Malaysian Journal of Chemistry, 2024, Vol. 26(3), 456-464
(Special Issue: *3rd Virtual International Symposium on Sciences 2023*)

Chemical Composition, Antibacterial Activity, and ADME Studies of Leaf Essential Oil of *Piper betle*

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This study aims to determine the chemical composition and antibacterial activity of the essential oil of *Piper betle* leaf and predict the physicochemical and ADME (absorption, distribution, metabolism and excretion) properties of the major compounds found from the oil. The essential oil was extracted by hydrodistillation technique and analysed using gas chromatography-flame ionisation detector (GC-FID) and gas chromatography-mass spectrometry (GC-MS). The antibacterial activity was screened using the disc diffusion method, while the physicochemical and ADME properties were predicted using the SwissADME web tool. Thirty-eight compounds were successfully identified, representing 94.1% of the total oil. Eugenol (18.9%), germacrene D (11.6%), chavibetol acetate (9.3%), β-caryophyllene (7.4%), and bicyclogermacrene (7.3%) were identified as the main compounds in the essential oil. The *P. betle* oil gave the diameter of inhibition zones against all tested bacteria in the range between 7.00 and 12.67 mm, with the largest inhibition zone observed for *B. cereus* (12.67 mm). The ADME studies revealed that eugenol and chavibetol acetate exhibited good drug-likeness properties by passing Lipinski's rule of five. These findings lead us to consider both compounds as a potential scaffold for enhancing antibacterial activity.

Keywords: *Piper betle*; essential oil; antibacterial; ADME; drug-likeness

Received: November 2023; Accepted: Februay 2024

Piper betle, commonly known as betel vine (in English), or sirih (in Malay), is an evergreen and perennial plant belonging to the family Piperaceae [1, 2]. The plant originates from Central and Eastern Peninsular Malaysia and distributed throughout East Africa and the tropical region of Asia. *P. betle* is extensively consumed in Asia, and its application is found in various traditional medicinal systems [2]. The leaves of this plant are the most valued plant part possessing diverse medicinal properties to treat ailments. The leaves of *P. betle* are utilised as a mouth freshener and for treating various disorders, including wound healing, pancreatic lipase stimulant, digestive problems, conjunctivitis, constipation, carminative, astringent, headaches, itches, rheumatism, ringworm, gum swelling, and bruising [2-5]. In addition, the leaves of *P. betle* are also chewed with mineral-slaked lime to increase salivation and act as a stimulant. Furthermore, the leaves of this plant have been documented to demonstrate an antibacterial effect that inhibits the growth of bacteria responsible for causing typhoid, cholera and tuberculosis [6].

The leaves of *P. betle* have a strong pungent aroma due to the presence of abundant terpenes and phenylpropanoids in its essential oil [7]. Examples of terpenes that are present in significant amounts are γ-muurolene, terpinen-4-ol, β-caryophyllene, β-cubebene, β-selinene, cubenol, α-selinene, and β-phellandrene [8-14]. Furthermore, several phenylpropanoids such as eugenol, chavibetol acetate, chavibetol, eugenyl acetate, estragole, chavicol, safrole, allyl-pyrocatechol monoacetate, allyl-pyrocatechol, (*E*)-isoeugenol, methyl eugenol, isoeugenol acetate, 4-allyl-1,2-diacetoxybenzene, and chavicol acetate are also detected in significant quantities [9, 11-23]. The essential oils of *P. betle* have been recognised to exhibit numerous remarkable pharmacological properties such as antiproliferative, anticancer, neuropharmacological, analgesic, antioxidant, anti-ulcerogenic, hepatoprotective, antiplatelet, and anti-fertility effects. In addition, the essential oil can also combat microbial and parasitic infections and show an inhibitory effect against insects and larvae [2, 23]. Although there are many reports available on the chemical compositions and pharmacological aspects of the leaf essential oils of *P. betle*, no studies have been conducted to predict the pharmacokinetic and drug-like properties of its compounds through *in silico* prediction. Hence, this research is conducted to

†Paper presented at the 3rd Virtual International Symposium on Sciences (3rd VISoS 2023)

determine the chemical composition and antibacterial activity of the leaf essential oil of *P. betle* and predict the ADME (absorption, distribution, metabolism and excretion) properties of the major compounds.

EXPERIMENTAL

Plant Material

The fresh leaves of *P. betle* were collected from Kampung Terusan, Juasseh, Negeri Sembilan, Malaysia (2°47'30.09"N 102° 17' 58.38"E), in September 2022 and verified by Dr. Shamsul bin Khamis. A voucher specimen (ID054/2022) was deposited at the Herbarium Universiti Kebangsaan Malaysia (UKMB).

Extraction of Essential Oil

The fresh leaves of *P. betle* (660 g) were cut into small pieces and subjected to hydrodistillation using a Clevenger-type apparatus for 6 hours. The oil phase of the distillate was separated with diethyl ether, dried over anhydrous magnesium sulphate, and stored at 4°C until further analysis [24].

Chromatographic Analysis

GC-FID analysis was conducted using a Shimadzu GC-2010 Plus gas chromatograph equipped with a flame ionisation detector (FID) and fitted with a ZB-5 capillary column (30 m × 0.25 mm; 0.25 µm film thickness). The oven temperature was programmed at 60°C with an initial hold time of 10 min, increasing at a rate of 3°C/min up to 230°C with a final hold time of 1 min. The injector and detector temperatures were maintained at 250°C each, while helium was used as a carrier gas with a flow rate of 30 mL/min. One microliter of essential oil in diethyl ether was injected manually using split mode (ratio 50:1). The relative content of each compound was measured by electronic integration and calculated based on the percentage of peak area. The injections were performed in three replications. GC-MS analysis was conducted using an Agilent Technologies GC-MS 7890A/5975C Series Mass Selective Detector (MSD) equipped with an HP-5MS fused silica capillary column (30 m × 0.25 mm; 0.25 µm film thickness). The same column and injector temperatures described for the GC-FID analysis were employed. The mass detector was operated in electron ionisation (EI) mode at 70 eV with a mass range of 50–550 amu at a rate of 2.91 scans/s. The total scan time was 67.667 min [24]. The identification of chemical composition was accomplished by matching their mass spectral data with the spectral data stored in the mass spectral databases (Wiley7NIST05.L, HPCH 2205. L and NIST05a.L), and further confirmed by comparing the experimental retention indices (RI) calculated using a series of *n*-alkanes (C_6–C_{28}) with those reported in the literature [25-32].

Antibacterial Activity

The antibacterial activity of the leaf essential oil of *P. betle* was tested against Gram-negative bacteria, *Escherichia coli* (ATCC 25922) and *Klebsiella pneumoniae*, (ATCC 700603) and Gram-positive bacteria, *Bacillus cereus* (ATCC 11778) and *Staphylococcus aureus* (ATCC 25923) using the disc diffusion method with slight modifications [33]. The 0.5 McFarland turbidity standard was used as a reference to adjust the turbidity of the bacterial suspension. The turbidity of the 0.5 McFarland standard and the bacterial suspension was compared by measuring their optical density at 600 nm. The optical density value that matches 10^8 colony-forming units/mL ranges between 0.08 and 0.132 [34]. The agar plate was inoculated individually with the bacterial suspension using a sterile cotton swab. Ten µL of the essential oil was applied to a sterile Whatman No. 1 sterile paper disc (6 mm diameter) and then placed on the surface of the inoculated agar plate. Streptomycin (10 µg per disc) was used as the standard antibiotic against the tested bacteria. The assay was conducted in triplicate, and all plates were incubated for 24 hours at 37ºC. The antibacterial activity was assessed by measuring the diameter of inhibition zone (in mm).

Drug-Likeness Prediction

The drug-likeness properties of selected major compounds were predicted using the SwissADME web tool (http://www.swissadme.ch/), as previously described [35]. The SwissADME software web server displayed the SwissADME submission page to estimate the individual ADME properties of selected compounds. A molecular sketcher based on Chem Axons Marvin JS was incorporated into the input zone, enabling users to draw and edit two-dimensional chemical structures. The chemical structures were transferred to the right-hand side of the submission page as a list and served as the actual input for the computation. Meanwhile, a simplified molecular-input line-entry system defined the list as an input molecule per line with multiple inputs (SMILES). Tables, graphs, and an Excel spreadsheet were used to illustrate the output data for each compound.

RESULTS AND DISCUSSION

Chemical Composition of *P. betle* Leaf Essential Oil

The extraction of the fresh leaves of *P. betle* using hydrodistillation technique yielded a yellowish oil (0.94 g, 0.14%). The GC-FID and GC-MS analysis of the essential oil identified 38 compounds representing 94.1% of the total oil (Table 1). Eugenol (18.9%), germacrene D (11.6%), chavibetol acetate (9.3%), β-caryophyllene (7.4%) and bicyclogermacrene (7.3%) were the major compounds in the oil. The *P. betle* oil

was dominated by sesquiterpene hydrocarbons (19 compounds) and phenylpropanoids (five compounds), contributing 47.6% and 34.8% of the oil, respectively. The oil also contained four oxygenated monoterpenes (9.7%) and nine oxygenated sesquiterpenes (1.6%). On the contrary, only one monoterpene hydrocarbon identified as (*E*)-β-ocimene (0.1%) was detected in this oil. In line with this study, eugenol, germacrene D and β-caryophyllene were also identified in a remarkable amount in the *P. betle* leaf oils growing in different regions of India and Vietnam [10-12, 16-18]. Chavibetol acetate was also detected in a significant quantity in the oils from Nepal and the Philippines [20, 21]. In contrast, the percentage of bicyclogermacrene in this study was higher than that of earlier studies [7, 11].

Table 1. Essential oil composition of the leaf essential oil of *P. betle*.

Compound	**RI[a]**	**Percentage(%)[b]**
1,8-Cineole	1030	0.3
(*E*)-β-Ocimene	1050	0.4
Linalool	1103	0.7
Terpinen-4-ol	1177	0.1
α-Terpineol	1192	0.5
δ-Elemene	1337	0.7
α-Cubebene	1349	0.2
α-Copaene	1375	2.6
Eugenol	1385	18.9
β-Elemene	1395	5.1
Methyl eugenol	1410	0.2
β-Caryophyllene	1422	7.4
β-Copaene	1429	0.4
(*E*)-α-Bergamotene	1437	0.2
Aromadendrene	1439	0.6
α-Humulene	1454	1.3
(*E*)-β-Farnesene	1461	0.7
Germacrene D	1486	11.6
β-Selinene	1489	1.2
Bicyclogermacrene	1500	7.3
α-Muurolene	1503	0.6
Premnaspirodiene	1508	1.5
(*E*,*E*)-α-Farnesene	1513	2.0
γ-Cadinene	1516	0.7
δ-Cadinene	1527	3.1
Eugenyl acetate	1535	2.0
Chavibetol acetate	1542	9.3
Germacrene B	1559	0.4
Spathulenol	1583	0.5
Globulol	1590	1.6
Viridiflorol	1597	1.8
Rosifoliol	1606	0.7
Junenol	1620	0.6
1-*epi*-Cubenol	1630	0.6
τ- Muurolol	1645	0.6
4-Allyl-1,2 diacetoxybenzene	1654	4.4
α-Cadinol	1659	3.1
Germacra- 4(15),5,10(14)-trien-1α-ol	1687	0.2
Total (%)		**94.1**

[a]Retention indices of the compounds were calculated relative to the retention times of a series of *n*-alkanes (C_6-C_{28}); relative percentages of the compounds were obtained electronically from the FID area percent data.

Table 2. Inhibition diameters of the leaf essential oil of *P. betle* (mean ± SD)[a].

Bacterial strain	Essential oil[b]	Streptomycin[c]
Gram-positive bacteria		
B. cereus	12.67 ± 3.21	15.00 ± 1.00
S. aureus	8.67 ± 1.53	13.67 ± 0.58
Gram-negative bacteria		
K. pneumoniae	7.67 ± 1.20	14.00 ± 0.00
E. coli	7.00 ± 0.00	12.00 ± 0.00

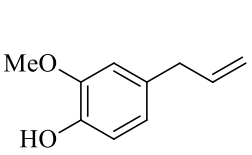
[a]Inhibition diameter (mm) including the diameter of the paper disc (6 mm); [b]Pure sample; [c]Positive control (10 μg/disc)

Antibacterial Activity

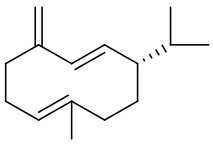
The leaf essential oil of *P. betle* was tested for its antibacterial potential by measuring the diameter of inhibition zone observed using a disc diffusion assay. As shown in Table 2, the oil demonstrated varying degrees of antibacterial activity against the tested bacteria, with the diameter of inhibition zones ranging from 7.00 to 12.67 mm. The highest inhibitory effect was observed against *B. cereus* (12.67 mm), followed by *S. aureus* (8.67 mm), *K. pneumoniae* (7.67 mm), and *E. coli* (7.00 mm). However, the bacteria were less sensitive to the essential oil than the positive control (streptomycin), which resulted in the inhibition zones ranging from 12.00 to 15.00 mm. In line with the present study, the leaf oils of *P. betle* from Sri Lanka and Trichy, India also demonstrated antibacterial activity against *S. aureus* [36, 37]. In addition, the leaf oil of *P. betle* from Mauritius also exhibited an antibacterial effect against *K. pneumoniae* and *E. coli* [15]. The antibacterial activity exhibited by *P. betle* oil may be attributed to its major compounds, eugenol and β-caryophyllene, which were previously reported to possess antibacterial properties [38].

Drug-Likeness Prediction of Five Major Compounds

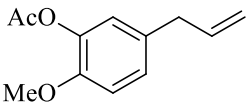
Evaluating ADME properties is an essential step in drug development. Hence, a drug-like product is absorbed within a desired time frame and distributed throughout the body for efficient metabolism [39]. The probable drug-likeness and ADME properties of the major compounds in *P. betle* leaf essential oil (Figure 1) were investigated using the SwissADME webserver. The results of the ADME studies are illustrated in Tables 3 and 4 and also in Figure 2. Absorption was predicted from human intestinal absorption (HIA), lipophilicity, and water solubility of the compound. Drug-likeness prediction was made using the Lipinski's rule of five, which states that the compound should not violate more than one rule to exhibit drug-like behaviour. According to Lipinski et al. [40], the rule of five for good absorption or penetration should have the molecular weight < 500 Da, the number of H donor bonds < 5, the number of H acceptor bonds < 10, the number of rotatable bonds < 10, lipophilicity (octanol/water partition coefficient, log *P*) < 5 and the total polar surface area < 140Å^2.



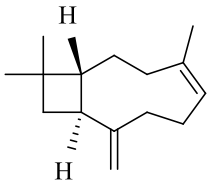
Eugenol (**1**)



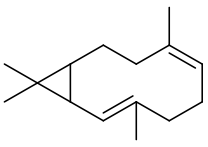
Germacrene D (**2**)



Chavibetol acetate (**3**)



β-Caryophyllene (**4**)



Bicyclogermacrene (**5**)

Figure 1. Chemical structure of the major compounds in *P. betle* essential oil.

Table 3. Drug-likeness prediction of the major compounds in *P. betle* essential oil.

Drug-likeness	Compound				
	1	**2**	**3**	**4**	**5**
Molecular weight (g/mol)	164.2	204.35	206.24	202.34	206.37
No of hydrogen donor	1	0	0	0	0
No of hydrogen acceptor	2	0	3	0	0
No of rotatable bonds	3	1	5	0	1
Total polar surface area (Å^2)	29.46	0	35.53	0	0
Log $P_{O/W}$	2.25	4.29	2.55	3.93	4.40
Lipinski's rule of five violation	0	1	0	1	1

Lipinski's rule of five; molecular weight < 500 Da; number of H donor bonds ≤ 5; number of H acceptor bonds ≤ 10; number of rotatable bonds < 10; lipophilicity (octanol/water partition coefficient, log P) < 5; total polar surface area < 140Å^2.

Based on Lipinski's rule (Table 3), eugenol and chavibetol acetate were highly considered for absorption as they have no violation of the rule, whereas, germacrene D, β-caryophyllene and bicyclogermacrene violated only one rule but would still be considered for absorption. The molecular weight of all major compounds ranged from 164.2 to 206.37 g/mol, which should be between 150 and 500 g/mol according to the drug-likeness prediction rule. The topological polar surface area (TPSA) values of chavibetol acetate and eugenol were found to be 35.53 Å^2 and 29.46 Å^2, respectively. The values suggested that both compounds showed good cell permeability compared to other compounds [41]. The compounds are considered to have good cell permeability if their TPSA falls between 20 and 130 Å^2 [42]. Based on Table 4, bicyclogermacrene and germacrene D displayed the highest skin permeant with log *Kp* values of -4.15 cm/s and -4.18 cm/s, respectively. In contrast, chavibetol acetate showed the least skin permeant with a log *Kp* value of -5.93 cm/s.

On the other hand, the octanol/water partition coefficient (log $P_{o/w}$) of bicyclogermacrene and germacrene D have high log $P_{o/w}$ values of 4.40 and 4.29, respectively. Meanwhile, other compounds showed moderate and low values (β-caryophyllene (3.93), chavibetol acetate (2.55), and eugenol (2.25)). All studied compounds showed log $P_{o/w}$ values less than five, indicating potential drug-like tendencies, good binding into hydrophobic pockets of enzymes, and metabolism's best distribution and excretion [39, 43].

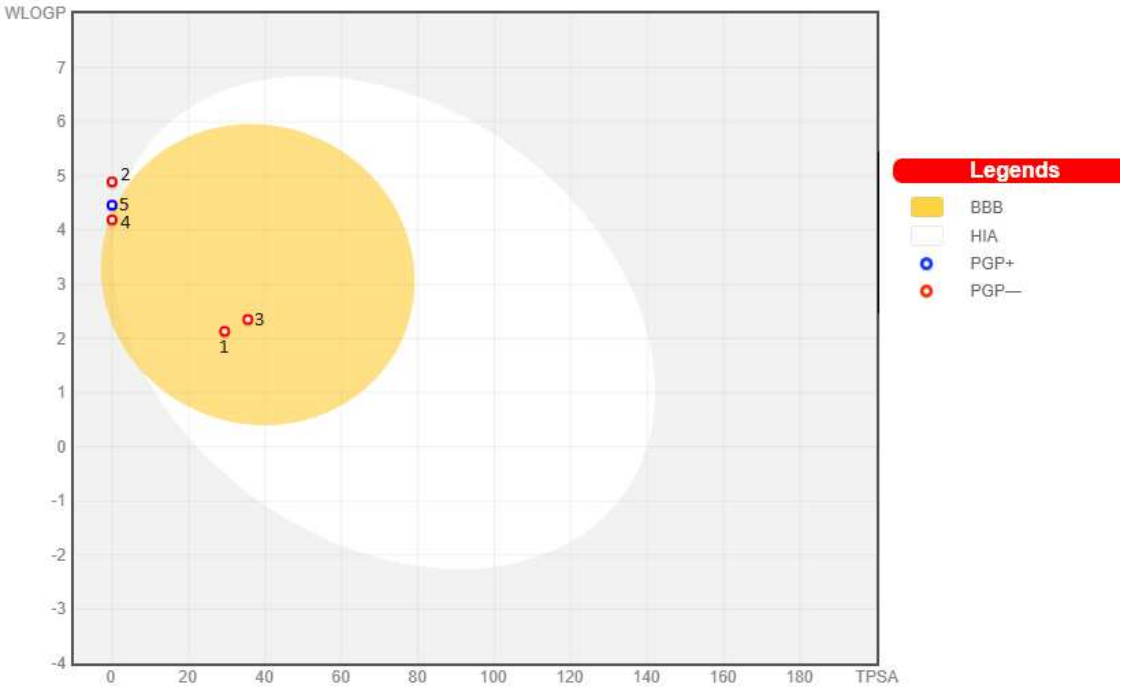


Figure 2. BOILED-Egg graph of the major compounds: 1: Eugenol, 2: Germacrene D, 3: Chavibetol acetate, 4: β-Caryophyllene, 5: Bicyclogermacrene.

Table 4. ADME prediction of the major compounds in *P. betle* essential oil.

ADME Properties	Compound				
	1	**2**	**3**	**4**	**5**
Pharmacokinetics					
GI absorption	High	Low	High	Low	Low
BBB permeability	Yes	No	Yes	Yes	No
Log *Kp* (cm/s)	-5.69	-4.18	-5.93	-4.88	-4.15
Inhibitor interaction					
P-gp substrate	No	No	No	No	No
CYP1A2 inhibitor	Yes	No	Yes	No	No
CYP2C19 inhibitor	No	No	No	Yes	Yes
CYP2C9 inhibitor	No	Yes	No	Yes	Yes
CYP2D6 inhibitor	No	No	No	No	No
CYP3A4 inhibitor	No	No	No	No	No

P-glycoprotein (P-gp); Gastrointestinal absorption (GI); Blood-brain barrier (BBB); Cytochromes (Cyp) of P450 family (CYP1A2, CYP2C19, CYP2D6, CYP2C9 and CYP3A4 inhibitors); Log *Kp* (skin permeability).

The distribution was also predicted using three parameters, namely P-glycoprotein (P-gp) substrate, gastrointestinal absorption (GI), and blood-brain barrier (BBB) permeability to maintain homeostasis in the central nervous system (CNS) to transfer nutrients, materials, and cells from the blood to the brain (Table 4) [44]. Through the analysis of the BOILED-Egg graph (Figure 2), eugenol and chavibetol acetate demonstrated high GI absorption, while other compounds exhibited low GI absorption. These findings suggested that the GI tract easily absorbed eugenol and chavibetol acetate [45]. The results also showed that eugenol, chavibetol acetate, and β-caryophyllene could easily cross the BBB, while germacrene D and bicyclogermacrene were predicted not to cross the BBB. When a compound has difficulty in crossing BBB, it is less likely to cause adverse effects on the CNS [46]. However, no compounds were found to be P-gp substrates, indicating that all the compounds have good intestinal absorption and bioavailability [35].

The metabolism was predicted based on the inhibitor interaction, i.e., the cytochromes of the P450 family (CYP1A2, CYP2C19, CYP2D6, CYP2C9, and CYP3A4 inhibitors). Cytochrome P450 (CYP450) enzymes are found in most species. They catalyse oxidative reactions of both endogenous and exogenous substances, with P450 enzymes from families CYP1, CYP2, and CYP3 particularly implicated in drug metabolism [46]. The oxidised molecules become polar and are excreted by the kidneys within a given time. Table 4 shows that eugenol and chavibetol acetate inhibited CYP1A2, while germacrene D, β-caryophyllene, and bicyclogermacrene were inhibited by CYP2C9. Meanwhile, β-caryophyllene and bicyclogermacrene were also predicted to inhibit another isoenzyme, CYP2C19. In contrast, all compounds were expected to be non-inhibitors of CYP2D6 and CYP3A4. The inhibition of this isoenzyme is the main cause of pharmacokinetic-related drug interactions. It can lead to toxic or unwanted side effects due to lower release and accumulation of the drug or its metabolites [47].

CONCLUSION

The current results showed that the essential oil extracted from the leaves of *P. betle* was rich in sesquiterpene hydrocarbons (47.6%) and phenylpropanoids (34.8%). In addition, the oil inhibited the growth of all tested bacteria, especially against *B. cereus*. Moreover, two major compounds in the *P. betle* oil, eugenol and chavibetol acetate, possessed good drug-likeness properties, low toxicity and good pharmacokinetic profiles. This leads us to consider both compounds as potential scaffolds for improving antibacterial activity. Future studies will be carried out on molecular docking to identify the binding sites on the target protein structure.

ACKNOWLEDGEMENTS

The authors wish to acknowledge Universiti Teknologi MARA (UiTM) Cawangan Negeri Sembilan, Kampus Kuala Pilah, Negeri Sembilan, for the financial assistance and research facilities. We would also like to thank ChM. Dr. Suhaidi Ariffin for providing the bacterial strains.

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