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EVALUATION OF *PIPER BETLE* L. EXTRACTS AND ITS ANTIVIRULENCE ACTIVITY TOWARDS *P. AERUGINOSA*

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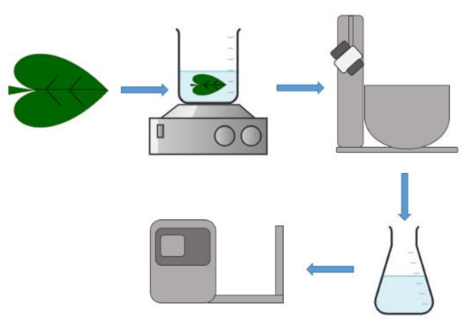
Graphical abstract



Abstract

The virulence factor of bacteria such as *P. aeruginosa* causes severe problems affecting human health and environmental quality. In this study, *Piper betle* undergoes an extraction process yielding extract to diminish the virulence factor of *P. aeruginosa*. The efficiency of *Piper betle* treatment on *P. aeruginosa* was measured using Pyoverdine assay. The different factors affected the *Piper betle* extract yield such as leaves to a solvent ratio (1:6 and 1:10), extraction method (maceration and sonication) and different solvents (methanol, ethanol, ethyl acetate and hexane) were tested. Pyoverdine assay illustrates ethyl acetate exhibits the lowest peak (OD_{630} = 0.2320) compared to methanol, ethanol and hexane due to the presence of a bioactive compound reducing the virulence factor. The ratio of 1:10 has a higher yield of 4.53±0.05 g and the ratio of 1:6 yields 2.86±0.05 g of extracts because of a better contact area. Maceration with agitation indicated the highest yield of 0.5210±0.05 g followed by maceration without agitation at 0.2660±0.05 g and 0.2792±0.05 g for sonication. The yield of *Piper betle* with different solvents showed the lowest yield is hexane 0.4741±0.05 g followed by ethyl acetate 2.4975±0.05 g, ethanol 3.7658±0.05 g and methanol 6.3331±0.05 g due to solvent polarity. This study aims to provide insightful knowledge of applied factor affecting *Piper betle* extracts and the ability of *Piper betle* as antivirulence and antibacterial agent against *P. aeruginosa*.

Keywords: *Piper betle*, *P. aeruginosa*, antivirulence, antibacterial, Pyoverdine assay

Abstrak

Faktor virulens untuk bakteria seperti *P. aeruginosa* menyebabkan masalah teruk yang boleh mempengaruhi kesihatan manusia dan kualiti persekitaran. Dalam kajian ini, *Piper betle* menjalani proses pengekstrakan bagi menghasilkan ekstrak untuk menurunkan faktor virulens *P. aeruginosa*. Kecekapan rawatan *Piper betle* terhadap *P. aeruginosa* diukur dengan menggunakan Pyoverdine assay. Faktor yang berbeza mempengaruhi jumlah ekstrak *Piper betle* seperti nisbah daun ke pelarut (1:6 dan 1:10), kaedah pengekstrakan (maserasi dan sonikasi) dan pelarut yang berbeza (metanol, etanol, etil asetat dan heksana) telah diuji. Pyoverdine assay menunjukkan etil asetat menunjukkan puncak terendah berbanding metanol, etanol dan heksana kerana adanya sebatian bioaktif yang mengurangkan faktor virulen. Nisbah 1:10 menghasilkan ekstrak yang lebih tinggi iaitu 4.53±0.05 g dan nisbah 1:6 menghasilkan 2.86±0.05 g ekstrak kerana mempunyai kawasan kontak yang lebih banyak. Macerasi dengan pergolakan menunjukkan hasil tertinggi 0.5210±0.05 g diikuti dengan maserasi tanpa pergolakan pada 0.2660±0.05 g dan 0.2792±0.05 g untuk sonikasi. Hasil *Piper betle* dengan pelarut yang berbeza menunjukkan hasil terendah ialah heksana 0.4741±0.05 g diikuti oleh etil asetat 2.4975±0.05 g, etanol 3.7658±0.05 g dan metanol 6.3331±0.05 g kerana kekutuban pelarut. Kajian ini bertujuan untuk memberi pengetahuan yang mendalam tentang faktor gunaan yang mempengaruhi ekstrak *Piper betle* dan keupayaan *Piper betle* sebagai agen antivirulen dan antibakteria terhadap *P. aeruginosa*.

Kata kunci: *Piper betle*, *P. aeruginosa*, antivirulen, antibacteria, Pyoverdine assay

1.0 INTRODUCTION

Piper betle taxonomical hierarchy comes from Kingdom: Plantae; Division: Magnoliphyta; Class: Magnolipsida; Order: Piperales; Family: Piperaceae; Genus: Piper and Species: betle [1, 2]. *Piper betle* common name differ from one to another based on locality such as Sirih in Malaysia and Indonesia, Paan in India and Kun in Myanmar [3, 4]. *Piper betle* substantially cultivated in East Asian countries such as Malaysia, Indonesia, Philippines, Vietnam, Laos, Thailand, Myanmar, Singapore, Bangladesh, Pakistan, India and Sri Lanka [3, 5]. *Piper betle* is a medium to large-sized climbers' plants that can grow up to 20 meters if left unattended [6]. *Piper betle* plants are shrubby herbs with a pile of heart-shaped leaves, thin and flexible stem, flower and seed [2, 7]. *Piper betle* leaves are smooth on the surface and veiny on the back. The leaves' colour transforms from dark green to lighter green and yellowish-green when it gets older. *Piper betle* leaves give off a pungent and savoury odour when the leaves break apart or are crushed [2, 6, 8]. The plant basically delicate plant to grow off the tropical region [4]. *Piper betle* preferred warm and humid condition with neutral soil bed between pH 7 to 7.5 [4, 5]. *Piper betle* is used in traditional medicine to treat open wounds and bruises [5, 9, 10]. The previous study exhibits *Piper betle* as a medium in antibacterial, antioxidant, antifungal, antidiabetic, and anticancer agents [5, 11, 12]

Piper betle have been actively researched on capability as antibacterial medium. Previous research of *Piper betle* against a different types of bacterial strain shown a positive outcome on antimicrobial activity. Nayaka *et al.* (2021) tested *Piper betle* ethanol extracts against *E. coli* and *P. aeruginosa* by agar well diffusion method. The concentration of *Piper betle* extracts lays between 50 µg/mL to 100 µg/mL [13]. *E. coli* have bigger area of inhibition range from 8.9 mm to 11.0 mm compared to *P. aeruginosa* range from 6.7 mm to 7.2 mm [13]. Widyaningtias *et al.* (2014) used two (2) types of solvent to extract the *Piper betle*; (1) n-hexane and followed by (2) ethanol at four (4) different concentration of 2.5 mg/mL, 5 mg/mL, 10 mg/mL, and 20 mg/mL [14]. The *Piper betle* extract tested against *P. acnes* using zone inhibition resulting in 7.01 mm, 8.92 mm, 13.28 mm and 21.08 mm respectively [14]. Hoque *et al.* (2012) had tested ethanol extracts of *Piper betle* for area of inhibition against *E. coli* and *S. aureus*. *E. coli* have a larger area of inhibition of (14.67±1.15) mm compared to *S. aureus* (14.67±0.57) mm [9]. Datta *et al.* (2011) performed zone of inhibition on antimicrobial activity of *Piper betle* ethanolic extracts on four (4) different bacterial strain such as *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumonia* and *Proteus vulgaris* [15]. At 0% of ethanolic dilution, the largest zone of inhibition is *Pseudomonas aeruginosa* (16±0.24) mm, followed by *Klebsiella pneumonia* (14±0.15) mm, *Staphylococcus aureus* (13±0.43) mm and *Proteus vulgaris* (10±0.5) mm [15]. Datta *et al.* (2011) also undergo Minimum Inhibitory Concentration (MIC) test on the bacterial strain are *Staphylococcus aureus* (40 µg), *Pseudomonas aeruginosa* (35 µg), *Klebsiella pneumonia* (25 µg), and *Proteus vulgaris* (25 µg) [15]. Kaveti *et al.* (2011) uses 50 µL *Piper betle* ethanol extracts to test four (4) bacterial strain of *B. subitilis*, *S. aureus*, *E. coli* and *P. aeruginosa*. The largest area of inhibition is *B. subitilis* (13.2+0.22) mm, followed by *S.aureus* (9.7+0.02) mm, *E. coli* (8.9+0.21) mm and *P. aeruginosa* at (7.2+0.42) mm [16].

Multiple extraction methods available to extract any plant components such maceration, soxhlet extraction, sonication, microwave-assisted extraction (MAE), supercritical fluid extraction (SFE) and ultrasound-assisted extraction (UAE) [5, 17, 18, 19]. The typical extraction method used in plant extraction is maceration and sonication due to simple experimental setup, less hassle, easier in material handling, can be operated in room temperature and is capable of extracting heat-sensitive volatile compounds [18, 20, 21]. Maceration technique used soaking method to extract plant components with/without additional support such as agitation or induce temperature at specific periods [7, 18]. In comparison, sonication uses electromagnetic waves to burst the air bubble produce by water ripples and causes the active pharmaceutical ingredients (API) or plant compounds to break apart from the plant and dissolve in the solvent [20, 22, 23, 24].

The solvent extracts the bioactive compounds or API from the plants. The solvent capability to extricate bio-active compounds depend on the polarity of the solvents, either polar or non-polar solvent. The polarity of solvent used in plant extraction affected the extract percentage of yield and the antioxidant activity [25, 26, 27]. The more polar the solvent, the more phenolic compound extracted out of plants [28]. The example of solvent based on higher polarity to lower polarity is methanol, ethanol, water, ethyl acetate and hexane. The number and types of components extracted from plants are undefined and variant depending on the polarity of solvent [29]. The bio-active compounds present in *Piper betle* are eugenol, flavonoids (quercetin), tannins, chavibetol and hydroxychavicol [5, 10, 30, 31, 32].

Bacterial infection faced by any living organism such as humans, animals and even plants causes difficulty performing the daily routine or can be deadly as well. Bacteria can infect both externally and internally depending on the route of infections. Skin infections causes by bacteria penetrated through open wounds that not treated carefully. The skin infection can be reduced and soothing the inflammation by applying antibacterial topical cream or gel on the infected skin with bacteria [33]. The treatment should relieve bacterial infection after a few applications depending on the level of severity. *Pseudomonas aeruginosa* (*P. aeruginosa*) commonly inhabits soil, water and vegetation. The ability of *P. aeruginosa* to adapt to the harsh condition and metabolic flexibility is proved by their evolution from mere bacteria into virulence bacteria such as PA14 and PA01 [34,35]. The virulence factor in bacteria in an environmental context focuses on *P. aeruginosa* in decreasing water quality by producing biofilm [36].

Biofilm formation started with bacterial communication. Quorum sensing (QS) is a form of communication between bacteria [37, 38]. The biofilm causes the virulence factor of bacteria to emerge and indirectly causes bacterial infection. The virulence factor of bacteria can be measured quantitatively by Pyoverdine assay (PA). Pyoverdine is a siderophore produced by *P. aeruginosa* that undergoes a stress environment and surroundings [39, 40, 41]. Siderophore is a small molecule responsible for producing the virulence factor of *P. aeruginosa* scavenging for iron (III) in biofilm formation [39, 42, 43]. Iron (III) is an essential compound required by *P. aeruginosa* in bacterial growth and virulence factor [44].

$$\text{Fe Dye}^{3-\lambda} + \text{L}^{X-} \text{-> Fe L}^{3-X} + \text{Dye}^{\lambda-}$$

In this study, the effectiveness of *Piper betle* extracts from different solvents uses a Pyoverdine assay by measuring the differences of the Optical density (OD) between t_0 and t_{10}. The other factor affecting the *Piper betle* extract yield is further discussed such as leaves to solvent ratio, extraction method and different solvent. The *Piper betle* extraction method focused on two (2) standard extraction techniques: maceration and sonication. Different ratios of *Piper betle* leaves to solvent determine by using two (2) different ratios (1:6 and 1:10). The total yield of *Piper betle* extract determines by using multiple solvents named methanol, ethanol, ethyl acetate and hexane. The results of this study allowed the researcher to gain knowledge on the substitution of raw material in antivirulence and antibacterial agents.

2.0 METHODOLOGY

2.1 Materials

Piper betle mature leaves (consistently purchased from a florist in Sungei Way, Petaling Jaya, Selangor), ethyl acetate, methanol, ethanol, hexane, *P. aeruginosa*, King's B media, Chrome Azurol S (CAS) reagent, Iron(III) chloride ($FeCl_3.6H_2O$) hexahydrate, Hydrochloric acid (HCl), hexadecyltrimethylammonium (HDTMA), Dimethyl sulfoxide (DMSO), distilled water.

2.2 *Piper betle* Extraction

Piper betle leaves was washed under running tap water thoroughly and rinse with distilled water [45]. The leaves dried in oven for 3 days at 50 °C. The leaves are then crushed using dried blender and sieved using mesh. 50 g of *Piper betle* leaves soaked in 500 ml of ethyl acetate with a ratio of 1:10 for 12 hours [18]. The extraction of *Piper betle* undergoes maceration with agitation using a magnetic stirrer for 8 hours at a speed of 800 rpm. The extraction was kept inside the fume hood with a cover to avoid the release of solvent fume into the surroundings. The mixture was filtered using a filter paper, and the filtrate is then evaporated using a rotary evaporator. The temperature was set at 40 °C until the evaporation process ended and extracts were collected. The extract scrapes out and was kept in the amber sample bottle. The whole procedure was repeated for all the other remaining solvents such as methanol, ethanol and hexane in order to obtain respective extracts.

2.3 Subculture of Bacteria

The bacteria undergo an agar subculture to recover bacteria [34]. A single colony from agar was subcultured then transferred into King's B media [46]. The bacterial cultured for 16 hours at 37 °C [34]. The bacterial culture was then measured using a spectrophotometer at $OD_{600} = 0.05$.

2.4 Preparation of CAS Solution

The Chrome Azurol S (CAS) solution was prepared [47,48]. 0.06 g of CAS reagent mixed with 50 mL of distilled water (Solution A). 0.0027 g of $FeCl_3.6H_2O$ was dissolved in 10 mL HCl (Solution B). 0.073 g of HDTMA dissolved in 40 mL of distilled water (Solution C). Solution A added with 9 mL of solution B, and the mixture was swirled gently to prevent air bubble formation. After that, solution C was added to the mixture. The mixture of solutions A, B and C will appear deep blue. The mixture was transferred into Scott bottle and autoclave for 20 min. The Scott bottle was wrapped with aluminium foil to minimise the penetration of light into the mixture solution. The solution was stored at 4 °C [34].

2.5 Culture Treatment

The bacterial subculture undergo treatment with *Piper betle* extracts from different solvents such as methanol, ethanol, ethyl acetate and hexane. The extract was prepared by diluting the solidified extracts with DMSO until the final concentration of extract was 200 mg/mL. A mixture of 20 µL of bacterial culture and 20 µL of *Piper betle* extract was added introduced into new 20 mL King's B broth [46]. The cultured treatment was then incubated at 37 °C for 16 hours [34]. The colour change can be monitored at the end of a cycle.

2.6 Pyoverdine Assay

The cultured treatment undergoes separation using refrigerated centrifuges (Eppendorf Centrifuge 5810R) at 4 °C, 3094 g for 30 minutes. Then, the supernatant was collected and filtered with a 0.5 mm syringe filter. The filtrate was analysed via Pyoverdine assay. 0.5 mL of filtrate was placed in 1 mL cuvette, and 0.5 mL CAS reagent was added to the cuvette. The reading was measured using a spectrophotometer at OD_{630} (Optical density used to measure bacterial biofilm production) [49]. The absorbance reading is taken at t_0 and t_{10}. The untreated *P. aeruginosa* was served as a control to differentiate the quorum sensing activity between *P. aeruginosa* with other extracts from different solvents such as methanol, ethanol, ethyl acetate and hexane. The experiment was carried in triplicate. The average data was recorded and tabulated.

3.0 RESULTS AND DISCUSSION

3.1 *P. aeruginosa* Agar Subculture

The agar subculture of virulence *P. aeruginosa* emits bluish-greenish colour (Figure 1) on the agar plate on areas inhabited by bacterial colonies [34,50]. Pyocyanin secreted by *P. aeruginosa* is a pigment responsible for producing bluish-greenish colour to signify the exhibition of a virulence factor in *P. aeruginosa* [34,50,51].

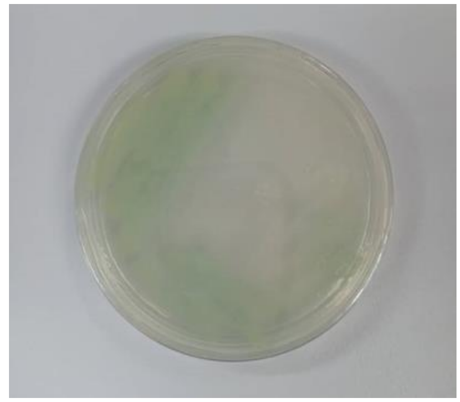


Figure 1 Agar subculture of *P. aeruginosa* in King's B agar shown the blue hues from the pyocyanin compound

3.2 *P. aeruginosa* Culture Treatment against *Piper betle*

The bacterial culture undergoes culture treatment with *Piper betle* extracts from multiple solvents (Figure 2). The culture treatment can be monitored qualitatively by observing colour variation between the type of solvent used at the end of treatment. The solvent used in *Piper betle* extraction process is methanol, ethanol, ethyl acetate and hexane. The solvent was selected based on the polarity of the solvent [29].

Figure 2 shows colour differences between the mixtures shifted from green to greenish-yellow and yellowish-chalky. Figure 2(a) contained only *P. aeruginosa* without treatment. The mixture's emphasis on green colour signifies pyocyanin's presence that emphasises the presence of *P. aeruginosa* virulence factor [34,50,51]. *P. aeruginosa* without treatment also served as a control throughout the experiment to differentiate the reaction between treated *P. aeruginosa* and non-treatment *P. aeruginosa*. Figure 2(b) mixtures colour appears greenish-yellow is a treatment between *P. aeruginosa* with *P. betle* extracts using hexane as solvent. The colour implies that hexane incapable or has insufficient ability to extract out the required component or bio-active compound responsible for blocking the formation of a virulence factor of *P. aeruginosa* due to hexane being a non-polar solvent. Figure 2(c), Figure 2(d) and Figure 2(e) are a mixture of *P. aeruginosa* treated with *Piper betle* extracts from methanol, ethanol and ethyl acetate respectively. All three (3) beaker shows a yellowish-chalky mixture indicated that the reaction *P. aeruginosa* broth subculture grows without the presence of virulence factor represented by a green colour (pyocyanin) [34,50,51].

Due to the lack of colour differences between the three of the mixture, further clarification or bio-assay is required to determine the strength and capabilities of methanol, ethanol and ethyl acetate extract on the virulence factor of *P. aeruginosa*. Therefore, in this study, further experiment was conducted by performing Pyoverdine assay.

Figure 2 *P. aeruginosa* subculture in *P. betle* extracts (a) *P. aeruginosa* without treatment (control), (b) *P. aeruginosa* in *P. betle* extracted from hexane, (c) *P. aeruginosa* in *P. betle* extracted from ethanol, (d) *P. aeruginosa* in *P. betle* extracted from methanol, (e) *P. aeruginosa* in *P. betle* extracted from ethyl acetate

3.3 Pyoverdine Assay

Pyoverdine assay is a method to measure the capabilities of the extract to treat *P. aeruginosa* virulence factor [35]. This method is a quantitative method as the mixture's optical density (OD) is measured at a time interval of 0 to 10 minutes using a spectrophotometer [49]. Figure 3 shows the graph of *P. aeruginosa* used to determine the quorum sensing (QS) activity after adding CAS reagent. The treatment culture incorporates CAS reagent measured at t_0 and t_{10}. The differences in absorbance reading between the OD are calculated and normalised (Table 1).

Virulence bacteria produce potent ligands during the formation of virulence factors. The high affinity towards iron III during quorum sensing chemical reaction leads to detection of siderophore (Pyoverdine) using CAS reagent as an indicator [47,48]. A potent ligand such as pyoverdine removed iron III from CAS reagent causes blue colour shifting between t_0 and t_{10} [49]. Thus, the more biofilm present, the more colour changes monitored and vice versa due to strong ligand (siderophore) to grab the iron III compound and remove the blue dyes from the mixtures. The absorbance was measured by differences in Optical density (OD) between t_0 and t_{10}. The larger the differences in OD between t_0 and t_{10} measured, the more interaction between the bacteria in the bacterial colonies and the more intense the virulence factor or biofilm production.

Table 1 Pyoverdine Assay

Solvent	Average±SD
PA01	1
Methanol	0.3863±0.05
Ethanol	0.4381±0.05
Ethyl Acetate	0.2320±0.05
Hexane	0.5567±0.05

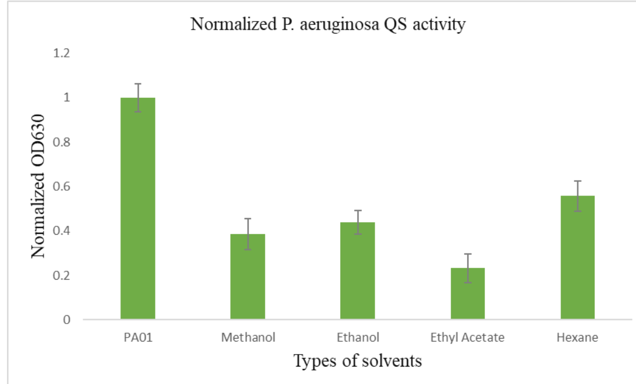


Figure 3 Graph of QS activity of *P. aeruginosa* against *P. betle* extracted from different solvent (a) *P. aeruginosa* without treatment (control), (b) *P. aeruginosa* in *P. betle* extracted from methanol, (c) *P. aeruginosa* in *P. betle* extracted from ethanol, (d) *P. aeruginosa* in *P. betle* extracted from ethyl acetate, (e) *P. aeruginosa* in *P. betle* extracted from hexane

P. aeruginosa has shown the highest peak (OD_{630} = 1) due to throughout QS activity because of nil treatment. Thus, the interaction between the bacteria to produce virulence factor was accomplished at the uttermost level. The second highest peak (OD_{630} = 0.5567) in the graph is hexane extract. Hexane is a non-polar solvent. Thus, many bioactive compounds do not mix or dissolve in hexane, resulting in lower phenolic bioactive compounds extracted [28]. The third highest peak (OD_{630} = 0.4381) is ethanol, whereas the fourth highest peak (OD_{630} = 0.3863) is methanol. Methanol is a more polar solvent than ethanol, resulting in a higher bioactive compound in methanol to lower the virulence factor. Finally, ethyl acetate has the lowest peak (OD_{630} = 0.2320) among the solvents. Ethyl acetate is a polar solvent. Even though the polarity of ethyl acetate was lower than both methanol and ethanol, ethyl acetate contains the active compound that minimises the virulence factor of *P. aeruginosa* [18,45]. Thus, the QS activity between bacteria lowered down and proved by the Pyoverdine assay.

3.4 Factor Affecting Yield of *Piper betle*: Different Ratio of *Piper betle* Leaves to Ethyl Acetate Solvent

In this experiment, *Piper betle* leaf to solvent was tested with two (2) different ratios of 1:6 and 1:10 [18,45,52]. The 1:6 ratio shows that the extracts' volume yields 2.86 mL while the 1:10 ratio manifests 4.53 mL of *Piper betle* extract (Table 2). Thus, the volume yields of *Piper betle* in ratio 1:10 almost double the yield extracted from 1:6 ratios. The more significant difference in *Piper betle* leaf ratio to solvent gives off a higher yield due to the higher surface volume of solvent contact with *Piper betle* leaves [45]. Thus, it increases the rate of extraction and produces a higher yield.

Table 2 Extraction of *Piper betle* using different ratios of leaf to solvent

Ratio of *Piper betle* leaf to volume of solvent	*Piper betle* leaf (g)	Volume of Ethyl Acetate (ml)	Average Volume of extract (g)
1:6	50	300	2.86±0.05
1:10	50	500	4.53±0.05

3.5 Factor Affecting Yield of *Piper betle*: Extraction Method

The experiment was carried out using two methods; sonication and maceration. Aside from stand still maceration techniques, the maceration with agitation is introduced to the system to test whether the agitation will improve the percentage yield of an extract. The experiment was used ethyl acetate as a solvent. Table 3 showed extraction yield using a different type of extraction method. The maceration without agitation yield 0.2792 g of yield, whereas maceration with agitation yield 0.5210 g of yield. Maceration took up to 12 hours for *Piper betle* extraction. Maceration techniques are the most appointed method for extraction due to simple procedure, less hassle, and cheaper [5,18].

Sonication yield 0.2660 g of extract. Sonication involves the electromagnetic waves that produce bubbles, vibrate at incredible speed, and burst the water bubble causes the bioactive compound to release to the surroundings and absorbed by solvent [22]. The sonication technique can preserve volatile bioactive compounds that disintegrate when exposed to heat [20,21]. Thus, this method will allow the volatile bioactive compound to be collected rather than disintegrate or break due to heat. However, due to the low yield of extracts, maceration with agitation is the preferable method to extract *Piper betle*. As the maceration with agitation without heat induced was applied during this study, the disintegration of volatile bioactive compound will be avoided.

Table 3 Extraction yield using a different type of extraction method

Extraction Method	*Piper betle* leaf (g)	Volume of Ethyl Acetate (ml)	Ratio of *Piper betle* leaf to volume of solvent	Average Yield of extract (g)
Sonication	10	100	1:10	0.2660±0.05
Maceration (Without Agitation)	10	100	1:10	0.2792±0.05
Maceration (With Agitation)	10	100	1:10	0.5210±0.05

3.6 Factor Affecting Yield of *Piper betle*: Types of Solvent

Piper betle leaf extracted with different solvents named methanol, ethanol, ethyl acetate and hexane based on 1:10 ratio of *Piper betle* leaves to solvent. The test was conducted to determine the *Piper betle* weight of yield extracted based on the type of solvent. Table 4 illustrates that methanol has the highest yield of 6.3331 g, followed by ethanol 3.7658 g, ethyl acetate 2.4975 g and hexane 0.4741 g. The methanol extracts exhibit the highest yield of extracts because methanol is the most polar compared to other solvents such as ethanol, ethyl acetate and hexane. The more polar the solvent used in the extraction method using *Piper betle* leaves, the better the extraction performance and the higher the average weight of yield of *Piper betle* extracts [25,26,27,28]. The yield does not represent the capabilities of the bioactive compound toward virulence factor but focuses solely on the yield of extracts. Based on the observation, the average weight of yield increased by the polarity of solvent started from methanol, ethanol, ethyl acetate and hexane [28].

Table 4 Total volume of extract using different solvent

Solvent	Piper betle leaf (g)	Volume of Solvent (ml)	Ratio of Piper betle leaf to volume of solvent	Average weight of extract (g)
Ethyl Acetate	50	500	1:10	2.4975±0.05
Ethanol	50	500	1:10	3.7658±0.05
Methanol	50	500	1:10	6.3331±0.05
Hexane	50	500	1:10	0.4741±0.05

4.0 CONCLUSION

The agar subculture of *P. aeruginosa* shown the blue hues from the pyocyanin compound on the agar plate proved the presence of virulence factor. The culture treatment of *P. aeruginosa* treated with *Piper betle* extracts from methanol, ethanol, ethyl acetate, and hexane showed that methanol, ethanol, and ethyl acetate positively react against virulence factors. The graph on Pyoverdine assay tested *P. aeruginosa* culture with methanol, ethanol, ethyl acetate and hexane *Piper betle* extracts shown highest peak is *P. aeruginosa* without the treatment followed by hexane, ethanol, methanol and ethyl acetate. Ethyl acetate has the lowest peak due to its capabilities to extract bioactive compounds to minimise the virulence factor of *P. aeruginosa* compared to other solvents.

The yield of *Piper betle* extract is affected by many factors such as the ratio of *Piper betle* leaves to solvent, type of extraction method and different solvents. The extraction method with the ratio of 1:10 producing a higher yield compared to 1:6 ratios due to the larger surface area of *Piper betle* leaves contact with the solvent. The extraction techniques of maceration and sonication show that maceration with agitation has a double yield compared to the other method. Weight yield of *P. betle* extract based on four (4) different solvents; hexane, ethanol, methanol and ethyl acetate exhibits methanol has the highest yield compared to other solvents.

Even though the ability of ethyl acetate *Piper betle* extract to minimise and decrease the biofilm formation and virulence factor of *P. aeruginosa* proved by Pyoverdine assay, the absolute compound that is still unknown. Thus, further investigation and research regarding the bioactive compound are required. The endless possibility of *Piper betle* can be discovered aside from its antibacterial effect such as antifungal, antioxidant, antidiabetic, and anticancer properties that will be beneficial for human society as *Piper betle* easy to grow, produce lushes leaves and requires minimum care to grow healthily.

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