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# Effect of extraction procedure on the yield and biological activities of hydroxychavicol from *Piper betle* L. leaves

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ABSTRACT

*Piper betle* Linn is one of the most common ethnomedicinal plants with its extract being popularly used in the modern product to enhance functionality. However, extraction methods always lead to differences in biological activities. The objectives of this study were to evaluate the effects of the extraction methods on the yield and biological activities of hydroxychavicol from *P. betle* L. extracts and to determine the correlation between the hydroxychavicol content and biological activities of *P. betle* L. extracts such as antioxidant, antimicrobial, and anticancer properties. The purity of the hydroxychavicol and its concentration (quantitative) in the crude extracts were also evaluated using a reverse-phase HPLC while GC–MS was employed to determine other components (qualitative). The results showed that only certain extraction procedures gave high yields of hydroxychavicol as well as remarkable biological activities. The chloroform extract following boiling with water (M2) gave the highest percentage of hydroxychavicol content based on the HPLC analysis. M2 and pure hydroxychavicol actively inhibited all the five cancer cell lines studied except A549. M2 showed more effective inhibition activity against MCF 7 with an $IC_{50}$ of 1.74 ug/mL. M2 extract also showed strong antibacterial activity against all the bacteria strains as well as a strong antifungal activity against *Candida albicans*. There was, however, a weak correlation between the hydroxychavicol content and the biological activities of *P. betle* L. extracts. In conclusion, extraction procedures greatly affect the yield and biological activities of hydroxychavicol from *P. betle* L. The designation of a single compound such as hydroxychavicol as a bioactive chemical marker compound in the *P. betle* L. extracts, however, is not enough to determine the biological activities of the extract.

## 1. Introduction

Herbal and natural raw materials have gained unprecedented attention in cosmetics, food additives, medicinal formulations, fragrances, and nutrition owing to the complex mixtures of several compounds in their matrices exhibiting synergetic and additive properties (Kharbach et al., 2020). *Piper betle L.* is one of the dicotyledonous plant species that grow heavily in Southeast Asia. In Malaysia, it is locally known as *sirih*. This species belongs to the Piperaceae family. It is a climber species and mainly cultivated for its leaves (Choudhary and Kale, 2002). This species is known in traditional folk medicine for oral care. In modern medicine, owing to its non-toxic properties both in vitro and in vivo, it is being intensively studied and found to have many strong biological activities such as antioxidant, anticancer, antibacterial, antifungal as well as anti-fertility, hepatoprotective, immunomodulatory, anti-allergic, gastro-protective, and wound healing (Dasgupta and De, 2004; Ma et al., 2013; Yadav et al., 2014; Venkadeswaran et al., 2016). To date, this plant has been known as a high-value herbal plant by the Malaysian government and worths further studies. Many health care products have been formulated with *P. betle* L. extract as one of the main ingredients (Ali et al., 2018). *P. betle* L. is rich with phenolic compounds from the class of phenylpropanoid (Rimando et al., 1986). One of the major and active compounds found in this species is hydroxychavicol. This compound has been reported to possess strong

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biological activities such as antinitrosation, antimutagenic, anticarcinogenic, antioxidant, antimicrobial, and anti-inflammatory (Ali et al., 2010; Kato et al., 2013). Polyphenols are not only good for the improvement of cognitive and memory functions but also for the prevention of cancer, diabetes, cardiovascular and chronic intestinal diseases as well as neurological disorders (Ou et al., 2019).

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated in the human body during normal oxygen metabolism, energy transfer processes, enzymatic reactions, and physiological activities such as inflammatory response and body defense against microbial invasion (Ribeiro et al., 2014; Mikail et al., 2016; Tao et al., 2019). An increase in the level of reactive oxygen species will lead to oxidative stress which, in turn, underlies various disorders and diseases such as atherosclerosis, neurodegenerative disorders, aging, and cancer (Gómez-Verjan et al., 2015; Ahmed et al., 2017). Nowadays, cancer has become a major public health problem worldwide. Since 2015, it has assumed the second leading cause of death which accounts for about 8.8 million mortalities all over the world (Mahmoud and Abdelrazek, 2019). There were about 9.6 million cancer deaths and 18.1 million new cancer cases in 2018 (Rattanaburee et al., 2019). According to the World Health Organization (WHO), the cancer statistic for 2018 revealed that the annual incidence of lung, breast, and colorectal cancer is around 2.09, 2.09, and 1.80 million cases, respectively, thus making the three the highest reported cases as compared with others (WHO, 2018a, b).

Though there are significant advances in the diagnosis, screening, and treatment of cancer, some of these cancers have been highly metastatic and highly resistant to various anticancer treatment strategies (Lima et al., 2016). The inhibition of ROS and other radical molecules might reduce these disorders and diseases. Phenolic compounds are known to have antioxidant properties, which will protect the cells from ROS and RNS. These phenolic compounds usually exhibit anti-oxidative activity via several mechanisms of action. The mechanisms are singlet oxygen quencher, hydrogen-donating antioxidant, free radical scavenger, and metal ions chelator (Ruhomally et al., 2015; Zamakshshari et al., 2019). These anti-oxidative activities are generally possessed by the plant extracts and are linked with the phenolic compounds, including hydroxychavicol present in the *P. betle* L. extracts (Gundala et al., 2014). On the other hand, infectious diseases are one of the leading causes of death worldwide (Ahmed et al., 2021). The development of drug-resistant microorganisms is emerging and spreading globally, making it difficult to treat common infectious diseases. The situation now leads to a prolonged illness, disability, and death (WHO, 2018a, b).

The use of herbs in commercial products as dietary supplements is gaining more popularity nowadays. Plant extract-based products are widely available in the market (Dagnon et al., 2019; Kithma et al., 2019; Quatrin et al., 2019; Ahmed et al., 2019, 2020; Allaq et al., 2021). The extraction of these herbs is important before the discovery, purification, and utilization of the active phenolic compounds for relevant biological activity (Haq et al., 2011; Ahmed et al., 2015). There are several extraction methods which include solvent extraction in which the solvent properties play a key role in the efficiency of the extraction techniques employed (Alcântara et al., 2019).

Solvent extraction is a solid-liquid leaching technique that involves the mass transfer of the solvent into the solid material (plant material) to dissolve the solutes (primary and secondary metabolites) and extract them out into the liquid phase (Ali et al., 2018). These solutes are then recovered by removing the liquid phase. The liquid phase usually consists of a volatile solvent such as hexane, methanol, or chloroform. It is hypothesized that hydroxychavicol can be designated as a bioactive chemical marker in the *P. betle* L. extracts to predict the plant's biological activity. Thus, the aim of this study was, thus, to determine the effect of the extraction method on the yield and biological activities of hydroxychavicol from *P. betle* L. A correlation analysis between the hydroxychavicol content and biological activities was carried out to evaluate the potential use of hydroxychavicol as a fingerprint bioactive chemical marker compound for *P. betle* L. extracts. We hypothesize that hydroxychavicol can be designated as a bioactive chemical marker in the *P. betle* L. extracts to determine its biological activity. Chemical profiling with GCMS was also done for all the extracts to understand the role of metabolite present in extract toward each biological activity.

## 2. Materials and method

### 2.1. Plant sampling

*P. betle* L. leaves were collected between March 2019 and April 2019 from Rimba Ilmu botanical garden at the University of Malaya, Kuala Lumpur Malaysia. The plant was authenticated and archived in the Rimba Ilmu herbarium by a botanist Dr. Kien-Thai, YONG with a voucher specimen no. KLU49902.

### 2.2. Chemicals and solvents

All chemicals were purchased from Sigma–Aldrich (Chemie, Steinheim, Germany) and Merck (Darmstadt, Germany) while all solvents used were either analytical or chromatographic grade.

### 2.3. Extract preparation

Different extraction procedures using different solvents were employed to obtain various crude extracts (Sharma et al., 2009; Haq et al., 2011; Liu et al., 2015; Rigane et al., 2017). It is assumed that different extraction methods would lead to different yields of hydroxychavicol and also affect their biological activities. The details of the procedures are itemized as follows:

i Extract 1 (M1): Freshly procured leaves of *P. betle* L. (200 g) were soaked in methanol (2 L) at room temperature (25 °C) for 72 h. This was repeated consecutively three times. Then, the yielded extracts were collected, filtered through filter paper (Whatman 1, cat NO 1001-150), and concentrated under reduced pressure at 50 ± 5 °C on a rotary evaporator to obtain a dark semi-solid extract. The methanol extract was subjected to column chromatography by using silica gel 60 (Merck, 0.063−0.200 mm) as stationary phase using a mobile phase containing chloroform: methanol (10 % methanol) and to obtain 11 fractions. Fraction 4 which contained hydroxychavicol was further purified. The purification of fraction 4 was done by column chromatography using silica gel 60 (Merck, 0.040−0.063 mm) as stationary phase, and the mobile phase used was chloroform: methanol (1 % methanol) to obtain 8 fractions. Then, further purification was done using column chromatography by combining fractions 4 and 5 (silica gel 60 Merck, 0.040−0.063 mm) as stationary phase and mobile phase containing hexane: ethyl acetate (20 % ethyl acetate) to obtain 6 fractions. Lastly, further purification of fraction 1 was carried out using column chromatography (Sephadex LH20, mobile phase: - 100 %methanol) to obtain pure hydroxychavicol. It took three weeks for the extraction and isolation of hydroxychavicol.

ii Extract 2 (M2): Freshly procured leaves of *P. betle* L. (200 g) were extracted in boiling water (1 L) with stirring for 4 h. The resulting extract was filtered through filter paper and concentrated to one-sixth of the original volume at 50 ± 5 °C using miVac Quattro Concentrator. The concentrated extract was then repeatedly extracted (five times) with 100 mL chloroform in a separating funnel. Subsequently, the chloroform extract was concentrated under reduced pressure to obtain a semisolid extract. This residue was subjected to column chromatography (Sephadex LH20, mobile phase: 100 %methanol) to obtain pure hydroxychavicol. It took one week for the extraction and isolation of hydroxychavicol.

iii Extract 3 (M3H, M3C, and M3M): Freshly procured leaves of *P. betle* L. (200 g) were dried overnight in the oven at 50 °C. Then, the dried

leaves were blended into fine particles (50 g) and soaked in hexane (200 mL) (M3H) for 72 h. This was repeated consecutively three times. All the three yielded extracts were combined and filtered through filter paper and concentrated to one-sixth of the original volume under reduced pressure at 50 ± 5 °C on a rotary evaporator. Further extraction was carried out on the residue using 200 mL chloroform (M3C), followed by 200 mL methanol (M3M) similarly for 72 h and three consecutive repetitions each. It took two months for the extraction and isolation of hydroxychavicol from each M3H, M3C, and M3M extract, following the purification methods as below:

a) M3H- The methanol extract was subjected to column chromatography by using silica gel 60 Merck, 0.063−0.200 mm as stationary phase and mobile phase comprising hexane: acetone (1 % acetone) yielding 9 fractions. Then, further purification on Fraction 6,7, and 8 were done by column chromatography using silica gel 60 Merck (0.040−0.063 mm) as stationary phase and mobile phase comprising, hexane: ethyl acetate (10 % ethyl acetate) yileding 4 fractions. Lastly, Fractions 1 and 2 were subsequently combined and purified using column chromatography (Sephadex LH20, mobile phase: 100 % methanol) to obtain pure hydroxychavicol.
b) M3C-The chloroform extract was subjected to column chromatography by using silica gel 60 Merck (0.063−0.200 mm) as stationary phase and mobile phase comprising chloroform: methanol (5 % methanol) yielding 13 fractions. Then, further purifications on Fractions 6 and 7 were done by column chromatography using silica gel 60 Merck (0.040−0.063 mm) as stationary phase and mobile phase comprising hexane: ethyl acetate (10 % ethyl acetate) yielding 5 fractions. Lastly, Fractions 1 and 2 were subsequently combined and purified using column chromatography (Sephadex LH20, mobile phase:- 100 % methanol) to obtain pure hydroxychavicol.
c) M3M- The methanol extract was subjected to column chromatography by using silica gel 60 Merck (0.063−0.200 mm) as stationary phase and mobile phase comprising chloroform: methanol (10 % methanol) to obtain 7 fractions. Then, further purifications of Fractions 2 and 3 were done by column chromatography using silica gel 60 Merck (0.040−0.063) mm as stationary phase and mobile phase comprising hexane: ethyl acetate (20 % ethyl acetate) to obtain 4 fractions. The purification on Fractions 1 and 2 from 4 was done by column chromatography using silica gel 60 Merck (0.040−0.063 mm) as stationary phase and mobile phase comprising chloroform: methanol (10 % ethyl acetate) yielding 6 fractions. Lastly, Fraction 4 was purified using column chromatography (Sephadex LH20, mobile phase:- 100 % methanol) to obtain pure hydroxychavicol.

iv Extract 4 (M4): Freshly procured leaves of *P. betle* L. (200 g) were dried in the oven at 50 °C overnight. Then, the dried leaves were blended into fine particles (48 g) and macerated in methanol (200 mL) for 72 h, repeated consecutively three times. Then the extract was filtered through filter paper, and concentrated under reduced pressure at 50 ± 5 °C on a rotary evaporator to obtain a dark semisolid extract. It took three weeks for the extraction and isolation of hydroxychavicol. The methanol extract was subjected to column chromatography by using silica gel 60 Merck, 0.063−0.200 mm as stationary phase and mobile phase containing chloroform: methanol (10 % methanol) gave 11 fractions. Then, fractions 5 and 6 were combined and purified by column chromatography using silica gel 60 Merck, 0.040−0.063 mm as stationary phase and mobile phase containing chloroform: methanol (1% methanol) to obtain 8 fractions. From these 8 fractions, further purifications were done by combining fractions 3,4,5 and 6 subjected them to column chromatography using silica gel 60 Merck, 0.040−0.063 mm as stationary phase and mobile phase containing hexane: ethyl acetate (20 % ethyl acetate) to obtain 5 fractions. Lastly, fractions 1 and 2 from 5 were combined and further purified using column chromatography (Sephadex LH20, mobile phase:- 100 % methanol) to obtain pure hydroxychavicol.

v Extract 5 (M5): Solvent-free microwave extraction (SFME) technique was carried out under atmospheric pressure with a 500 W Milestone NEOS system (Italy). Freshly procured leaves of *P. betle* L. (200 g) were sliced into small pieces. Then, the sample was subjected to microwave heating at 500 W for 30 min in the presence of 50 mL ultrapure water. The condensate obtained was then dried over anhydrous sodium sulfate to obtain an orange-yellowish extract. No isolation of hydroxychavicol was performed on this extract.

vi Extract 6 (M 6): Freshly collected leaves of *P. betle* L. (200 g) were sliced into small pieces and were subjected to hydro-distillation for 270 min using a Clevenger apparatus as described in the European Pharmacopeia. The distillate obtained was then fractionated with ethyl acetate and dried over sodium sulfate anhydrous to obtain a green-yellowish extract. No isolation of hydroxychavicol was performed on this extract.

### 2.4. Quantitative analysis of hydroxychavicol using HPLC

Quantitative analysis was performed using HPLC to determine the amount of hydroxychavicol (Sharma et al., 2009). The protocol for HPLC was established using HPLC-GILSON GX271 reverse-phase on a C18 column (5 μm pore size; 250 by 4.6 mm internal diameter) and UV detection at 280 nm. The column solvent gradient system consisted of solvent A (deionized water: formic acid, 100:1.8, v/v) and solvent B (acetonitrile: formic acid, 100:1.8, v/v). Sample (100 μL) was injected into the HPLC column at a flow rate of 1 mL/minute. The sample was run under an isocratic system with solvent A (50 %) for 10 min. The identification of hydroxychavicol was done by comparing the retention times between the standard and the sample on the HPLC chromatogram. A calibration curve was prepared by using the multipoint calibration curve method. Pure hydroxychavicol concentrations (10 – 0.0390 mg/mL) were prepared and injected (100 μL) into the HPLC. A calibration curve was obtained by plotting a graph of concentration against the area under the curve (supplementary data). Hydroxychavicol is a brownish oil with EI-MS *m/z*: 150, 131, 103, 77, and 51. $^{1}$H and $^{13}$C- NMR spectra (supplementary data) are consistent with the literature (Jitesh et al., 2006).

### 2.5. Isolation of hydroxychavicol

The plant extracts were subjected to isolation and purification process to obtain hydroxychavicol (Sharma et al., 2009; Liu et al., 2015). The yields of hydroxychavicol isolated from each extract were compared. Column chromatography was used for the isolation and purification techniques of hydroxychavicol. The extracts were subjected to series of column chromatography using silica gel 60 Merck, size 0.063−0.200 mm and 0.040−0.063 mm as the stationary phase and mobile phases of hexane, chloroform, ethyl acetate, and methanol to obtain hydroxychavicol. Sephadex LH20 was used in the final step of the column chromatography technique for the purification of the compound.

### 2.6. Cytotoxic activities

The cytotoxic assays were performed using MTT assays as described by Mosmann (1983). The extracts were tested against five cancer cells; two breast cancer cell lines (MCF7 and MDA-MD-231), two colon cancer cell lines (SW948 and HT29), and a lung cancer cell line (A549). All the cancer cells were purchased from the ATCC (American Type Culture Collection, USA). Briefly, all the cells were cultured and allowed to grow to the log phase in RPMI1640 medium together with 5% fetal bovine serum. The cancer cells were seeded in 96-well plates with specific cell concentrations. The concentrations for both SW948 and MCF7 cells were $1 \times 10^5$ cells per well in 100 μL aliquots of the medium while the others were at $2 \times 10^5$ cells per well. Each sample was introduced into the 96

well plates within a range of concentrations. The plates were incubated for 72 h at 37 °C in a 5 % $CO_2$ humidifier incubator. After 72 h of exposure to the extract, 20 µL of MTT solution (5 mg/mL) was added to each well. Then, the plate underwent another 3 h of incubation at 37 °C in the 5 % $CO_2$ humidifier incubator. Eighty percent of media from each well were discarded, followed by the addition of the same amount of DMSO into each well to dissolve any purple formazan crystals formed. Finally, the absorbance of the content of each well was determined at 550 nm using a microplate reader. The results were calculated as the percentage of cell inhibition following the formula below:

Percentage of cell inhibition = [(A–B)/A] * 100 %

where A represents the average absorbance of the cell without treatment and B represents the average absorbance of the cell with treatment.

A graph of percentage cell inhibition against the concentration of the extracts was plotted and the cytotoxicity of the compounds was expressed as $IC_{50}$ values. Each experiment was performed in triplicates. Tamoxifen and paclitaxel were used as positive controls in the present study.

### 2.7. Bacterial and fungal strains

Bacterial and fungal stock cultures were preserved on Muller-Hinton agar and potato dextrose agar, respectively, and kept at 4 °C. The antimicrobial activities were studied against six bacteria; three Gram-positive strains [*Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (B145), *Staphylococcus epidermidis* (a clinical isolate)] and three Gram-negative strains [*Pseudomonas aeruginosa* (ATCC27853), *Escherichia coli* (a clinical isolate), *Klebsiella pneumoniae* (ATCC 2513)]. Meanwhile, two fungal strains involved in this study were *Candida albicans* (C2213) and *Aspergillus niger* (A121). All the bacteria strains were obtained from the Microbiology Laboratory, Medical Faculty, Universiti Putra Malaysia, and all fungi strains were obtained from the Institute for Medical Research (IMR), Kuala Lumpur.

### 2.8. Antibacterial and antifungal assays

The diffusion method was used for the antibacterial and antifungal assays (Al-Madhagi et al., 2019) with each extract concentration at 10 mg/mL. The antimicrobial activities were evaluated by measuring the inhibition zone diameter after incubating the plates at 37 °C for 24 h (for antibacterial assay) and 25 °C for 48 h (for antifungal assay). Streptomycin sulfate (100 µg/mL) was used as a positive control for the antibacterial assay while ketoconazole was used as a positive control for the antifungal assay. Dimethyl sulfoxide (DMSO) was used as a negative control. The minimum inhibitory concentration (MIC) of each plant extract was determined by using the broth microdilution assay. Serial dilutions of the plant extracts (10 mg/mL - 19.53 ug/mL) were used for the assay. Then, each sample was added to 20 µL of an aqueous solution of 2, 3, 5-triphenyltetrazolium chloride (TTC, 5 mg/mL), and the mixture was incubated at 37 °C for 1 h. The appearance of a pink color indicated the presence of microbial growth. The MIC value was determined from the lowest concentration that remained colorless.

### 2.9. Antioxidant assay

The antioxidant activities of the extracts were also evaluated. Four antioxidant assays were employed, namely; β-carotene bleaching (BCB), ferric reducing power (FRAP), total antioxidant capacity (TAOC), and ABTS 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid). The β-carotene bleaching assay was conducted according to the protocol by Kassim et al. (2013). Gallic acid was used as a standard in this experiment. The absorbance was measured at 470 nm at 0 h and 2 h. The extract (10 mg/mL) and positive control (100 µg/mL) were compared for the BCB assay. The reducing power (FRAP) of the extracts and gallic acid were determined following the method described by Nordin et al. (2014). A standard curve of gallic acid was constructed and a standard equation was determined to find the reducing power (FRAP) for the extracts. The reducing power results were expressed relative to gallic acid equivalence (GAE, µg of gallic acid/mg of extract). TAOC assay is a non-enzymatic assay and was carried out according to a modified protocol by Sun et al. (2011). The extracts were screened at a concentration of 10 mg/mL and analyzed at 695 nm. The total antioxidant activity was expressed as an equivalence of ascorbic acid. Free radical scavenging assay was performed by using the ABTS method described by Dudonné et al. (2009) with slight modification. Briefly, 10 µL of the sample (10 mg/mL) or DMSO as a blank was added to the 96-well microplate, followed by 300 µL of ABTS+● solution. The plate was kept for 10 min at 30 °C. The absorbance was recorded at 743 nm. ABTS was decolorized as a result of the scavenging activity of the antioxidant agent.

### 2.10. Gas chromatography-mass spectrometry (GC–MS)

The methodology was adopted from the work of Muruganandam et al. (2017) with modification. The GC–MS column used was RTX-5MS fused-silica capillary column (30 m ×0.25 mm i.d.; 0.25 µm film thickness) with helium as the carrier gas and was run at a constant pressure of 100.0 kPa. The injection was conducted using the splitless mode at an injector temperature of 300 °C. The oven temperature was ramped from 40 to 160 °C (5 min hold) at a rate of 4 °C/ minute, and 160−280 °C (15 min hold) at 5 °C/ minute (rate). The total run time for each sample was approximately 74 min n. The GC–MS interface temperature was set to 280 °C. MS mode was used during analytical scanning from 45 to 500 atomic mass units (amu). The ion source temperature was set to 280 °C. The identification of the peaks was conducted against the National Institute of Standard and Technology Mass Spectral Library (NIST08 and 08 s) as well as Pubchem©.

### 2.11. Statistical analysis

The results obtained from the quantitation of hydroxychavicol in *P. betle* L. extract and the antioxidant and antimicrobial assays were represented as the mean ± standard deviation of triplicate independent analyses. Meanwhile, the results obtained from the cytotoxic assays were represented as the mean ± standard error of three independent experiments. Data were analyzed using one-way ANOVA by Tukey post hoc test (SPSS 14.0) to determine the significant differences among samples. The independent sample T-test (SPSS 14.0) was used to determine any significant difference between samples and standard drugs. Pearson correlation (SPSS 14.0) was also used to determine the relationship between quantitative analysis with antioxidant, cytotoxic, and antimicrobial activities. The significance level was set at $p < 0.05$.

## 3. Results

### 3.1. Yields of hydroxychavicol

The quantitative analysis of the amount of hydroxychavicol was determined using HPLC. The highest yield of hydroxychavicol was obtained from M2 (98 %) while M6 contained the lowest hydroxychavicol content (2 %). The extracts except M5 and M6 were further processed for isolation and purification to obtain hydroxychavicol. The two extracts were exempted due to the presence of a little amount of hydroxychavicol content based on the HPLC analysis. The other five extracts (M1, M3H, M3C, M3M, and M4) were fractionated using column chromatography and eluted with hexane, chloroform, ethyl acetate, and methanol to obtain hydroxychavicol. However, M2, with the highest yield of hydroxychavicol, was further subjected to column chromatography using Sephadex LH20 as a stationary phase and methanol as a mobile phase to obtain pure hydroxychavicol. The purification of hydroxychavicol from M2 extract gave the highest yield percentage (80 %).

Though M1 gave the highest crude extract weight, the percentage yield of purified hydroxychavicol obtained from this extract was only 5 %. This might be due to the loss of hydroxychavicol during the various purification processes to obtain pure hydroxychavicol. The yields of the various crude extracts, the quantitative determination of hydroxychavicol content (Fig. 1a, b, and supplementary data) in each extract using HPLC, and the yield of the purified hydroxychavicol are shown in Table 1.

### 3.2. Cytotoxic activities

The plant extracts were tested against 5 cancer cell lines which were two breast cancer cell lines (MCF7 and MDA-MD-231), two colon cancer cell lines (SW948 and HT29), and a lung cancer cell line (A549) as representatives of the three leading death-causing cancer types. The $IC_{50}$ values of the extracts are summarized in Table 2. M2 and pure hydroxychavicol actively inhibited all the cancer cell lines except A549. M2 also showed more effective inhibition activity against MCF 7 compared to tamoxifen with $IC_{50}$ of 1.74 ug/mL. Meanwhile, M2, M4, M5, and hydroxychavicol showed active inhibition against MDA-MD-231 with $IC_{50}$ of 3.58, 5.47, 7.32, and 4.15 μg/mL, respectively. However, the extracts and pure hydroxychavicol were not as effective as the standard drug, tamoxifen. None of the extracts showed strong inhibition towards A549. For the SW948 cell line, all the extracts showed strong inhibition except M3M. However, for the other colon cancer cell line (HT29), only M1, M2, M3H, M3C, M5, and hydroxychavicol were more effective compared to other extracts. All the extracts were, however, not as effective against SW948, HT29, and A549 as paclitaxel.

### 3.3. Antimicrobial activity

The preliminary screening against the bacteria and fungi studied was done by using the well diffusion method and the results are shown in Table 3. The extracts showing an inhibition zone less than 10 mm were classified as weak antibacterials and antifungals (Fu et al., 2007) while the extracts with an inhibition zone of more than 15 mm were classified as strong antibacterials and antifungals (Al-Madhagi et al., 2019). Strong antimicrobial activity was shown by M2 extract against all the bacteria strains similar to pure hydroxychavicol. Meanwhile, both the pure compound and M2 extract showed strong antifungal activity only toward *Candida albicans*. Surprisingly, M6 extract shows a strong antifungal activity towards *Aspergillus niger*. These active extracts and pure hydroxychavicol were further tested for their MIC, MBC, and MFC (Supplementary data).

### 3.4. Antioxidant assay

Four antioxidant assays were performed to evaluate the antioxidant activity of the extracts and pure hydroxychavicol. The results of the antioxidant activities are shown in Table 4. All the crude extracts and pure hydroxychavicol except for M3M showed remarkable ABTS scavenging activity. Meanwhile, the highest percentage of BCB inhibition was exhibited by M1 with 79.93 % inhibition. However, it was not as effective as gallic acid (88.58 % inhibition). The highest FRAP value was also demonstrated by M1 with the value of 11.45 μg gallic acid/ mg extract while the highest TAOC was exhibited by M3M with a value of 24.10 μg ascorbic acid/ mg extract.

### 3.5. Correlation studies between the amount of hydroxychavicol in the extracts and the biological activities

Correlation studies were carried out to determine the role or mechanism of action of phenolic compounds in the extracts towards antioxidant and cytotoxic activities. The results of the correlation analysis between the hydroxychavicol content and the various antioxidant and cytotoxic activities are shown in Table 5. The correlation coefficients ranging from 0.8 to 0.9 indicate strong correlations while 0.5 – 0.7 suggest moderate correlations, and 0.1 – 0.4 suggest weak correlations (Lai and Lim, 2011). The quantity of hydroxychavicol in the extracts strongly influenced the antibacterial activities against two bacteria which were *Bacillus subtilis* and *Staphylococcus epidermidis*. However, hydroxychavicol showed a moderate and weak correlation in the cytotoxicity activity against selected cancer cell lines. Meanwhile, the hydroxychavicol yield in the extracts showed a weak correlation with the antioxidant assay, thus did not influence the antioxidant activities.

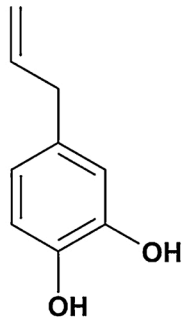
### 3.6. GC–MS analysis

Phytochemical analysis of the volatile components was performed using GCMS for all extracts, as shown in Table 6. All compounds were assigned with a compound name while three compounds were labeled as 'unknown' since the SI (selectivity index) of these compounds did not achieve 80 % when the mass spectrum was compared with the NIST library (SD 15 – SD 22). The profiling of all *Piper betle L.* extracts with GC–MS revealed that hydroxychavicol appeared in each extract. Besides hydroxychavicol, several phenylpropanoids such as eugenol, 2-methoxy-3-allylphenol, chavicol, chavibetol, 2-methoxy-4-vinyl phenol, acetoxychavicol acetate, 2-methoxy-4-vinylphenol, 4-hydroxy-2-methylacetophenone, and methyleugenol, at certain extract. Meanwhile, terpenes such as stigmasterol, β-sitosterol, β-sitosterol acetate, β-stigmasterol, and stigmast-5-en-3-ol were present in the extracts obtained by the maceration extraction method employed for M1, M3H, M3C, and M4. Campesterol, which is from the class of steroid was found to be present in the non-polar (M3H) and semi-polar (M3C) extracts obtained through the maceration extraction method.

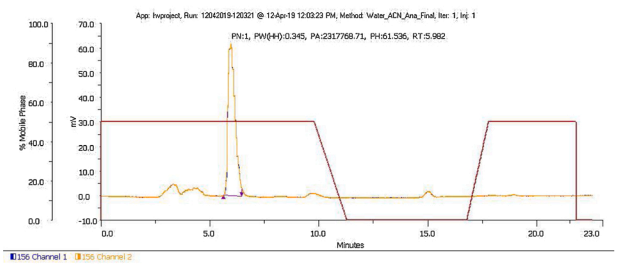
## 4. Discussion

Plants are known for their medicinal uses and 80 % of the world population rely on plants for their primary healthcare (Luqman et al., 2009). Most of these plants, known as ethnomedicinal plants, are very rich in natural antioxidants that can protect humans from oxidative stress, thus, playing an important role in the chemoprevention of diseases (Ahmed et al., 2020; Allaq et al., 2021). *P. betle L.* is one of such known ethnomedicinal plants widely used to prevent and treat diseases, particularly in Asia. Furthermore, hydroxychavicol is one of the main active compounds in *P. betle L.* with very high biological activities. Naturally isolated active compounds are very expensive owing to the high cost of the isolation and purification process as well as the requirement of skilled workers. Therefore, the optimization of the isolation and purification of hydroxychavicol is highly needed to obtain high yields of active compounds. From the extraction and isolation studies, M2 extract gave the highest percentage of hydroxychavicol content, with almost up to 90 percent of the final extract containing hydroxychavicol based on the HPLC analysis. The boiling process in M2 was also able to remove chlorophyll and, thus, containing only phenolic compounds. Hydroxychavicol is a semi-polar compound that needs a semi-polar solvent such as chloroform to attract hydroxychavicol from the water extract. Meanwhile, the other extracts, involving no boiling process, naturally contained a variety of compounds including chlorophyll, and thus needed to be further fractionated several times to obtain the pure hydroxychavicol. Furthermore, hydroxychavicol is a non-volatile compound that exerts a low vapor pressure and has a slow rate of evaporation (Lucchesi et al., 2004).

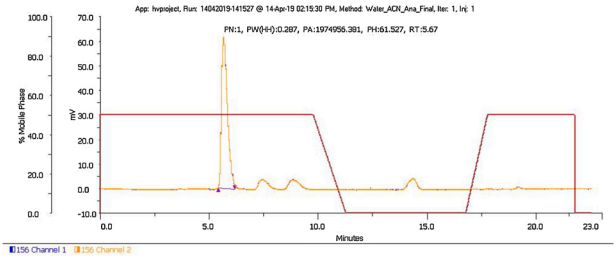
On the other hand, cancer is one of the most prominent death-causing diseases worldwide (Lima et al., 2016; Lee et al., 2018). Many chemotherapeutic drugs have been developed based on organic and inorganic compounds. However, some of the commercially available chemotherapeutic drugs have led to severe side effects resulting in functional loss of human organs. For instance, the use of cisplatin as a chemotherapeutic drug reportedly led to kidney and liver damage (Mavligit et al., 1995). As a result, there is an urgent need to search for a



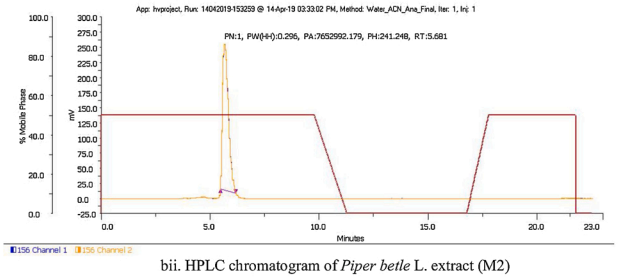

a: Structure of hydroxychavicol



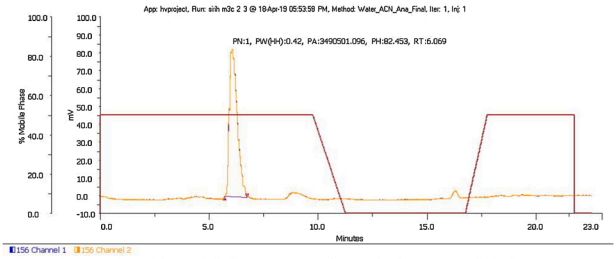
bi. HPLC chromatogram of *Piper betle* L. extract (M1)



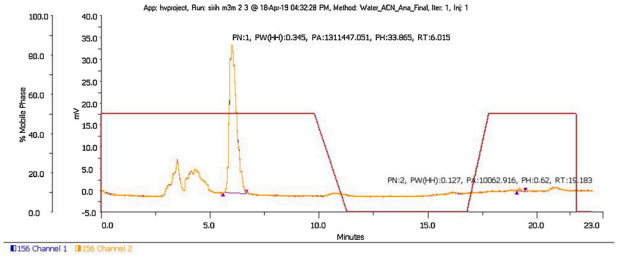
biii. HPLC chromatogram of *Piper betle* L. extract (M3)



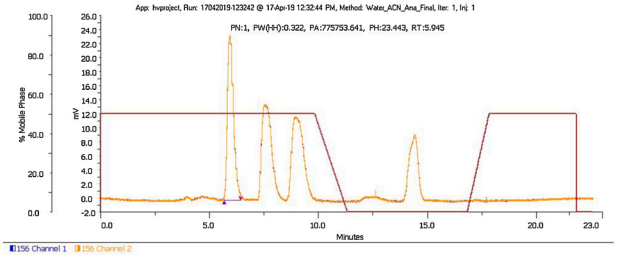
bii. HPLC chromatogram of *Piper betle* L. extract (M2)



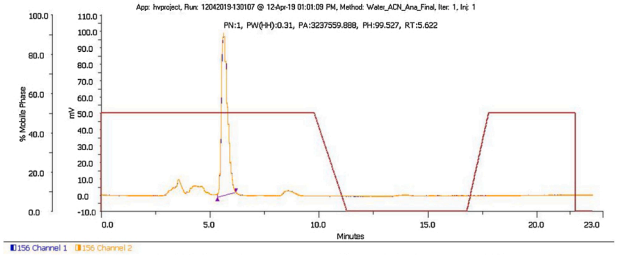
biv. HPLC chromatogram of *Piper betle* L. extract (M3C)



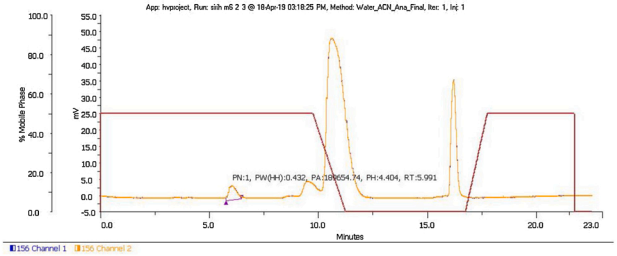
bv. HPLC chromatogram of *Piper betle* L. extract (M3M)



bvii. HPLC chromatogram of *Piper betle* L. extract (M5)



bvi. HPLC chromatogram of *Piper betle* L. extract (M4)



bviii. HPLC chromatogram of *Piper betle* L. extract (M6)

**Fig. 1.** a Structure of hydroxychavicol. bi. HPLC chromatogram of *Piper betle* L. extract (M1). b ii. HPLC chromatogram of *Piper betle* L. extract (M2). b iii. HPLC chromatogram of *Piper betle* L. extract (M3). b iv. HPLC chromatogram of *Piper betle* L. extract (M3C). b v. HPLC chromatogram of *Piper betle* L. extract (M3M). b vi. HPLC chromatogram of *Piper betle* L. extract (M4). b vii. HPLC chromatogram of *Piper betle* L. extract (M5). b viii. HPLC chromatogram of *Piper betle* L. extract (M6).

**Table 1**
Yields of *Piper betle* L. crude extracts, hydroxychavicol content (HPLC), and purified hydroxychavicol isolated from each extract.

| Extracts | Crude extract (g) | Hydroxychavicol content (mg hydroxychavicol/ mg extract) |
|---|---|---|
| M1 | 13.498 | $0.30 \pm 0.01^{e}$ |
| M2 | 0.850 | $0.98 \pm 0.00^{h}$ |
| M3H | 1.765 | $0.26 \pm 0.02^{d}$ |
| M3C | 2.643 | $0.44 \pm 0.02^{g}$ |
| M3M | 2.750 | $0.17 \pm 0.01^{c}$ |
| M4 | 3.740 | $0.40 \pm 0.02^{f}$ |
| M5 | 0.457 | $0.10 \pm 0.04^{b}$ |
| M6 | 0.783 | $0.02 \pm 0.01^{a}$ |

(*a-h*) denote a significant difference between samples using a Tukey's post hoc test (SPSS 14.0) at $p < 0.05$.

new chemotherapeutic drug that is safe for consumption with no side effects. The present study showed that most of the *P. betle* L. extracts possess positive inhibition effects against all the cancer cell lines except lung cancer. The M2 extract containing 90 % hydroxychavicol showed a comparable cytotoxic effect as the pure hydroxychavicol. It shows that the other minor compounds such as chavicol and 2-methoxy-4-vinylphenol (from GCMS) contained in the M2 extracts did not influence its cytotoxic activity. However, the fact that the other extracts such as M5 with less amount of hydroxychavicol showed high cytotoxic activity towards all cancer cell lines except A549 implies that the extracts might contain other compounds with high anticancer potential. The GCMS profiling showed that M5 consists of other compounds such as acetoxychavicol acetate and chavibetol. According to the literature, acetoxychavicol actively inhibits several cancer types such as breast cancer, colon cancer, and cervical cancer (Da'I et al., 2019). The combination of two major compounds, acetoxychavicol acetate (40.32 %) and hydroxychavicol (40.49 %) in M5 has led to the synergistic interaction between M5 and the cancer cell line. The weak correlation between the biological activities and hydroxychavicol content might be due to the antagonistic or synergistic effect between the various compounds contained in the extracts.

Infectious diseases are also one of the leading causes of death worldwide besides cancer (Ahmed et al., 2021; Allaq et al., 2021). Nowadays, drug resistance towards bacteria and fungus has become one of the greatest threats to humanity. Antifungal and antibacterial resistance decreases the effectiveness of drugs, thus, increasing the risk of morbidity and mortality and, therefore, compromising human health (Collignon et al., 2009). For instance, in 1989 a drug-resistant microorganism was observed when enterococci species was found to be resistant to the antibiotic, vancomycin (Blondelle and Lohner, 2000). A similar pattern was observed with antifungal azole resistance among non-*Candida albicans* isolates, azoles resistance in *Aspergillus fumigatus*, and echinocandin resistance in *C. glabrata* (Wiederhold, 2017). Due to this problem, the discovery of novel antibacterial and antifungal agents had become a necessity. The herbal extract is one of the main sources to find these novel compounds. *P. betle* L. extracts are known for their antibacterial and antifungal activities as well as for hydroxychavicol content (Foo et al., 2015; Garg and Jain, 1992). From the current study, the amount of hydroxychavicol in the extracts influenced the inhibition

**Table 2**
Cytotoxic effect of *Piper betle* L. extracts against MCF7, MDA-MD-231, SW948, HT29, and A549.

| Extracts | $IC_{50}$ (μg/mL) | | | | |
|---|---|---|---|---|---|
| | MCF7 | MDA-MD-231 | SW948 | HT29 | A549 |
| M1 | 5.95 ± 0.17* | 16.64 ± 0.57* | 4.83 ± 0.06* | 9.56 ± 0.64 | >50* |
| M2 | 1.74 ± 0.10* | 3.58 ± 0.46 | 2.17 ± 0.26* | 4.45 ± 0.38 | 15.73 ± 0.23* |
| M3H | 4.79 ± 0.23* | 11.70 ± 0.68* | 5.81 ± 0.05* | 9.05 ± 0.05* | 23.20 ± 3.67* |
| M3C | 3.34 ± 0.35 | 10.35 ± 0.17 | 4.34 ± 0.24 | 9.92 ± 0.77* | 22.75 ± 1.83 |
| M3M | 21.57 ± 1.93 | 41.97 ± 1.54 | 15.76 ± 0.77 | >50 | >50* |
| M4 | 5.01 ± 0.16* | 5.47 ± 0.16* | 3.46 ± 0.36 | 10.41 ± 0.36 | 35.60 ± 3.11* |
| M5 | 3.90 ± 0.07* | 7.32 ± 0.32* | 2.78 ± 0.14* | 5.47 ± 5.35 | 27.53 ± 1.58* |
| M6 | 14.10 ± 1.83 | 35.55 ± 0.56 | 7.77 ± 0.70 | 16.98 ± 0.26 | >50* |
| Hydroxychavicol | 2.57 ± 0.20 | 4.15 ± 0.20* | 2.51 ± 0.08 | 5.67 ± 0.51* | 16.84 ± 1.14* |
| Paclitaxel | ND | ND | 0.26 ± 0.02 | 0.163 ± 0.01 | 2.23 ± 0.01 |
| Tamoxifen | 2.28 ± 0.10 | 3.15 ± 0.04 | ND | ND | ND |

* denotes a significant difference between a sample and standard drug; ND; not determined.

**Table 3**
Inhibition diameters of crude extracts of *Piper betle* L. hydroxychavicol, and controls against selected microbes.

| | Inhibition zone (mm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Bacteria strains tested | | | | | | Fungi strains tested | |
| Extract | S.A | K.P | E.C | P.A | S.E | B. S | A. N | C.A |
| M1 | 12.33 ± 0.58* | 11.33 ± 0.58* | 11.33 ± 0.58* | 11.33 ± 0.58* | 10.00 ± 0.00 | 10.67 ± 0.58* | 10.00 ± 1.00* | 10.00 ± 1.00* |
| M2 | 15.67 ± 0.58* | 15.00 ± 1.00* | 14.33 ± 1.15* | 17.67 ± 0.58* | 15.00 ± 1.00* | 14.67 ± 0.58* | 11.00 ± 1.00* | 16.00 ± 1.00* |
| M3H | 11.33 ± 0.58* | 13.67 ± 1.53* | 11.33 ± 1.53* | 13.00 ± 1.00* | 10.33 ± 0.58* | 10.33 ± 1.15* | 9.67 ± 1.15* | 13.00 ± 1.00* |
| M3C | 13.00 ± 1.00* | 13.00 ± 1.00* | 11.67 ± 0.58 | 11.00 ± 1.00* | 12.33 ± 0.58* | 12.33 ± 0.58* | 14.00 ± 1.00* | 11.33 ± 0.58* |
| M3M | 12.33 ± 0.58* | 11.33 ± 0.58* | 10.67 ± 0.58* | 11.33 ± 1.53* | 7.33 ± 0.58* | 7.33 ± 0.58* | NA | NA |
| M4 | 10.67 ± 0.58* | 13.00 ± 1.00* | 12.00 ± 1.00* | 13.00 ± 1.00* | 10.33 ± 0.58* | 10.33 ± 1.15* | NA | 11.00 ± 1.00* |
| M5 | 12.67 ± 1.53* | 12.33 ± 0.58* | 13.00 ± 1.00* | 10.67 ± 0.58* | 7.33 ± 0.58* | 9.33 ± 0.58* | 9.67 ± 0.58* | 11.67 ± 0.58* |
| M6 | 10.33 ± 1.53* | 14.00 ± 1.00* | 12.33 ± 1.53* | 12.67 ± 1.53* | 8.67 ± 0.58* | 9.00 ± 1.00* | 15.33 ± 0.58* | 13.67 ± 0.58* |
| Hydroxychavicol | 16.33 ± 1.53* | 15.33 ± 0.58* | 15.00 ± 1.00* | 16.00 ± 1.00* | 14.67 ± 0.58* | 15.33 ± 0.58* | 9.00 ± 1.00* | 15.00 ± 1.00* |
| Streptomycin (1 mg/mL) | 24.33 ± 0.58 | 29 ± 1.00 | 28.67 ± 0.57 | 26.00 ± 1.73 | 30.67 ± 0.58 | 29.67 ± 0.58 | ND | ND |
| Ketoconazole (1 mg/mL) | ND | ND | ND | ND | ND | ND | 25 ± 1.00 | 26.33 ± 1.53 |
| DMSO | NA | NA | NA | NA | NA | NA | NA | NA |

NA = Not Active, ND = not determined; (*) denote a significant difference between a sample and standard drugs. SA= *Staphylococcus aureus* (ATCC 25923); BS= *Bacillus subtilis* (B145); SE = *Staphylococcus epidermidis*, PA= *Pseudomonas aeruginosa* (ATCC27853); EC= *Escherichia coli*; KP= *Klebsiella pneumoniae* (ATCC 2513); CA= *Candida albicans* (C2213) and AN= *Aspergillus niger*.

**Table 4**
Antioxidant activities of *Piper betle* L. extracts, hydroxychavicol, and positive control.

| Sample | Total antioxidant capacity (μg ascorbic acid/ mg extract) | ABTS % of scavenging of extract at 10 mg/mL | FRAP (μM ferrous sulphate / mg dry extract) | Beta carotene bleaching (% of β-carotene bleaching of extract at 10 mg/mL) |
|---|---|---|---|---|
| M1 | $21.38 \pm 0.29^{d,e}$ | 91.50 ± 0.24* | $11.45 \pm 0.11^{b}$ | 76.93 ± 21.30* |
| M2 | $21.73 \pm 0.12^{e}$ | 94.50 ± 0.29 | $11.34 \pm 0.53^{b}$ | 31.89 ± 2.57* |
| M3H | $19.22 \pm 0.43^{c}$ | 95.16 ± 0.17* | $10.33 \pm 0.69^{b}$ | 15.71 ± 2.92* |
| M3C | $20.52 \pm 0.30^{d}$ | 81.29 ± 2.24 | $10.96 \pm 0.10^{b}$ | 34.17 ± 7.64 * |
| M3M | $24.10 \pm 0.50^{f}$ | 69.69 ± 0.62* | $11.01 \pm 0.93^{b}$ | 39.89 ± 11.96* |
| M4 | $19.34 \pm 0.26^{c}$ | 93.03 ± 0.41* | $8.14 \pm 0.98^{a}$ | 18.45 ± 8.31* |
| M5 | $17.71 \pm 0.36^{b}$ | 95.31 ± 0.16* | $10.61 \pm 0.09^{b}$ | 26.84 ± 6.05* |
| M6 | $13.46 \pm 0.13^{a}$ | 95.41 ± 0.09* | $11.04 \pm 0.04^{b}$ | 58.37 ± 20.13* |
| Hydroxychavicol | $12.92 \pm 0.20^{a}$ | 95.18 ± 0.83 | $11.04 \pm 0.43^{b}$ | 19.31 ± 0.86* |
| Gallic acid (100 μg/ mL) | ND | 94.05 ± 0.25 | ND | 88.58 ± 1.42 |

ND = not determined; (*a-f*) denote significant difference between sample; (*) denote a significant difference between a sample and standard drugs using Tukey's post hoc test (SPSS 14.0) at $p < 0.05$.

**Table 5**
Correlation analysis between hydroxychavicol content and biological activities of *P. betle* L. extracts.

| Biological activities | Correlation with hydroxychavicol content |
|---|---|
| $IC_{50}$ of MCF7 | r(24)= -0.537, p = 0.007 |
| $IC_{50}$ of MDA-MD-231 | r(24)= -0.581, p = 0.003 |
| $IC_{50}$ of SW948 | r(24)= -0.457, p = 0.025 |
| $IC_{50}$ of HT29 | r(24)= -0.362, p = 0.083 |
| $IC_{50}$ of A549 | r(24)= -0.636 p = 0.001 |
| Inhibition against *Pseudomonas aeruginosa* | r(24) = 0.747, p = 0.000 |
| Inhibition against *Bacillus subtilis* | r(24) = 0.851, p = 0.000 |
| Inhibition against *Staphylococcus epidermidis* | r(24) = 0.906, p = 0.000 |
| Inhibition against *Staphylococcus aureus* | r(24) = 0.708, p = 0.000 |
| Inhibition against *Escherichia coli* | r(24) = 0.453, p = 0.026 |
| Inhibition against *Klebsiella pneumoniae* | r(24) = 0.379, p = 0.68 |
| Inhibition against *Candida albicans* | r(24) = 0.393, p = 0.057 |
| Inhibition against *Aspergillus niger* | r(24)= p = 0.41, p = 0.850 |
| BCB | r(24)= -0.191, p = 0.371 |
| TAOC | r(24) = 0.465, p = 0.022 |
| FRAP | r(24) = 0.061, p = 0.776 |
| ABTS | r(24)= p = 0.106, p = 0.622 |

properties of Gram-positive bacteria but very selective towards Gram-negative bacteria and the two fungi species. Therefore, in some regards, hydroxychavicol can be considered as a bioactive chemical marker in the extract for an antibacterial product specifically to inhibit Gram-positive bacteria. Meanwhile, the M6 extract showed a strong antifungal activity towards *Aspergillus niger* due to the presence of two major compounds, chavibetol (63.78 %) and chavicol (15.74 %). These two compounds reportedly possess good anti-fungal activities (Dwivedi and Tripathi, 2014). In general, the results are comparable with other effective plant extracts such as the crude extracts of the stems of *Anabasis articulata* (Belyagoubi-Benhammou et al., 2019), leaves of *Bruguiera gymnorrhiza* (Haq et al., 2011), some Greek aromatic plants (Proestos et al., 2006), and leaves of *Pistacia atlantica* (Rigane et al., 2017).

The principle of BCB assay is to measure the ability of an antioxidant to inhibit lipid peroxidation (Othman et al., 2014). The BCB results showed that hydroxychavicol did not act as a good inhibitor of lipid peroxidation. M6 extract with less amount of hydroxychavicol (2.71 %) but rich in chavibetol (63.78 %) and chavicol (15.74 %), however, showed a moderate activity compared to other extracts. Meanwhile, the principle of ferric reducing antioxidant power is based on the reduction of $Fe^{3+}$ complex to intensely blue-colored $Fe^{2+}$ complex by an antioxidant compound in an acidic medium (Antolovich et al., 2002). Therefore, higher FRAP values demonstrate greater reducing power of the test compound, thus having a high antioxidant activity. Most of the extracts showed values above 10 μM ferrous sulphate/mg dry extract except M4. This might be due to the antagonistic effect between the component compounds in M4 such as hydroxychavicol (59.73 %), eugenol (8.52 %), 4-hydroxy-2-methylacetophenone (0.88 %), phytol (4.35 %), and other triterpenes (4.7 %). The ABTS•+ assay was based on the activation of

**Table 6**
GC–MS analysis of the compounds present in each *Piper betle* L. extract.

| No | Compounds | % compound in extract (in 10 mg extract) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | M1 | M2 | M3H | M3C | M3M | M4 | M5 | M6 |
| 1 | Hydroxychavicol | 58.64 | 87.67 | 25.31 | 63.73 | 33.47 | 59.73 | 40.49 | 2.71 |
| 2 | Eugenol | 5.71 | 0.81 | N.D | 5.09 | N.D | 8.52 | N.D | N.D |
| 3 | 2-Methoxy-3-allylphenol | 2.68 | N.D | N.D | N.D | 0.41 | N.D | N.D | N.D |
| 4 | Chavicol | N.D | N.D | N.D | 4.86 | 1.50 | N.D | N.D | 15.74 |
| 5 | Chavibetol | N.D | N.D | 7.1 | N.D | N.D | N.D | 7.7 | 63.78 |
| 6 | 2-Methoxy-4-vinylphenol | N.D | 0.4 | N.D | N.D | N.D | N.D | N.D | N.D |
| 7 | Acetoxychavicol acetate | N.D | N.D | 44.38 | 7.55 | N.D | N.D | 40.32 | 11.13 |
| 8 | 2-Methoxy-4-vinylphenol | N.D | N.D | N.D | N.D | N.D | N.D | N.D | N.D |
| 9 | 4-Hydroxy-2-methylacetophenone | N.D | N.D | N.D | N.D | N.D | 0.88 | N.D | N.D |
| 10 | Methyleugenol | N.D | N.D | N.D | N.D | N.D | N.D | N.D | 0.67 |
| 11 | Campesterol | N.D | N.D | 1.34 | 0.53 | N.D | N.D | N.D | N.D |
| 12 | Phytol | N.D | N.D | 5.19 | N.D | 2.93 | 4.35 | N.D | N.D |
| 13 | α-Tocopherol | 0.66 | N.D | 0.28 | N.D | N.D | 0.53 | N.D | N.D |
| 14 | Cholenic acid | 1.62 | N.D | 0.43 | N.D | 3.41 | 1.16 | N.D | N.D |
| 15 | Stigmasterol | 1.29 | N.D | 0.47 | N.D | N.D | 0.88 | N.D | N.D |
| 16 | β-Sitosterol | 5.77 | N.D | 1.64 | 1.33 | N.D | 3.82 | N.D | N.D |
| 17 | β-Sitosterol acetate | 2.11 | N.D | N.D | 2.36 | N.D | N.D | N.D | N.D |
| 18 | β-stigmasterol | N.D | N.D | 1.55 | 0.69 | N.D | N.D | N.D | N.D |
| 19 | Stigmast-5-en-3-ol | N.D | N.D | 4.83 | N.D | N.D | N.D | N.D | N.D |

N.D = not detected.

metmyoglobin with hydrogen peroxide in the presence of ABTS to produce the radical cation, in the presence or absence of antioxidants (Robert et al., 1999). Meanwhile, the total antioxidant capacity (TOAC) is the measure of the moles of a given free radical scavenged by a test solution (Mauro and Daniele, 2004). The extract M3M showed the highest TOAC but weak ABTS activities. The weak correlation between the hydroxychavicol content and these two-antioxidant assays showed that hydroxychavicol did not influence both antioxidant activities. Each antioxidant assay measures the antioxidant activity based on different reaction mechanisms, thus, leading to different antioxidant results. A single antioxidant assay is, thus, not enough to evaluate the antioxidant potentials of extracts. The weak correlation between the amounts of hydroxychavicol in the extracts and the antioxidant activities implies that hydroxychavicol alone does not influence the antioxidant activity of *P. betle* L. extracts.

## 5. Conclusion

The extraction method of *P. betle* L. plays an important role as it affects the yield of hydroxychavicol as well as the extracts' biological activities. The biological activities of the extracts, however, suggest the presence of antagonistic and synergistic effects between hydroxychavicol and other components in the various extracts. Thus, the hypothesis is rejected. The designation of a single compound such as hydroxychavicol as a bioactive chemical marker in the *P. betle* L. extracts might not be enough to determine its biological activity.

## Authors' contribution

Nor Hisam Zamakshshari, Idris Adewale Ahmed, and Muhammad Nazil Afiq Nasharuddin were involved in the manuscript conceptualization. Nor Hisam Zamakshshari wrote the first draft of the manuscript. Nor Hisam Zamakshsharia and Idris Adewale Ahmed were involved in the first review and subsequent completion of the review. Najihah Mohd Hashim, Mohd Rais Mustafa, Rozana Othman, and Mohamed Ibrahim Noordin supervised the project and were responsible for the critical review of the manuscript and its further enhancement. All the authors were then involved in the final review and editing. All the authors agree to the submission of the manuscript.

## 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 data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jarmap.2021.100320.

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