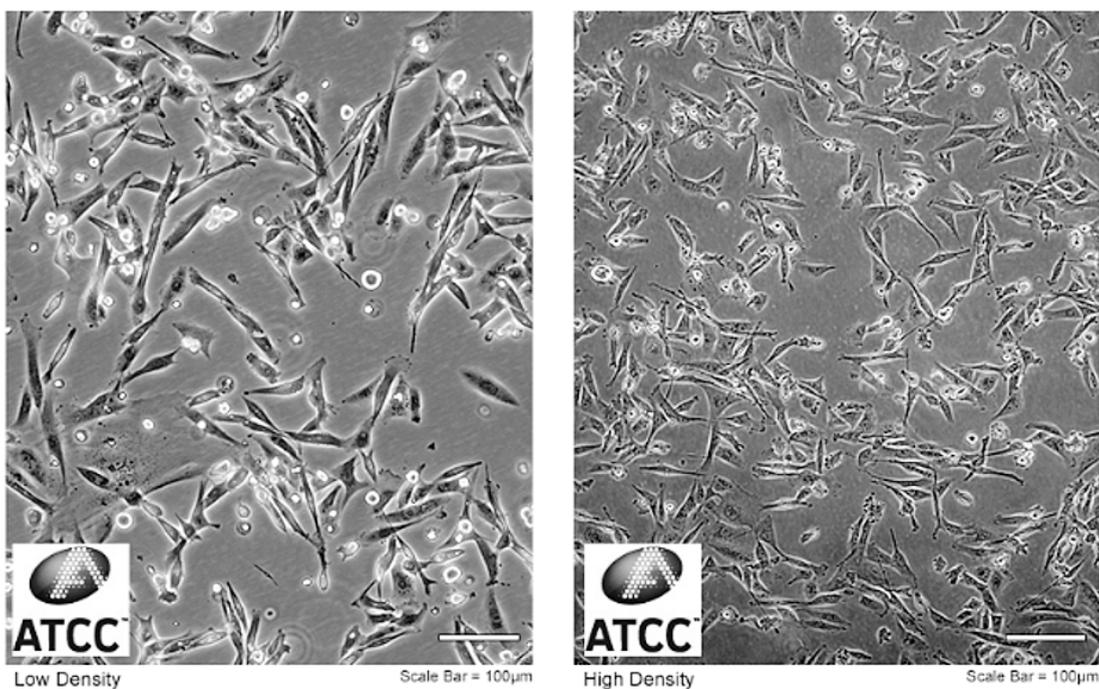

Fig. 3: Cell morphology of MDA-MB-231 under low and high density from ATCC

The evaluation of drug-likeness results revealed that both vitexin and β-sitosterol displayed 'drug-like' properties, satisfying all four criteria of the Rule of Five. In comparison to orientin and kaempferol, vitexin and β-sitosterol demonstrated acceptable blood-brain barrier (BBB) permeability, as illustrated in Figure 5. Table 4 presents the SwissADME web-based predictions for the physicochemical properties, including lipophilicity, pharmacokinetics, drug-likeness, and medicinal chemistry compatibility of these compounds. The *In-silico* predictions considered various factors, as shown in Figure 5, such as lipophilicity, molecular weight, and water solubility, all falling within acceptable ranges: lipophilicity (-0.7 to +5.0), molecular weight (150 to 500 g/mol), and water solubility (≤6). In contrast, orientin, vitexin, and kaempferol exhibit polar surface areas exceeding the recommended threshold (>130 Å²), which is associated with lower gastrointestinal absorption rates. Additionally, all compounds were predicted to have carbon saturation in Csp3 values within acceptable limits (≥0.25). The number of

rotatable bonds, which reflect compound flexibility, also remained within the recommended range (≤9) for these four flavonoid compounds. Furthermore, the results from the boiled egg prediction analysis (Figure 6) evaluated the passive gastrointestinal absorption (HIA) and brain penetration (BBB) of the compounds using WLOGP (lipophilicity) and TPSA (topological polar surface area).
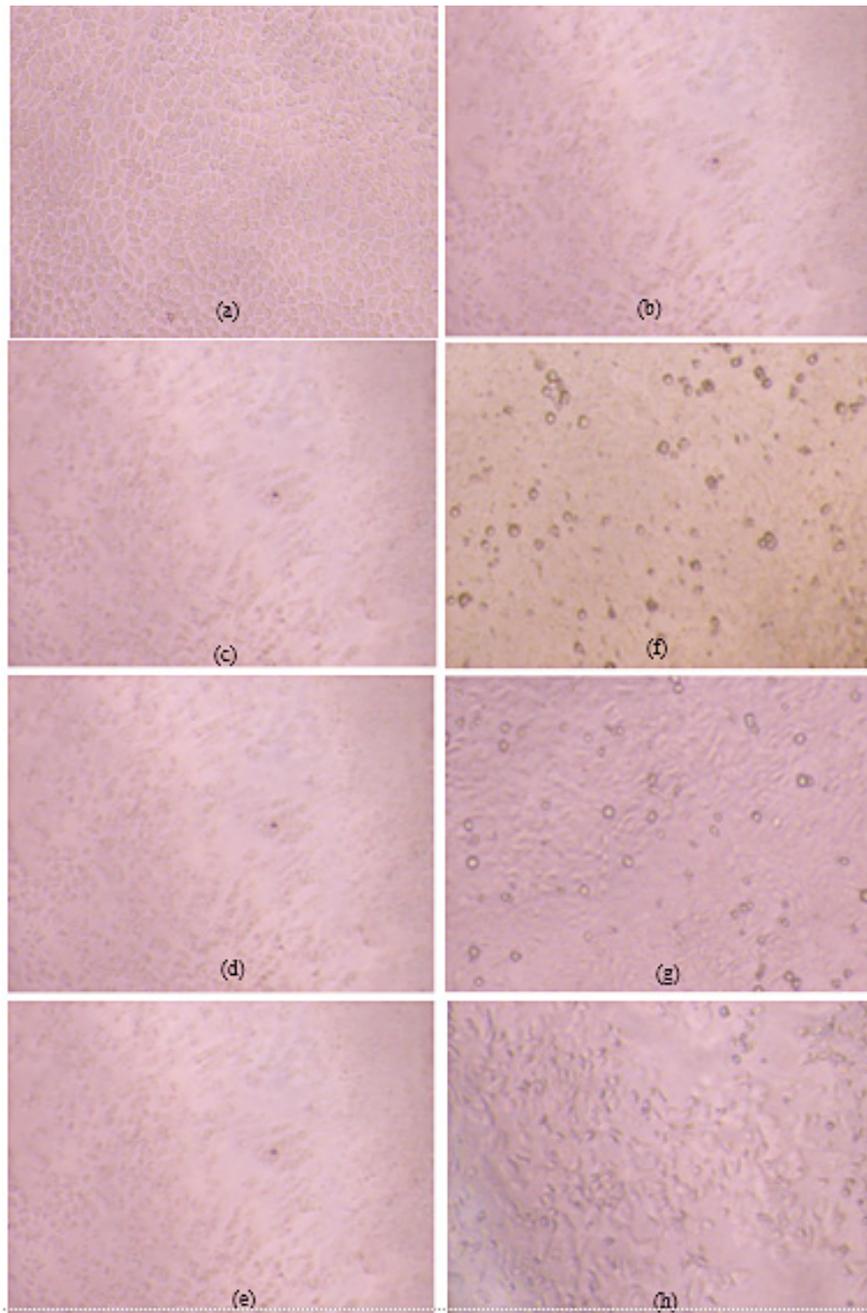

Fig. 4: Cell morphology for (a) negative control (b) positive control (c) concentration of 500 mg/ml FemiVine V jelly (d) concentration of 250 mg/ml FemiVine V jelly (e) concentration of 125 mg/ml FemiVine V jelly (f) concentration of 500 mg/ml raw extract (g) concentration of 50 mg/ml raw extract (h) concentration of 125 mg/ml raw extract

Table 4: *In silico* analysis for orientin, vitexin, kaempfolienol and β- Sitosterol

Parameter	Orientin	Vitexin	Kaempfolienol	β-Sitosterol
Lipophilicity (XLOGP3)	-0.60	1.62	-5.89	8.79
Size (MV)	434.35 g/mol	432.38 g/mol	364.35 g/mol	400.68 g/mol
Polarity (TPSA)	201.28	181.05	202.30	20.23
Solubility				
(Log S)	-3.16	-3.57	2.31	-9.10
Water soluble	Soluble	Soluble	High soluble	Poorly soluble
Saturation Csp3	0.25	0.29	0.87	0.93
Num.rotatable bonds	2	3	1	6
GI absorption	Low	Low	Low	Low
BBB permeant	No	No	No	No
P-gp substrate	No	No	Yes	No
Lipinski (Drug likeness)	No	Yes	Yes	Yes
Ghose (Drug likeness)	No	Yes	No	No
Bioavailability Score	0.17	0.55	0.55	0.55
Synthetic accessibility	5.12	5.19	5.3	6.17

*MW: molecular weight; GI: gastrointestinal; BBB: blood-to-brain barrier; P-gp: Glycoprotein Substrate XLOP3 Lipophilicity between -0.7 and +5.0 Size: MW between 150 and 500 g/mol; Solubility: TPSA between 20 and 130 Å2; solubility: Log S ≤ 6; Saturation: fraction of carbon in sp3 hybridization ≥ 0.25; Flexibility: ≤ 9 rotatable bonds Synthetic Accessibility: 1-10 (very easy to very difficult to synthesize)
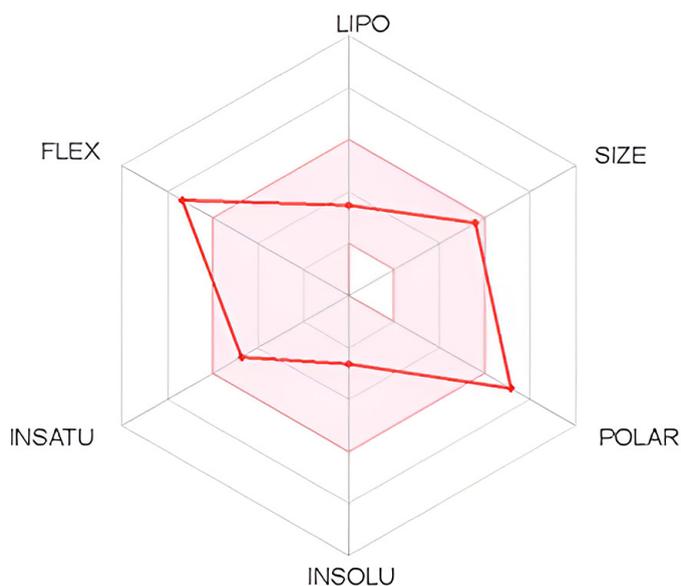
Fig. 5: The bioavailability radar allows a first look at the drug-likeness of a molecule. The pink area represents the optimal range for each property (lipophilicity: XLOGP3 between -0.7 and +5.0, size: MW between 150 and 500 g/mol, polarity: TPSA between 20 and 130Å2 , solubility: log S not higher than 6, saturation : carbon fraction in sp3 hybridization not less than 0.25, and flexibility: not more than 9 rotatable bonds this example, the compound is predicted to be unavailable orally, because it is too flexible and too polar
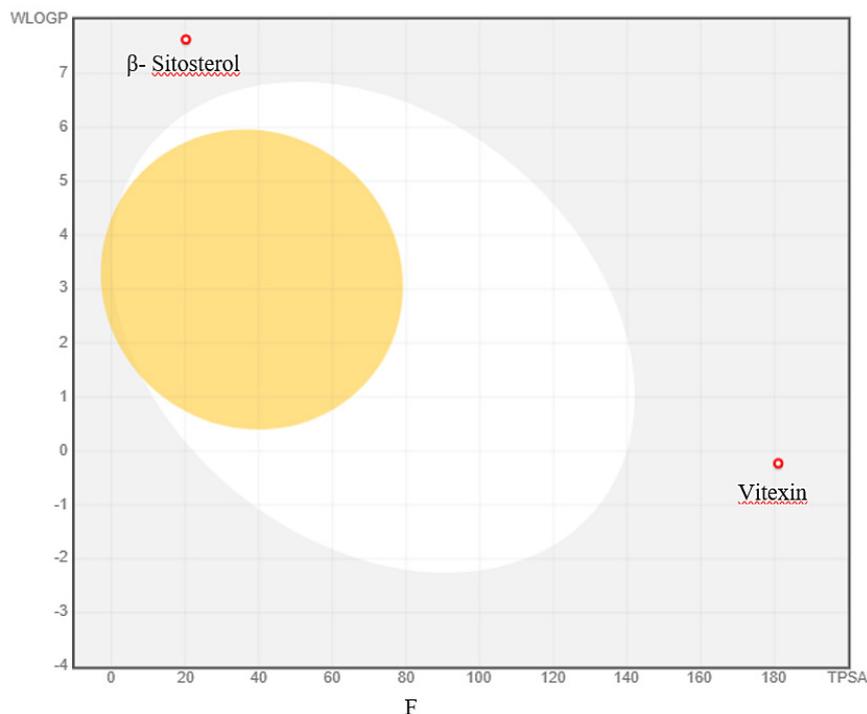
Fig. 6: Prediction analysis of boiled eggs to estimate the gastrointestinal absorption and brain penetration of vitexin and β- Sitosterol molecules. *Blood-brain barrier (BBB) penetration, assess passive active gastrointestinal absorption (HIA), PGP+ is a glycoprotein substrate and PGP- is not a glycoprotein substrate

Discussion

Flavonoid Identification and Glycosylation Patterns in *K. Angustifolia*

Flavonoids are synthesized through a series of condensation reactions involving hydroxycinnamic acid, which interacts with the B-ring and carbon atoms 2, 3, and 4 of the C-ring, and malonyl residues, which contribute to the A-ring. This process results in a base structure characterized by a C_6–C_3–C_6 backbone. The three-carbon bridge connecting the phenyl rings typically undergoes cyclization, forming a third ring (C-ring). In plants, flavonoids exist in various modified forms, often involving hydroxylation, methylation, and glycosylation. Additional modifications, such as attachment of aromatic or aliphatic acids and groups like sulfate, prenyl, methylenedioxyl, or isoprenyl, can further diversify the flavonoid nucleus and its glycosides.[20] Glycosylation plays a crucial role in modifying flavonoids, reducing their reactivity and increasing their water solubility. This modification serves as a protective mechanism in plants, preventing

cytoplasmic damage and enabling the safe storage of flavonoids in vacuoles. Glycosylation occurs through the attachment of sugar molecules to the flavonoid core, often via acid-resistant C–C bonds, resulting in the formation of flavonoid C-glycosides. These C-glycosides are categorized into mono-C-glycosylflavonoids, di-C-glycosylflavonoids, and C-glycosylflavonoid-O-glycosides. For flavones and flavonols, glycosylation typically occurs at the 7-hydroxyl group, while C-glycosylation usually takes place at the 6C or 8C positions.

For vitexin, retention times of 20.664 minutes in FemiVine V jelly and 20.619 minutes in the raw species were recorded. The molecular ion [M + H]+ was detected at m/z 432.24 and 435.36, with a molecular formula of $C_{21}H_{20}O_{10}$. Fragmentation primarily involved the sequential loss of $C_4H_8O_3$, water, and carbon monoxide, resulting in fragments [M – C4H8O3 – H], [M – C4H8O3 – H2O – H], and [M – C4H8O3 – CO – H], respectively.[21] For β-sitosterol, the molecular ion [2M + H]+ was detected at m/z

414.7 in FemiVine V jelly and 413.32 in the raw extract, corresponding to a molecular formula of $C_{29}H_5O_{10}$.

The compound formed an ammonium adduct, and its fragmentation yielded cholestanol and sitostanol ions, with water loss producing [M + H – H_2O]+.[22] Retention times were 15.793 minutes for FemiVine V jelly and 22.113 minutes for the raw extract. Kaempferol was identified in the mass chromatograms with m/z values of 354.5 and 289.05, at retention times of 1.064 minutes and 1.062 minutes, respectively. The fragmentation pattern suggested the presence of kaempferol glucoside, with the loss of [M – H]− to [A – H].[21]

Antioxidant Properties of *K. angustifolia* Extracts
The total phenolic content in the fresh, edible portion of *K. angustifolia* was quantified as milligrams of gallic acid equivalents (GAE) per 100 grams. The results revealed that FemiVine V Jelly contained significantly higher levels of phenolic compounds (31.93 ± 0.20 mg/g) compared to the raw extract (12.00 ± 0.23 mg/g, $p < 0.05$). This difference suggests that the processing involved in the production of FemiVine V Jelly may enhance the concentration of bioactive phenolic compounds, which are known for their antioxidant properties.

To further assess the antioxidant capacity, the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was utilized. This assay evaluates the ability of antioxidants to scavenge free radicals through either a single electron transfer (SET) or a hydrogen atom transfer (HAT) mechanism.[23,24] The DPPH radical, a stable free radical, is used as a chromogenic reagent that changes color upon reacting with antioxidants, allowing for the quantification of their scavenging ability. Both the chloroform and methanol extracts of *K. angustifolia* showed similar effectiveness in scavenging DPPH radicals. However, FemiVine V Jelly exhibited a significantly higher inhibition of 67.6 ± 0.15%, compared to just 30.54 ± 0.15% for the raw extract ($p < 0.05$). This indicates that FemiVine V Jelly has superior radical-scavenging ability, potentially due to its higher concentration of phenolic compounds, which contribute to its enhanced antioxidant activity.

The Ferric Reducing Antioxidant Power (FRAP) assay was also employed to evaluate the antioxidant capacity by measuring the reduction of Fe^{3+}-TPTZ (ferric-tripyridyltriazine) to Fe^{2+}-TPTZ. This method provides a direct assessment of an antioxidant's reducing power. FemiVine V Jelly demonstrated stronger reducing power than the raw extract, with values of 5.08 ± 0.01 mg/100 g ascorbic acid compared to 4.81 ± 0.05 mg/100 mL Vitamin C equivalent antioxidant capacity (VCEAC), respectively ($p < 0.05$). This result further supports the observation that FemiVine V Jelly has superior antioxidant activity, making it a potentially valuable source of natural antioxidants.

Taken together, these findings highlight that FemiVine V Jelly not only contains higher levels of phenolic compounds but also exhibits greater antioxidant efficacy than the raw extract. This enhanced antioxidant potential could have implications for the product's therapeutic use, particularly in mitigating oxidative stress-related diseases, where antioxidant activity plays a critical role. The difference in antioxidant capacity between the jelly and the raw extract underscores the importance of processing methods in improving the bioavailability and effectiveness of natural products. Further exploration of these properties could pave the way for developing more potent antioxidant-rich formulations for health benefits.

Cytotoxicity and Antiproliferative Effects
A recent study examined the antiproliferative effects of these compounds across various cancer cell lines, indicating that structural features such as methoxy substituents, α-methylation of the enone moiety, and 2'-oxygenated substituents contribute to cytotoxic activity due to their antimitotic properties. Additionally, methoxylated chalcones have demonstrated cytotoxic activity by inhibiting tubulin polymerization in multiple tumor cell lines, with IC_{50} values generally in the high micromolar range (IC_{50}: >50 µM). Phenolic compounds, including flavonoids, are known for their antioxidant properties, as they donate electrons to free radicals, thereby converting them into stable metabolites and halting radical chain reactions.[25]

This mechanism may contribute to further damage and the eventual death of cancer cells. The observed antiproliferative activity was not solely due to individual compounds but rather a combination of several flavonoids, including vitexin, orientin,

kaempferol, and β-sitosterol, all of which are present in the product.[26] Although other organic solvents, such as methanolic leaf extracts, have been used to extract these phenolic constituents and have demonstrated significant efficacy in killing cancer cells, subacute oral administration in rats showed no recorded toxicity.[19, 23, 27]

In silico Evaluation of Drug-Likeness, BBB Permeability and Therapeutic Potential of Flavonoid Compounds

Several descriptor properties were employed to assess the molecular characteristics of orientin, vitexin, kaempferol, and β-sitosterol, focusing on the 'Rule of Five' (ROF) and blood-brain barrier (BBB) permeability.[26,28] The prediction analysis of boiled eggs (Figure 6) indicates that both vitexin and β-sitosterol are likely to be absorbed passively through the gastrointestinal tract but do not appear to penetrate the brain. Additionally, these two flavonoid compounds are not substrates for P-glycoprotein (P-gp), suggesting that they are not actively transported back into the gut after absorption.[29,30] *In silico* analysis thus suggests that vitexin and β-sitosterol possess favourable pharmacokinetic properties for targeting MDA-MB-231 breast cancer cells. Their good absorption profiles, lack of BBB penetration, and non-substrate status for P-gp highlight their potential as promising candidates for therapeutic development.[31,32] However, further research is needed to assess their cytotoxic effects on normal fibroblast cells to confirm their clinical suitability. With additional studies, these compounds could emerge as targeted agents with minimal side effects.

Conclusion

In conclusion, the study investigated four key flavonoids, orientin, vitexin, kaempferol, and β-sitosterol found in FemiVine V Jelly and its raw extract, using Q-TOF LC-MS analysis to identify their presence. Using Q-TOF LC-MS analysis, these compounds were successfully identified in both FemiVine V Jelly and the raw extract, with m/z values of 447.69 (orientin), 432.24 (vitexin), 354.5 (kaempferol), and 414.7 (β-sitosterol). Antioxidant potential was evaluated through Total Phenolic Content (TPC), DPPH, and FRAP assays. FemiVine V Jelly exhibited a significantly higher phenolic content (31.93 ± 0.20 mg/g) compared to

the raw extract (12.00 ± 0.23 mg/g, $p < 0.05$). In the DPPH radical scavenging assay, FemiVine V Jelly demonstrated superior scavenging activity (67.60 ± 0.15%) compared to the raw material (30.54 ± 0.10%, $p < 0.05$). Similarly, in the FRAP assay, FemiVine V Jelly displayed stronger reducing power (5.08 ± 0.01 mg/100 g ascorbic acid) than the raw extract (4.81 ± 0.05 mg/100 ml Vitamin C, $p < 0.05$). Cytotoxicity screening revealed that compounds isolated from *K. angustifolia* exhibited antagonistic effects against MDA-MB-231 breast cancer cells. However, the tested products showed limited activity, with IC_{50} values of 125 ± 0.20 mg/ml for FemiVine V Jelly and 82 ± 0.06 mg/ml for the raw extract. The raw extract demonstrated stronger cytotoxic potential ($p < 0.05$). Additionally, *In-silico* analysis suggested that vitexin and β-sitosterol possess favorable pharmacokinetic properties, including good gastrointestinal absorption and no brain penetration, making them promising candidates for peripheral cancer treatments such as MDA-MB-231 breast cancer. Their non-substrate status for P-glycoprotein (P-gp) could enhance intracellular retention. However, further studies are required to evaluate their selective cytotoxicity against cancer cells versus normal fibroblast cells. To emphasize practical implications, future studies could explore the development of FemiVine V Jelly or similar formulations as adjuncts to cancer therapies, particularly in peripheral cancers, by assessing their ability to selectively target and kill cancer cells without harming healthy cells. This would be important in optimizing the use of these compounds in clinical settings and advancing their therapeutic application.

Conflict of Interest
The author(s) do not have any conflict of interest.

Ethics Statement
This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement
This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration
This research does not involve any clinical trials.

Data Availability Statement
No data were used elsewhere to support this study and it was entirely a new set of data.

Permission to Reproduce Materials from Other Sources
Not Applicable.

Author Contributions
- **Farah Nadiah Mohd Fazil:** Conceptualization, Methodology, Writing – Original Draft.
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