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Studies on Phytochemical and Biological Activities of *Mentha piperita* (Peppermint)

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M. piperita or peppermint is well-known among medical herbs for its own healing properties, which could be used to cure many diseases. The phytochemical and biological activities of *Mentha piperita* were studied. The plant was extracted by using three different polarities of solvents such as n-hexane, ethyl acetate, and methanol through the cold extraction method. The result has shown that the highest percentage yield was ethyl acetate leaves extract which was 25.93%. The phytochemical screening analysis was done on each extract and it revealed there were many secondary metabolic presences in *M. piperita* such as flavonoids, alkaloids, tannins, saponins, carbohydrates, terpenoids and phenols. Antibacterial study was conducted by using disc diffusion method against four types of selected bacteria such as *Bacillus cereus, Staphylococcus aureus, Escherichia coli* and *Klebsiella pneumonia*. The study has shown that the highest antibacterial activity was recorded on ethyl acetate and methanol against *Klebsiella pneumonia* with a diameter inhibition of 18 mm. In addition, antioxidant study has revealed the highest antioxidant activity on methanol extract with percentage inhibition of 83.49%. The extracts of *Mentha piperita* have high antibacterial and antioxidant potential and could be used as medicine.

Keywords: *Mentha piperita*; phytochemical; antibacterial; antioxidant

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M. piperita, a species of the Lamiaceae family is well accustomed to the peppermint fragrance of perennial mint family herbs. *M. piperita* is a hybrid of *M. spicata* and *M. aquatica,* that has been bred in Europe, Asia, and North America [1]. It grows best in a wet environment with moist soil. Mint thrives in chilly and moist environments. However, this plant prefers cold temperatures for growth, with the ideal temperature around 30 ºC to 80 ºC [2].

According to a study conducted by Meamarbashi (2014) [3], peppermint oil has a vasodilating effect on animals as it helps lower the heart rate and systolic pressure and smooths the muscles and relaxes the bronchial tubes. The essential oil was proven to be effective against a number of Gram-positive and Gram-negative bacteria [4]. According to the authors, the membrane- damaging impact of the monoterpene components aid in the destruction of microorganisms. Furthermore, peppermint oil has the highest total phenolic content (TPC) value as discovered in other research, implying that *M. piperita* essential oil is rich in phenolic compounds and has excellent antioxidant capabilities [5].

M. piperita is a medicinal plant that also contains various kinds of phytochemicals that can be used to treat various diseases. The types of compounds mainly found in *M. piperita* are flavonoid**,** phenolic acid, and other substances such as limonene and menthol [6]. This herbal plant exhibits good antioxidant properties due to the presence of polyphenols [6]. Apart from that, compounds such as caffeic acid and γ-terpinene also contribute to the antioxidant properties [7]. These biomolecules avert inflammation activity and other chronic diseases that affect tissues and organs, such as cardiovascular disease and diabetes. *M. piperita* essential oil has been proven to have a broad antibacterial spectrum against pathogenic bacteria such as *Escherichia coli, Aeromonas hydrophile, Staphylococcus aureus, and Enterococcus faecium* [8]. Based on a previous study, 80% of the human population relies on plant extract and essential oils as a basic need [8]. Most of the research data that was available came from extracted essential oil of the plant with no specific and proper information regarding the peppermint crude extract [9]. This study, therefore, was aimed at extracting and studying the phytochemical constituent and biological activities of *M. piperita*.

EXPERIMENTAL

Materials and Methods

Plant Materials

Mentha piperita or peppermint was purchased from local market in Hulu Langat, Selangor, Malaysia. The plant was washed thoroughly with water to discard soil and other unwanted particle. The leaves were air

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dried. The leaves were then ground into fine powder using a blender and stored in an airtight container for further usage [10].

Extraction of Plant

The powdered leaves were extracted using three different polarity solvents-: hexane, ethyl acetate and methanol using the maceration method. Then, the extract was concentrated using rotary evaporator to produce crude extracts. The extracts were subsequently used to screen the phytochemical and biological activities of this plant.

Phytochemical Analysis

Selected phytochemical tests were used to observe the presence of flavonoids, alkaloids, tannins, saponin, carbohydrate, terpenoid, phenols, and glycoside in the crude extracts of *Mentha piperita*.

Flavonoids (Lead Acetate Test)

A volume of 2 mL of crude extract was treated with a few drops of 5% lead acetate solution. Formation of a yellow precipitate indicated the presence of flavonoids [11].

Alkaloids (Wagner's Test)

2 mL of crude extract was dissolved in dilute hydrochloric acid (HCl) and then filtered. A few drops of Wagner's solution were subsequently added. The formation of reddish-brown precipitate indicated the presence of alkaloids [12].

Tannins (Ferric Chloride Test)

A volume of 2 mL of plant extract was mixed with distilled water and heated in a water bath. The mixture was filtered, and ferric chloride was added. Formation of dark green color indicated the presence of tannins [11].

Saponins (Foam Test)

The crude extract (50 mg) was shaken with 20 mL distilled water. for about 15 minutes. The presence of saponin was detected by the formation of a stable foam [13].

Carbohydrates (Benedict's Test)

A mass of 0.5 mg of extract was dissolved in 5 mL distilled water and filtered. 0.5 mL of Benedict's reagent was added into 0.5 mL filtered extract. Then the mixture was heated in boiling water bath for 2 minutes. The presence of changing color indicated the presence of carbohydrate (sugar) [13].

Terpenoids

A volume of 1 mL crude extract was treated with 2 mL chloroform and evaporated in water bath. The mixture was then boiled with 2 mL of concentrated sulfuric acid H_2SO_4. Red brick formation indicated the presence of terpenoids [13].

Phenols

A mass of 50 mg of crude extract was dissolved in 5 mL distilled water. Then, a few drops of 5% ferric chloride were added. A dark green color formation indicated the existence of phenolic compound [13].

Glycosides

50 mg of crude extract was mixed with 3 mL of chloroform and shaken until it separated and formed a chloroform layer. Then, a few drops of 10% of ammonia solution were added. The appearance of pink color on the sample indicated the presence of glycosides [14].

Antioxidant Assay

2 mg of DPPH powder was diluted with 100 mL methanol in a volumetric flask and covered with an aluminum foil. The crude extract of *Mentha piperita* was weighed (0.001 g) and mixed with 1 mL of methanol.

Determination of Antioxidant Activity

The prepared crude sample was serially diluted to a concentration of 1000, 500, 250,125, and 65.3 μg/mL. Then, 0.2 μg/mL of each concentration was mixed with 3.8 mL of DPPH solution each and kept in the dark for 30 minutes. The activity of the antioxidant of the extract was shown by a change in color of DPPH from purple, a stable free radical and became yellow color of diphenyl-picrylhydrazine [15]. The absorbance of the solution was measured using a UV-Vis spectrometer at 517 nm.

The equation above was used to calculate the percentage of scavenging DPPH free radical. The IC_{50} was determined by plotting the result of percent inhibition against concentration by using a non-linear regression algorithm. The experiment was repeated in triplicate and results were recorded as mean ± standard deviation [15].

$$\% \text{ scavenging activity} = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100$$

Table 1. Mass of *M. piperita* leaves sample.

Leaves sample	Mass of sample (g)
Fresh sample	5000
Dried sample	376.90

Table 2. Results of sample extraction.

Solvent	Weight of sample (g)	Weight of extract (g)	Percentage yields (%)
Hexane	376.90	69.34	18.40
Ethyl acetate	360.50	93.50	25.93
Methanol	345.90	45.75	13.22

Antibacterial Assay

Mentha piperita crude (500 mg) was pipetted and dissolved in 100% DMSO. The agar was prepared by dissolving the nutrient agar powder (23 g) in distilled water (500 mL) and autoclaved. The broth was prepared by dissolving 30 g of nutrient broth powder in 500 mL of distilled water.

Disc Diffusion Method

A bacterial culture was disseminated to agar plate evenly using a sterile swab. The disc that had been soaked with 10 µL of plant extract was placed on an agar surface [16]. The DMSO was used as a negative control while Streptomycin 5µg/disc as a positive control [17], which was slightly modified by using Ciprofloxacin. The petri dish was incubated for 24 hours at 37 ºC and subsequently, the incubated plates was examined for inhibition zone. The assay was conducted in triplicate [17].

RESULTS AND DISCUSSION

The sample of *M. piperita* was bought from local market in Selangor. The leaves sample was cleaned and left to air dried for about a week. The dried leaves were then finely grounded into powder. The mass of fresh and dried leaves is shown in Table 1.

The plant sample must be kept out of direct sunlight as it dried. This was due to the plant sample's bioactive compound's extreme sensitivity to heat. Some of the compound could be destroyed when high temperature was applied. This method was preferable because samples were not exposed to any high temperature hence the compounds would not be degraded. Applying a high drying temperature often results in the loss of volatile chemicals, and it might also encourage the heat-labile compounds' breakdown [18].

Extraction of Sample

Different polarity of solvent was used to extract the samples, starting with non-polar, semi-polar and ending with polar solvent. This is because plant materials contain different bioactive chemicals with varying solubility qualities in different solvents, the best solvent for extraction will depend on the specific plant material and the molecules that need to be extracted [19]. The selected solvent used includes hexane, ethyl acetate, and methanol. The maceration method was used to extract bioactive compounds from the leaves. In Table 2 below, hexane was the most effective solvent extraction for leaves, followed by ethyl acetate and methanol, based on the extraction data.

Based on the table, ethyl acetate gave the highest percentage yield with 25.93%. This implies that *M. piperita* contains many semi-polar compounds in the leaves itself. When extraction using ethyl acetate as solvent, less phytochemical compound inside the leaves degraded. This agreed with a study by Berktas and Cam (2020)[20] that found ethyl acetate had a higher yield than ethanol, a polar solvent.

Phytochemical Screening of Crude Extract

M. piperita contains many active compounds such as flavonoid, tannin, alkaloid and saponin [21]. In this study, phytochemical screening was carried out to detect the presence of secondary metabolite in the leave crude extracts. Table 3 shows the results of the phytochemicals screening of those extracts.

Table 3. Results of phytochemical screening.

Phytochemical test	Hexane crude	Ethyl acetate crude	Methanol crude
Alkaloid	+	+	+
Flavonoid	-	-	+
Tannins	-	+	+
Saponins	+	+	+
Terpenoids	-	-	+
Benedict's	-	-	+
Phenols	-	-	+
Glycosides	-	-	+

Key: + = present, - = absent

Based on the results obtained, alkaloid and saponin can be found in all three extracts. The appearance of reddish-brown color indicated the presence of alkaloids and formation of foam and bubbles indicated the present of saponins. However, the appearance of tannins can be found in ethyl acetate and methanol extract and absence in hexane extract. The formation of dark green color indicated the presence of tannins.

Other screening tests that were conducted in this study such as flavonoid, terpenoid, Benedict's, phenol, and glycosides only presented in methanol extract. Yellow precipitate indicated the presence of flavonoids. While color from blue to green indicated presence of carbohydrate. On the other hand, dark green color indicated the presence of phenol and formation of pink color indicated the presence of glycosides in the compound.

As comparison, the presence of alkaloid, flavonoids, saponins and tannins in this study were also found in the leaves of plant collected from Oman [22]. There are other methods that can be used to screen phytochemicals in plant samples. Not every method would produce the same finding and favorable outcomes. If the results were negative, the method used requires to be attempted again and modified.

Antibacterial Activity

The study shows *M. piperita* extract has the ability in inhibitory effect against Gram-positive bacteria and Gram-negative bacteria. It depends on the solvent extraction that was used. The Gram-positive bacteria are type of bacteria that does not have an outer membrane but surrounded by thick layer of peptidoglycan. Meanwhile, the Gram-negative bacterium has an outer membrane and surrounded by a thin layer of peptidoglycan [23].

Ciprofloxacin was used as positive control while a 10% DMSO as the negative control. The inhibition zone was measured to evaluate the potential of *M. piperita* extract against different types of organisms. Diameter of inhibition zones obtained were between 6 mm to 18 mm. The leaves crude ethyl acetate extract showed highest inhibition zone against Gram-positive *B. cereus* which was 10 mm. Meanwhile for *S. aureus* the leaves crude of methanol and hexane extract both showed 15 mm diameter of inhibition zone. This study was comparable to one carried out by Gigante (2019) [24] on Gram-positive bacteria, which found that hexane had the highest zone of inhibition (mm) against *S. aureus* and that ethyl acetate exhibited the highest zone of inhibition against *B. cereus*.

Antibacterial activity against *E. coli* on the methanol and hexane crude extracts showed 10 mm diameter of inhibition zone. While for *K. pneumonia*, the ethyl acetate and methanol crude extracts displayed the highest diameter of inhibition zone with 18 mm each. According to a research carried out by Singh et al. (2015) [9] the ethyl acetate extract showed the highest diameter of inhibition zone, 12 mm, against *K. pneumonia*. Meanwhile, hexane and methanol extract exhibited the highest inhibition zone which was 8 mm against *E. coli* [22]. Tannins and terpenoids are active against microbes and they were present in methanol crude extract, Strong antibacterial properties found in *Mentha piperita* crude extract make it efficient at eliminating the bacterial pathogens [25].

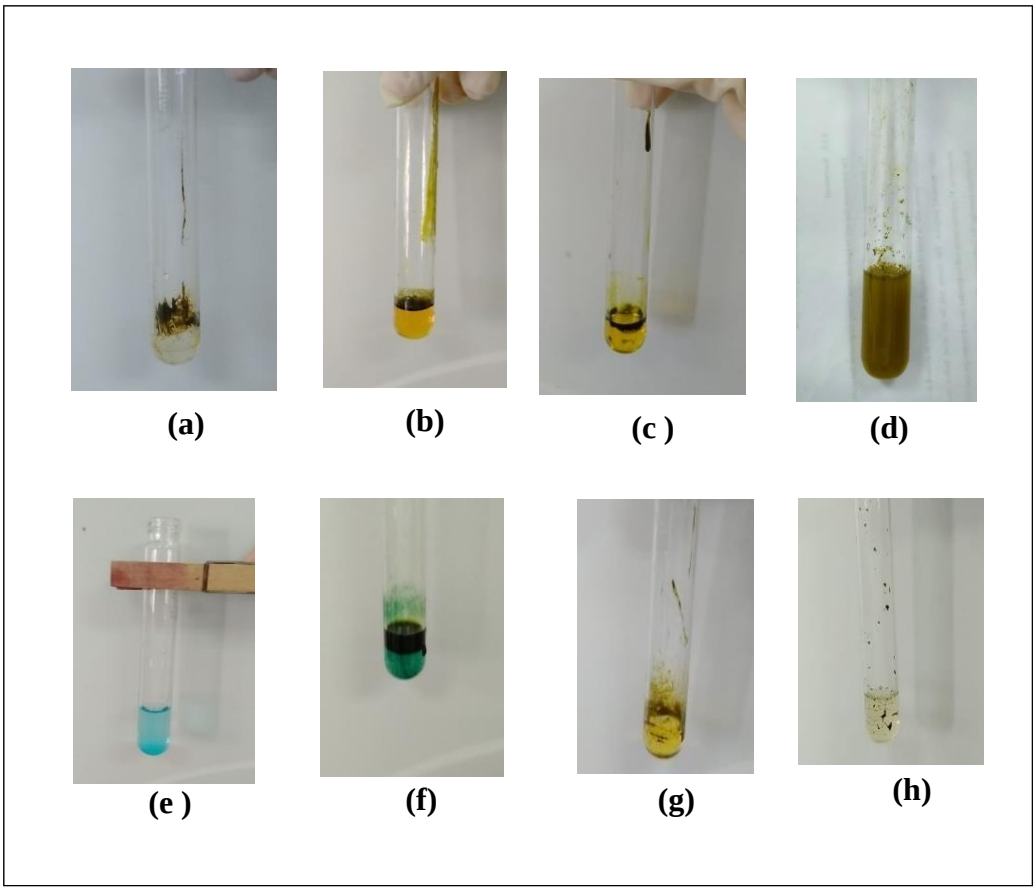

Figure 1. Phytochemical screening of hexane crude extract.

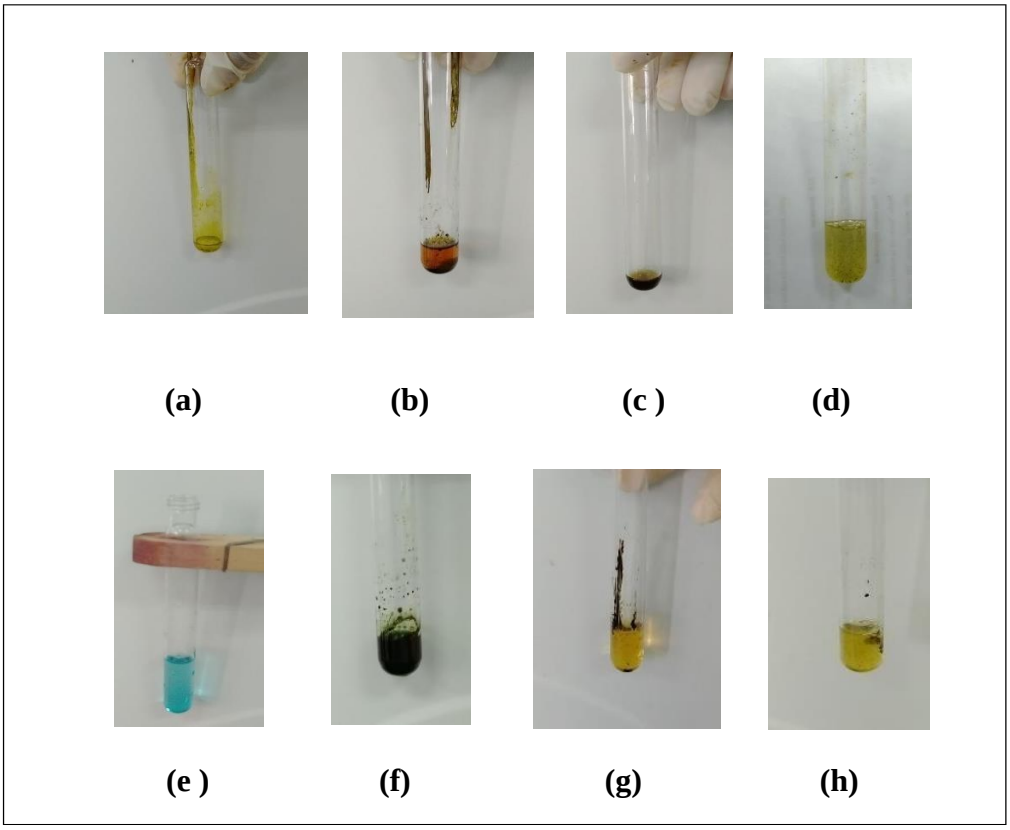

Figure 2. Phytochemical screening of ethyl acetate crude extract.

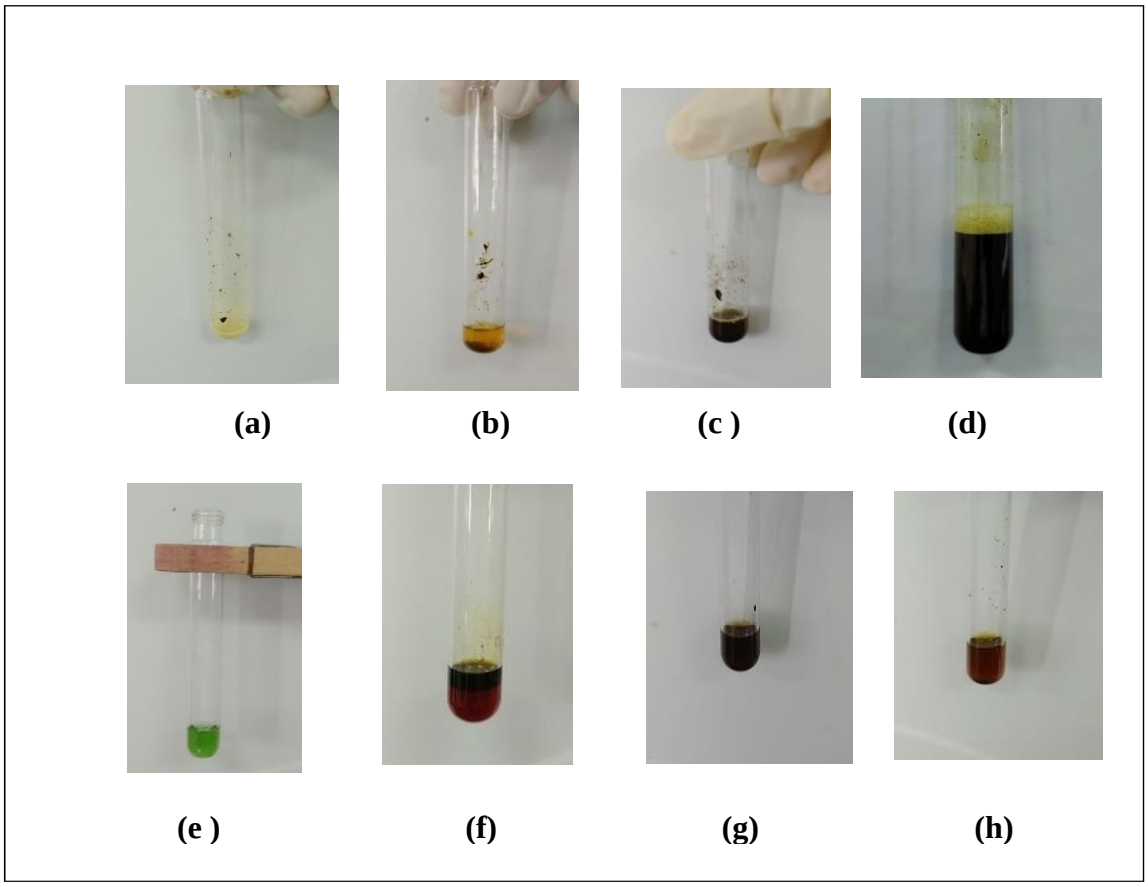

Figure 3. Phytochemical screening of methanol crude extract

Note:

(a) Test for flavonoids
(b) Test for alkaloids
(c) Test for tannins
(d) Test for saponins
(e)Test of carbohydrates
(f)Test for terpenoids
(g)Test for phenols
(h)Test for glycosides

Table 4. Antibacterial activity of difference *M. piperita* extracts.

	Diameter of inhibition zone (mm)			
Bacteria/ Sample	***B. cereus***	***S. aureus***	***E. coli***	***K. pneumonia***
Ciprofloxacin	20.0	25.0	35.0	23.0
Negative control (10% DMSO)	0.0	0.0	0.0	0.0
Hexane	6.0	15.0	10.0	11.0
Ethyl acetate	10.0	10.0	7.0	18.0
Methanol	9.0	15.0	10.0	18.0

Table 5. Percentage inhibition of *M. piperita* leave extracts.

Extract	Percent inhibition of DPPH
Hexane	$38.65 \pm 11.54 \times 10^{-4}$
Ethyl acetate	$41.23 \pm 0.58 \times 10^{-3}$
Methanol	$83.49 \pm 0.57 \times 10^{-3}$
Ascorbic Acid (standard)	96.71 ± 0.08

Antioxidant Activity

The antioxidant activity of extract of *M. piperita* were evaluated using DPPH radical scavenging assay, While, ascorbic acid was used as reference standard in evaluation of antioxidant activity. Antioxidant DPPH is a persistent free radical molecule that absorbs particularly at 517 nm in wavelength. The transfer of an electron or hydrogen atom to DPPH occurs due to interaction of antioxidant with DPPH where it neutralizes free radical and due to this interaction the color of mixture change to yellow from purple [26]. All the sample extract were tested at concentration of 1000 µg/mL. The percentage inhibition of leaves extract *M. piperita* are shown in Table 5.

Based on the table, methanol crude extract showed the highest antioxidant activities with value 83.49 % and followed by ethyl acetate 41.23 % and hexane with value of 38.65 % This result was in line with previous study which reported that methanol had the strongest radical scavenging activity [27]. A study conducted in 2016 by Benabdallah et al. [28]. on the antioxidant activity of different Mentha species revealed that *M. piperita* was one of those species that exhibited efficient antioxidant activity. The present results were also supported by Riachi and De Maria, (2015) [29] who discovered that peppermint extract showed good antioxidant activity. The phenolic component found in *Mentha piperita* makes it a possible candidate for antioxidant properties [30].

CONCLUSION

In conclusion, different polarity of solvent was used during the extraction process which produced various results that affect the qualitative composition of the phytochemical constituent and bioactivity studies of *M. piperita*. The highest percentage yield (25.93 %) was the ethyl acetate extract. In addition, phytochemical screening analysis showed that *M. piperita* contained alkaloids, flavonoids, tannins, saponins, terpenoids, carbohydrates, phenols, and glycosides. The methanol extract contained the majority classes of phytochemicals compared to ethyl acetate and hexane.

The crude extracts of *M. piperita* were able to inhibit the tested bacteria with the highest diameter of inhibition zone. The methanol and hexane crude extracts on *B. cereus* exhibited the largest diameter of the inhibitory zone, measuring 10 mm, while the methanol and ethyl acetate crude extract on *K. pneumonia* had the largest diameter, measuring 18 mm, in the case of Gram-negative bacteria. In addition, *M. piperita* leave extracts also displayed antioxidant properties with, methanol crude extract demonstrated the highest DPPH radical scavenging activities which were 83.49% of inhibition.

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