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Research Article
Phytochemical Analysis, Identification and Quantification of Antibacterial Active Compounds in Betel Leaves, *Piper betle* Methanolic Extract

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Abstract
Background and Objective: The problems of bacterial diseases in aquaculture are primarily controlled by antibiotics. Medicinal plants and herbs which are seemed to be candidates of replacements for conventional antibiotics have therefore gained increasing interest. Current study was performed to investigate the presence of phytochemical constituents, antibacterial activities and composition of antibacterial active compounds in methanolic extract of local herb, *Piper betle*. **Methodology:** Qualitative phytochemical analysis was firstly carried out to determine the possible active compounds in *P. betle* leaves methanolic extract. The antibacterial activities of major compounds from this extract against nine fish pathogenic bacteria were then assessed using TLC-bioautography agar overlay assay and their quantity were determined simultaneously by HPLC method. **Results:** The use of methanol has proved to be successful in extracting numerous bioactive compounds including antibacterial compounds. The TLC-bioautography assay revealed the inhibitory action of two compounds which were identified as hydroxychavicol and eugenol. The β-caryophyllene however was totally inactive against all the tested bacterial species. In this study, the concentration of hydroxychavicol in extract was found to be 374.72±2.79 mg g^{-1}, while eugenol was 49.67±0.16 mg g^{-1}. **Conclusion:** Based on these findings, it could be concluded that hydroxychavicol and eugenol were the responsible compounds for the promising antibacterial activity of *P. betle* leaves methanolic extract. This inhibitory action has significantly correlated with the amount of the compounds in extract. Due to its potential, the extract of *P. betle* leaves or it compounds can be alternative source of potent natural antibacterial agents for aquaculture disease management.

Key words: *Piper betle*, methanolic extract, phyto-constituents, antibacterial activity, phenolic compounds, hydroxychavicol, eugenol, TLC, HPLC, quantification, aquaculture

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Bacterial diseases is a serious problem in aquaculture and most of the time antimicrobial drugs particularly antibiotics were applied as a mitigative solution. These conventional approaches however, have been reported to adversely affect the fish and may cause the suppression of growth and immune system. Furthermore, the improper use of antibiotics has led to the emergence of antibiotic-resistance of pathogenic bacteria[1]. Thus, many existing antibiotics have been modified to yield new and more potent derivatives. Nevertheless, this only provides temporary solutions because existing resistance mechanisms often rapidly adapt to accommodate the new derivatives[2]. Whereas, the use of chemotherapeutants possess negative impact that result in drugs residues in treated organisms which eventually be detrimental to public health[3]. Due to many undesirable side effects, scientists are now having shifted the search for development of new synthetic antimicrobial drugs to the search for antimicrobials from alternative sources. With the increasing interest of natural therapy in aquaculture, attention has focused on medicinal plants and herbs, as well as their derivatives, which could be ideal candidates of replacements for conventional antibiotics. Moreover, plants product are generally recognized as safe or non-toxic, environmental friendly and more practical to be administered in fish disease management such as supplemented in preparative feed[4].

Numerous studies have reported on the potentials of natural plant products on their biologically activities for wide variety of purposes[5]. Amongst other plants, *Piper betle* have long been documented to have many beneficial health effects. *Piper betle* Linn. (Betel vine) or locally known as sireh is economically important plant belongs to the genus *Piper* of the family Piperaceae. It is native to Malaysia and currently the plant is widely cultivated in India, Sri Lanka, Indonesia, Philippine and East Africa[6]. The betel vine is an evergreen and perennial creeper, smooth with glossy heart-shaped leaves and white catkin[7]. In traditional culture, *P. betle* leaves were used as a masticatory as it is very nutritive and contain substantial amount of vitamins, minerals and also the enzymes like diastase and catalase[8]. It is known medicinally as a carminative, stimulant, digestive, an antiseptic and an expectorant which useful for the treatment of various diseases like bad breath, boils and abscess, conjunctivitis, constipation, headache, mastitis and leucorrhoea[9-12]. The pharmacological actions demonstrated by this plant are related to their active phytochemical constituents[13].

There are several of phytochemical constituents found in *P. betle* leaves that gained interest among researchers such as tannins, saponins, alkaloids, flavonoids, steroids, terpenoids and phenolic compounds[14]. However, these phytochemical constituents have been reported to be vary due to geographical factors[15]. In addition, the extraction of active constituents is considered as the most essential steps in acquisition of target compounds[16]. It is mainly depends on the polarity of the diluent since polar compounds are easily extracted using polar solvent[17]. Hence, the solvent used for the extraction of bioactive compounds must be critically selected as it will influence the quantity and quality of the yield[18].

To date, most of the study related to *P. betle* involves biological activities with the crude extract, but the correlation of these activities to the represented active compounds has yet been studied in detail. *Piper betle* consist of important active compounds of eugenol, eugenol acetate, allylpyrocatechol, allylpyrocatechol monoacetate, chavibetol acetate[19], chavibetol[20], hydroxychavicol, hydroxychavicol acetate[21], piper betol, piperol A and B[22], caryophyllene[23], isoeugenol, methyl eugenol[24] and phytol[25]. Amongst all, hydroxychavicol and eugenol from propenylphenol group and β-caryophyllene (belonging to terpene/sesquiterpene group) are stated as major compounds in betel leaves[26]. In the previous study, we reported that the crude methanolic extract of *P. betle* leaves exhibited successful antibacterial activity against several fish pathogenic bacteria[27]. Therefore, the aim of the present study was to investigate the phytochemical constituents of *P. betle* leaves methanolic extract, identify and quantify of it major active compounds which responsible for antibacterial activities. Qualitative phytochemical analysis was firstly carried out to confirm the presence of the possible active compounds in the crude extract of *P. betle* leaves. The antibacterial activities towards aquaculture pathogens were then evaluated by means of TLC bioautography technique with series of standards compounds, followed with HPLC assay to determine the content of the antibacterial active compounds. The results of this study will elucidate the relation of its efficacy as an antibacterial agent. To best of our knowledge, there was no earlier report on the antibacterial activities from active compounds of *P. betle* leaves against aquaculture pathogens.

MATERIALS AND METHODS

Preparation of herbal extract: Fresh *P. betle* leaves were collected from Herbal Garden of University's Agriculture Park, Universiti Putra Malaysia, Selangor. The leaves were first washed with running tap water to remove dirt and dried in a laboratory oven at 40°C. The leaves were then milled into fine powder using laboratory grinder. Several methanolic extracts were prepared by macerating 500 g of herb powder with 1500 mL of 80% methanol (Grade AR) in Schott's bottle

wrapped in aluminum foil. The preparation were allowed to stand for a week at room temperature[28]. The extract was filtered using Whatman No. 1 membrane filter paper and dried under vacuum using rotary evaporator at 50°C, 150 rpm. The obtained crude extracts were stored at -20°C until further use. The yield percentage of the extract was determined by using the equation of Anokwuru *et al.*[29]:

$$\text{Yield (\%)} = \frac{W_2 - W_1}{W_0} \times 100$$

where, W_2 is the weight of the extract and container, W_1 is the weight of the empty container and W_0 is the weight of the initial dried sample.

Phytochemical analysis of *P. betle* extract: The methanolic extract of *P. betle* leaves was analyzed for the presence of active phyto-constituents such as alkaloids, flavonoids, phenols, tannins, saponins, glycosides, terpenoids and steroids. Phytochemical test was carried out according to the standard procedures of plant analysis as previously described by Trease and Evans[30], Sofowora[31], Evan[32] and Trease and Evans[33]. All the tests were rerun 3 times.

Identification of major antibacterial active compounds: The assay was performed using Thin Layer Chromatography (TLC)-agar overlay bioautography assay following the method as described earlier by Rahalison *et al.*[34]. The methanolic extracts of *P. betle* leaves were identified for its antibacterial active compounds with reference to the major compounds: Hydroxychavicol, (Chromadex, A1036B), eugenol (Aldrich, E51791) and β-caryophyllene (Aldrich, 7-44-5) against nine species of Gram-positive and Gram-negative bacteria, namely *Bacillus* sp., *Entrococcus feacalis*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Aeromonas hydrophila*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Vibrio alginolyticus*. The standard compounds used were chosen based on preliminary study using GC-MS and several literature searches[23, 26,35-41].

Inoculum preparation: Microbial inocula were revived from stock cultures by streaking onto Mueller Hinton Agar (MHA). After an overnight incubation, a single colony was used to inoculate sterile broth using Mueller Hinton Broth (MHB). Inoculated broths were incubated overnight at 35°C. The microbial cultures were diluted to optical density of 0.11-0.12 by using Shimadzu model 160-A spectrophotometer. The resultant cultures corresponded to an approximate concentration of 10^6-10^7 CFU mL^{-1}. The cultures were further serially diluted 10X and incubated for a further 15 min to permit the bacteria to enter into early exponential growth phase.

Chromatography development: The TLC of *P. betle* leaves methanolic extract was performed on 10×4 cm commercial aluminum sheets, silica gel 60 F_{254} of layer thickness 0.2 mm (Merck, Art., 5554). Several plates were prepared and each plate was marked with pencil at 1.0 cm from the top and 0.5 cm at the bottom. One microliter of 100 mg mL^{-1} *P. betle* extract and the standard compounds hydroxychavicol (HC), eugenol (EU) and β-caryophyllene (β-c) at concentration of 20 µg mL^{-1} were applied as spots onto the bottom line of the TLC plate. The plates were developed with suitable solvent systems (hexane, dichloromethane and ethyl acetate) in development tanks. The TLC plates were kept in the tanks without solvent touching the bottom line and left to separate and develop. When the solvent movement reached the top line, the TLC plate was removed quickly from the tank. The spot detected for each separated compound of TLC plate was then circled to mark the spot position and calculated for retention factor (R_f) by using the following equation[42]:

$$R_f \text{ value} = \frac{\text{Distance travelled by substance}}{\text{Distance travelled by solvent front}}$$

Three chromatograms were developed to be used for each bacterial species and two chromatograms were prepared as reference chromatograms in triplicates.

Bioautographic agar overlay assay: After complete removal of solvents, each chromatogram used to detect the antibacterial active compounds of *P. betle* was rapidly overlaid with 5 mL of 10^6 CFU mL^{-1} bacteria-inoculated molten agars (35°C) and was allowed to solidify. The TLC plates were then kept in sterile petri dishes lined with moist filter papers and incubated at 35°C for 24 h. After which, the plates were sprayed with a solution of 0.5% iodonitrotetrazolium chloride or 2-(4-iodo-phenyl)-3-(4-nitrophenyl)-5-phenyl-tetrazolium chloride (SIGMA, I-8377) in water and reincubated for another 4 h to reveal living organisms as dark peach colonies. The active compounds were detected as the clear zones against a dark background. Reference chromatograms were analyzed by staining with vanillin/H_2SO_4 and $FeCl_3$ spraying reagents. Comparison between the reference chromatograms and bioautograms were carried out and the TLC characteristics of the detected antibacterial active compounds were recorded. The inhibition zones represented by certain R_f value were also measured. Three series of determinations were run against each bacterial species and reference compounds.

Quantification of antibacterial active compounds: The composition of antibacterial active compounds in the methanolic extract of *P. betle* leaves was determined by quantifying the identified compounds using reversed phase High Performance Liquid Chromatography (HPLC). All compounds were analyzed simultaneously using the validated method developed by Singtongratana *et al.*[26] and Singgih *et al.*[43] with some modifications.

Instruments and chromatographic condition: An agilent 1100 series HPLC system (Agilent, Waldbronn, Germany) equipped with a degasser, a binary pump and an autosampler (model ALS, DS11116519) was used for analysis. The output signal was detected by Diode Array Detector (DAD) and integrated using Excel 2010 (Microsoft office software package, Microsoft, USA). The chromatographic separation was performed using agilent eclipse C_{18} column (150×4.6 mm, 5 µm) coupled with C_{18} analytical guard column. The whole HPLC system was controlled by agilent Chemstation for LC 3D Rev., A. 10.02 [1757] software.

Firstly, the mobile phase consisted of acetonitrile and 1% acetic acid with ratio of 40:60 (v/v) was prepared fresh in 1000 mL volumetric flask. The solution was filtered using sartorius filtration system set over 0.45 µm nylon disk filter and then sonicated for 15 min in an ultrasonic water bath to remove the air bubbles. The mobile phase was delivered using isocratic method at a flow rate of 1 mL min^{-1}. The column temperature was set at 30°C and detection was monitored at wavelength of 280 nm. All the standards and sample were prepared and filtered using 0.45 µm nylon membranes into 1.5 mL screw-capped sample vial prior to injection on the HPLC system. The injection was performed with a consistent volume of 10 µL by using 1 mL injection loop. Quantification was achieved by direct comparison of peak area ratios of the sample to authentic standard compounds used.

Preparation of standard stock solutions and calibration curve: Standard stock solutions of HC and EU were prepared by weighing 5 mg of each compound in a 50 mL volumetric amber glass flask and filled up with 50 mL of methanol to get a respective concentration of 100 ppm. These stock solutions were further diluted to five different concentrations of 1, 5, 10, 15 and 20 ppm to establish calibration curves. Standard compounds were analyzed simultaneously by mixing the preparative standard solutions in the same sample vial according to their concentrations. All the mixture was filtered and injected 3 times for each concentration into the HPLC. The calibration curve for each standard compound was constructed by plotting the concentrations on the x-axis and the peak area on the y-axis.

Preparation of sample stock solution: A quantity of extract sample equivalent to 50 mg was put in a 50 mL volumetric amber glass flask and methanol was added up to the mark to get a concentration of 1000 ppm of stock solution. This solution was further diluted to bring the final concentration of 50 and 100 ppm. The resulting solution was then sonicated for 15 min and filtered through 0.45 µm nylon syringe filter. Each diluted *P. betle* leaves methanolic extract was injected 3 times into the HPLC. The data of peak areas was collected and used for analyte quantification.

Statistical analysis: All the results reported in each assay are the averages of three measurements. The quantitative results were analyzed using Excel 2010 (Microsoft office software package, Microsoft, USA) and presented as Mean±SE.

RESULTS AND DISCUSSION

Plant-based antibacterial preparations are known to have enormous therapeutic potential due to the presence of several antibacterial substances[44]. In order to identify the antibacterial active compounds of the herbs or medicinal plants, such factors should be taken into consideration including the extractions and bioassay techniques employed. Generally, the type of solvent used for the extraction plays a significant role in the solubility of the active principles of plant materials that not only affected the amount of representative compounds where consequently will influence the antibacterial activity of the extract[45].

As shown in Table 1, an amount of 10.28% yield extract could be obtained from 50 g of dried leaves sample macerated with methanol. The result revealed that methanolic extract of *P. betle* leaves displayed a moderate percentage extraction yield as compared to the earlier studies that used other solvents subjected to the same amount of dried leaves

Table 1: Percentage yield of crude methanolic extract of *Piper betle* leaves

	Yield percentage			
Extract	Weight of the initial dried sample	Weight of the empty container	Weight of the extract and container	Yield (%)
Maceration with methanol (80%)	50.01±0.00	176.97±2.32	182.11±2.33	10.28±0.01

Data are expressed as Mean±SE of triplicate experiments

Table 2: Qualitative analysis of phyto-constituents in the methanolic extract of *Piper betle* leaves

Phyto-constituents	*P. betle* leaves extract	Observation
Alkaloids	+	Reddish-brown coloration
Phenols	+	Blue-green to black coloration
Flavonoids	+	Yellow coloration
Tannins	+	Blue-black coloration
Saponins	+	Stable persistent froth
Glycosides	+	Greenish coloration
Terpenoids	+	Reddish brown coloration
Steroids	+	Blackish-green coloration

+: Presence

powder. A study by Annegowda *et al.*[39] indicated that the ethanolic extract of *P. betle* leaves yielded of about 9.1% of extract with maceration, 10.25% with Soxhlet extraction and 8.1% with sonication, whereas, Singtongratana *et al.*[26] reported that the *P. betle* leaves extract showed better yield when ethyl acetate was used as a solvent with a percentage of 15.6% through liquid-liquid extraction. In contrast, Shafiei[46] found that, the highest yield percentage accounted for 1.70% was obtained in maceration with methanol, followed by ethyl acetate at 1.28% and n-hexane at 0.93%, in a study done on *Psidium guajava* leaves extract, suggested that methanol was the best solvent for solubility of several compounds. It was believed that the observed variation in the extraction yield also reflected the way of the extraction techniques applied. Nevertheless, the preferred extraction method should be simple, fast, economical and importantly able to retain the important phyto-constituents[39].

Bioactivity properties of herbs were closely related to their phytochemical constituents which are classified into various major groups[47]. In the current study, the qualitative phytochemical analysis carried out for methanolic extract of *P. betle* leaves showed the presence of alkaloids, phenols, flavonoids, tannins, saponins, glycosides, terpenoids and steroids, as summarized in Table 2. However, it is important to highlight that the type of diluent used was the main factor that could influence in variation of phyto-constituents being extracted. For example, a study by Chakraborty and Shah[48] on several extracts of *P. betle* leaves using methanol, petroleum ether, aqueous and ethyl acetate produced different results in which all the tested solvents, except for water extract had indicated the presence of flavonoids, tannins, sterols and phenol, but lack of alkaloids. While, the aqueous extract showed the absence of two constituents namely, alkaloids and sterols. Other study that evaluated the existence phytochemicals of petroleum ether, chloroform, ethanol and aqueous extracts also revealed the difference in solubility of active compounds. In comparison, petroleum ether and chloroform extracts were found incapable to extract more than two phyto-constituents tested[49]. These results might be explained by the fact that phytochemical compounds were more soluble in moderate polar organic solvent such as methanol[50]. Furthermore, as previously reported by Chan *et al.*[51], this active compounds also could be effectively extracted with aqueous methanol rather than absolute methanol due to the higher polarity. Therefore, it can be deduced that 80% methanol was the effective solvent to extract all of the examined bioactive constituents as indicated in the present study.

There were some investigations which correlated the antibacterial activities of herbal extracts with the presence of observed phytochemical constituents[52-56]. According to Burt[57] and Witkowska *et al.*[58], phenolic compounds were the most common secondary metabolites implicated with microbial growth inhibitory action in herbs. Study carried out by Cetin-Karaca[59] had showed the effectiveness of antibacterial activities of phenolic compounds against Gram-positive and Gram-negative bacteria such as *Bacillus* sp., *Listeria monocytogenes*, *Clostridium* sp., *E. coli* and *Salmonella* sp. This could be explained by the action of carboxyl group in the aromatic hydrocarbons which present in the phenols of the plant extracts that formed complexes with extracellular and soluble proteins of bacteria which made the later incapable of infection[50]. Likewise, flavonoids which are classified as polyphenolic compounds exhibited antibacterial action also due to this attribute[60]. On the other hand, other studies conducted by Akiyama *et al.*[61], Funatogawa *et al.*[62] and Banso and Adeyemo[63] revealed *in vitro* antibacterial properties of tannins. As reported by Akiyama *et al.*[61] an inhibitory effect of tannins was due to tannic acid. Its potential antibacterial activity was demonstrated when tested against intestinal bacteria such as *Bacteroides fragilis*, *Clostridium perfringens*, *E. coli* and *Enterobacter cloacae*. Moreover, several herbs which were rich in tannins have been shown to possess strong antibacterial effect against a number of bacterial strains with increasing concentration[63]. Whereas, investigations on the effects of another major phyto-constituent, terpenoids upon bacterial membranes also showed its antibacterial potential for microbes. Terpenoids have been shown to induce leakage of reducing sugars and proteins thus destroying the permeability of bacterial membrane[64].

In the previous studies, the phytochemical constituents detected in the plant materials clearly demonstrated antibacterial activities against a wide variety of pathogens. Hence, in-depth investigation on pure compounds was carried out since phytochemical constituent groups consisted of several active compounds, that possibly responsible for antibacterial action. In the current study, three major compounds derivatives of terpenoids and phenolics groups,

Table 3: TLC characteristics of major compounds of *Piper betle* leaves methanolic extract

	TLC characteristics				
	$R_f \times 100$ in solvent systems			Vanillin/	
Compounds	Hex 100%	Hex:DCM (1:1)	DCM:EA (99:1)	H_2SO_4	$FeCl_3$
β-caryophyllene	88.9	100.0	100.0	Purple	No color
Eugenol	5.6	51.6	83.3	Peach	Dark blue
Hydroxychavicol	2.8	9.7	33.6	Peach	Dark blue

R_f: Retention factor, Hex: Hexane, DCM: Dichloromethane, EA: Ethyl acetate, H_2SO_4: Sulfuric acid, $FeCl_3$: Ferric chloride

Table 4: Antibacterial activities of different compound of *Piper betle* leaves methanolic extract evaluated by using TLC agar overlay bioautography assay

	Inhibition zone (cm^2)		
Bacterial species	β-caryophyllene	Eugenol	Hydroxychavicol
Gram-positive			
Bacillus sp.	-	++	++++
E. faecalis	-	+	++++
S. aureus	-	+	++
S. agalactiae	-	++	++
Gram-negative			
A. hydrophila	-	++	++
E. coli	-	++	+++
K. pneumoniae	-	++	+++
P. aeruginosa	-	+	+++
V. alginolyticus	-	++	++++

IZ: Inhibition zone (cm^2), -: No inhibition zone, +: 0.5-1.0 cm^2, ++: 1.1-2.0 cm^2, +++: 2.1-3.0 cm^2, ++++: 3.1-4.0 cm^2

specifically known as β-caryophyllene, eugenol and hydroxychavicol were evaluated for antibacterial active compounds by TLC. From the result presented in Table 3, β-caryophyllene, eugenol and hydroxychavicol were best resolved in screening system of hexane (100%), hexane:dichloromethane (1:1) and dichloromethane:ethyl acetate (99:1) with R_f values of 88.9, 51.6 and 33.6, respectively. The TLC visualization of reference chromatograms using vanillin/H_2SO_4 and $FeCl_3$ showed different spot colors i.e., purple and no color for β-caryophyllene and peach as well as dark blue for eugenol and hydroxychavicol. Meanwhile, the appearance of various fractions on TLC plate confirmed the presence of numerous phytochemical constituents in the *P. betle* leaves methanolic extract. Although, the TLC analysis is the simplest and cheapest method in getting the fractionation and separation in short time[46], it is proven that a suitable solvent system is necessary to obtain the best separation. A good separation obtained from *P. betle* leaves methanolic extract was resulted from solvent mixture used, with its various polarities in different ratio. According to Lavanya and Brahmaprakash[42] R_f values for active phytochemical constituents generally relied on the mobile phase uses, where compound that possessed higher R_f value denoted low polarity while compound with lower R_f value indicated high polarity. In addition, Shafiei[46] stated that the different visualization techniques either viewed under UV (long and short UV) or normal light as well as assisted by chemicals, also gave different range of R_f values. Thus, the use of appropriate visualization aid needs to be consistent.

The TLC agar overlay bioautography assay was tested against nine fish pathogens of Gram-positive and Gram-negative bacteria showed varying antibacterial activities (Table 4). This bioautography technique allows outlining the chemical profile contained in the *P. betle* methanolic extract thus, the active substances that presented antibacterial activities can be identified by matching the location of the standard compounds as explained by Gupta *et al.*[65]. Generally, the active compounds could be seen as clear spots against the background of growing bacteria. In this study, the assay exhibited clear inhibition zones corresponding to eugenol and hydroxychavicol, but none for the β-caryophyllene. This revealed that the antibacterial activity demonstrated by methanolic extract of *P. betle* leaves was represented by eugenol and hydroxychavicol, as it shown the inhibition zones against all the bacteria tested (Fig. 1, 2). On the other hand, β-caryophyllene was found to be totally inactive against all the tested bacterial species although it was expected to has antibacterial action due to its high content in *P. betle* as reported by several studies[15,66].

Based on zone of inhibitions, it was showed that hydroxychavicol possessed higher sensitivity against the investigated bacterial species as compared to eugenol. The differences of inhibition zones of these two compounds was about two folds i.e., in the range of 0.5-2.0 cm observed for eugenol and 2.0-4.0 cm attained by hydroxychavicol. Besides, the inhibition response produced by each bacteria species appeared to be diverse according to active compounds. For instance, eugenol showed weak antibacterial activity to *E. faecalis*, in contrary a very strong activity was manifested by hydroxychavicol. This finding was in agreement with report by Jesonbabu *et al.*[67] in which hydroxychavicol showed a remarkable antibacterial activity when *P. betle* extract was tested against several gastrointestinal pathogens, suggested of its major role in antibacterial action. However, no previous data regarding the antibacterial activity of this compound towards aquaculture pathogens could be found in the current literatures. To best of our knowledge, the antibacterial activities of hydroxychavicol and eugenol against *S. agalactiae*, *A. hydrophila*, *Bacillus* sp., *E. faecalis*, *K. pneumoniae*, *P. aeruginosa* and *V. alginolyticus* are reported for the first time. The results of this study were very encouraging as the two pure compounds from *P. betle* were verified as

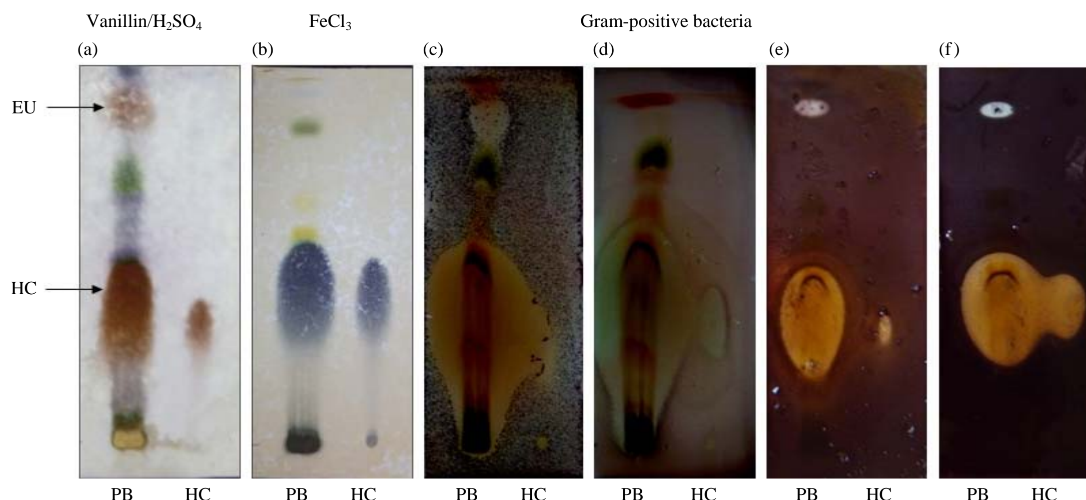

Fig. 1(a-f): Bioautography of methanolic extract of *Piper betle* leaves showing clear zone of growth inhibition that match the location of hydroxychavicol (HC), (a) Reference chromatogram stained with vanillin/H_2SO_4 reagent, (b) Sprayed with $FeCl_3$ reagent, (c) Bioautogram with *Bacillus* sp., (d) *E. faecalis*, (e) *S. aureus* and (f) *S. agalactiae*

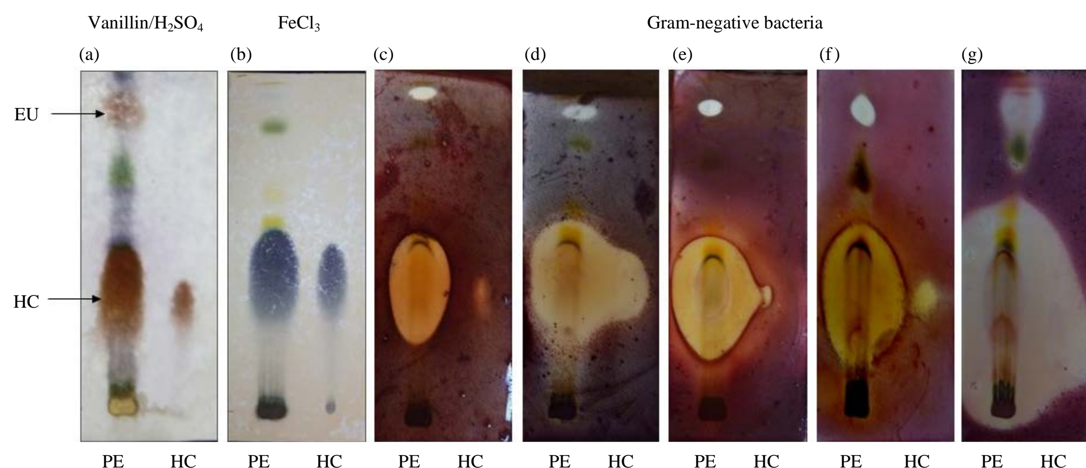

Fig. 2(a-g): Bioautography of methanolic extract of *Piper betle* leaves showing clear zone of growth inhibition that match the location of hydroxychavicol (HC), (a) Reference chromatogram stained with vanillin/H_2SO_4 reagent, (b) Sprayed with $FeCl_3$ reagent, (c) Bioautogram with *A. hydrophila*, (d) *E. coli*, (e) *K. pneumoniae*, (f) *P. aeruginosa* and (g) *V. alginolyticus*

antibacterial active compounds with promising antibacterial activity. According to Nalina and Rahim[37], isolated group of phytocompounds demonstrated their antibacterial action by interrupting the bacterial plasma cell membrane and rendering them more permeable. The researchers suggested that the compounds penetrated into the bacteria cells and coagulated the nucleoid. In the current investigation, the susceptibility of bacteria to the phytocompounds with respect to the varied inhibition strength was postulated to be affected by concentration of the compounds in the extract.

Usually, while evaluating on antibacterial activities of medicinal plants or herbal extracts, it was expected that a greater number of compounds would be active against Gram-positive rather than Gram-negative bacteria[68]. This was due to the fact that Gram-positive bacteria was more susceptible to the inhibitory effects of the plant extracts owed to its single layer and lacks natural sieve effect against large molecules, whereas Gram-negative bacteria has multi layered and complex cell wall structure[21] as cited by Scherrer and Gerhardt[69]. Despite that, the results obtained in the present

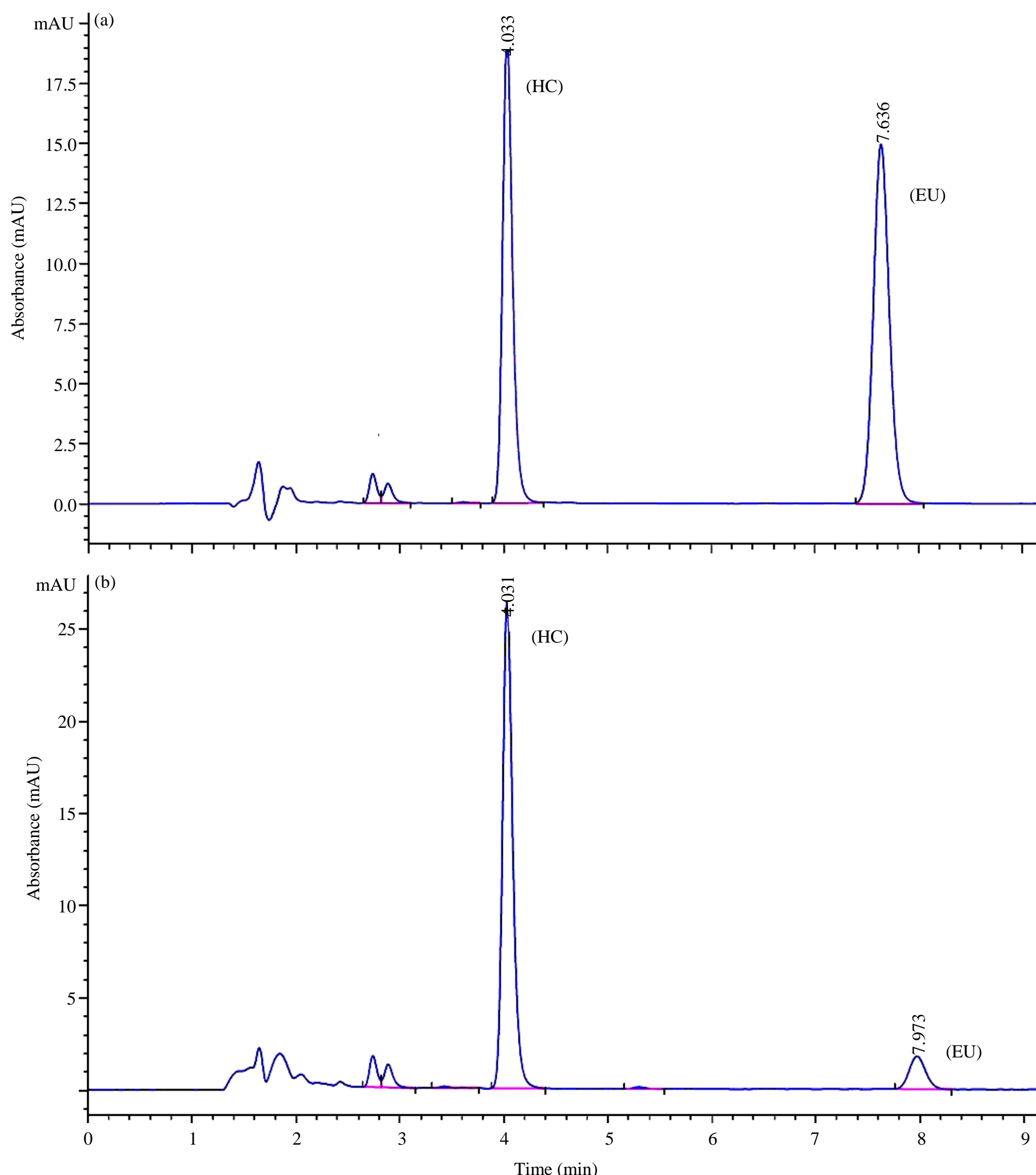

Fig. 3(a-b): Representative HPLC chromatograms of (a) Standard solution of hydroxychavicol and eugenol and (b) Extract of *Piper betle* leaves

study illustrated that the compounds of hydroxychavicol and eugenol of *P. betle* methanolic extract seemed to be sensitive to both Gram-positive and Gram-negative bacteria. It was interesting to note that the crude extracts and single compounds were different in their composition, where the crude extracts contain a number of phyto-constituents includes active and non-active compounds. Hence, this tends to produce different sensitivities to the types of bacteria amongst them. The degree of antibacterial sensitivity in present study was assumed to be greatly dependent on the amount of active compounds, where it was later quantitated by means of HPLC.

It was well reported that HPLC is an efficient method in terms of simplicity, precision, rapid and accurate for the simultaneous determination of bioactive compounds in the extracted sample[26]. Therefore, in this current study, HPLC method to quantify the content of hydroxychavicol and eugenol in *P. betle* methanolic extract was employed. The linear regression for both analytes has showed good linearity in the investigated ranges with correlation coefficients of 0.9990 for hydroxychavicol and 0.9959 for eugenol, as illustrated in Table 5. Typical chromatogram of the standards is shown in Fig. 3a. The average retention time of hydroxychavicol and eugenol was found at 4.02 ± 0.002 and

Table 5: Linearity parameters for the calibration curve of the hydroxychavicol (HC) and eugenol (EU)

Compounds	Slope (a)	Intercept (b)	Correlation coefficient (r^2)
HC	10.3830	19.2350	0.9990
EU	11.5740	8.9902	0.9959

Working range: 1-20 ppm

Table 6: Standard compounds quantified by HPLC from *Piper betle* leaves

Compounds	Content of standard compound in extract (mg g^{-1})	Content of standard compound (mg) in 50 g dried leaves
Hydroxychavicol	374.72±2.79	1927.39±14.39
Eugenol	49.67±0.16	255.47±0.82

Data are expressed as Mean±SE of triplicate experiments

7.61±0.005 min, respectively. The identity of the major compound peaks in the chromatogram, Fig. 3b which has confirmed by their retention times showed the highest spike corresponded to hydroxychavicol, followed by eugenol. Based on the content of these two active compounds in the extract, hydroxychavicol presented a greater concentration, 374.72% as compared to eugenol at 49.67% (Table 6). The content of hydroxychavicol indicated about 7.5 folds higher than eugenol, revealing that hydroxychavicol was the most dominant and main antibacterial compound in *P. betle* leaves methanolic extract. The estimated content of hydroxychavicol and eugenol in raw material also indicated that the use of 50 g dried leaves contained 1927.39±14.39 mg of hydroxychavicol and 255.47±0.82 mg of eugenol. Results from this study also showed the success of extraction method used in extracting important bioactive compounds, as the obtained amount of hydroxychavicol demonstrated much higher proportion compared to earlier report[40]. Furthermore, it was agreed that combination of bioautography and chromatography techniques for determining and quantifying the bioactive compounds were a great tool for consistency evaluation of herbal active compounds as mentioned by Jothy *et al.*[70].

It was evidenced that there was a correlation between antibacterial properties with the concentrations of hydroxychavicol and eugenol which were tested against pathogens. As the trends showed hydroxychavicol exhibited greater inhibition zones than eugenol on the all aquaculture pathogens tested, thus, it was undoubtedly attributed by its higher concentration. Although, the exact inhibitory mechanisms were not determined, the results of this study suggested that both phenolic compounds were responsible for the promising growth inhibitory effect of *P. betle* leaves methanolic extract against Gram-positive and Gram-negative bacteria. Their application either as an extract or pure compounds itself, hence, could be more efficient as it have broad spectrum of antibacterial activities, rather than certain commercial antibiotics which were species specific.

CONCLUSION

The present study revealed that the use of 80% methanol was able to extract numerous bioactive compounds from *P. betle* leaves including antibacterial ingredients. Two compounds namely, hydroxychavicol and eugenol from phenolic group were identified as active antibacterial compounds with promising antibacterial activities. The concentrations of these active compounds in *P. betle* leaves methanolic's extract have a profound effect as antibacterial and we have provided important scientific support regarding their estimated quantities in *P. betle* leaves sample. The results also could be considered as a new finding since no antibacterial study of these compounds has been done towards aquaculture pathogens. Furthermore, the data would serve valuable information for future isolation study of pure antibacterial compounds from *P. betle* leaves extract. Since hydroxychavicol and eugenol demonstrated potent antibacterial action against wide variety of fish pathogens in *in vitro*, it suggested that the existence of these two compounds contained in methanolic extract of the leaves could provide effective outcomes when used as antibacterial agents in fish culture. Findings from this study also beneficial to the researchers and aquaculturist to innovate the application of this extract or its pure compounds for aquaculture purposes for example as feed additive and eco-friendly medication. We believed that the hydroxychavicol and eugenol have great potential in preventing and controlling bacterial diseases, comparable to synthetic antimicrobial drugs and antibiotics. Therefore, this alternative therapy could effectively prevent the antimicrobial resistance development of bacterial pathogens, protect the fish and save the aquaculture industry from disastrous disease outbreak.

SIGNIFICANCE STATEMENTS

As reported in earlier studies, antimicrobials from plant have been shown to have a variety healing potentials. They have been shown to be able to treat contagious diseases and to alleviate some of the adverse reactions frequently associated with synthetic drugs. Our findings revealed that *Piper betle* methanolic extract is a promising antibacterial agent. It showed significant inhibitory activities on numerous

species of fish pathogens due to presence of active compounds viz., hydroxychavicol and eugenol and this was correlated with the compound concentrations. We postulate that these compounds have great potentials to become a natural source of therapeutic agent for bacterial infection especially for aquaculture. Thus, inevitably avoids the development of aquaculture superbugs when using artificial antibiotics.

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