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Short Communication: Antioxidant activity and metabolite profiles of leaves and stem extracts of *Vitex negundo*

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Abstract. *Alfarabi M, Turhadi, Suryowati T, Imaneli NA, Sihombing PO. 2022. Short Communication: Antioxidant activity and metabolite profiles of leaves and stem extracts of* Vitex negundo*. Biodiversitas 23: 2663-2667.* Many plant species could be used as natural sources of antioxidants, one of which is *Vitex negundo* or *lagundi* which grows widely in Southeast and South Asia. Due to the wide distribution of growth areas, so different habitats could be a factor causing variations in *V. negundo* bioactivity, especially antioxidant activity. In addition to environmental factors, extract derived from different plant parts could also contribute to variations in the content of metabolites that affect bioactivity. The objective of this study was to determine the antioxidant activity of leaf and stem extracts of *V. negundo* and evaluate the metabolites contained in the extracts. Antioxidant activity analysis was performed using the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), and GC-MS analysis was used to identify bioactive metabolites. Leaf and stem extract of *V. negundo* showed antioxidant activity directly proportional to the increase in extract concentration. A total of 8 metabolites were identified from the three extracts, i.e., acrolein, hydroquinone, sitosterol, naphthalene, pyrogallol, squalene, phytol, and hexadecenoic acid. There were correlations between antioxidant activity and the metabolites contained in the three extracts. It is suggested that the bioactivity of the extract is due to the interaction of these metabolites.

Keywords: Bioactivity, metabolite profiling, natural product, phytochemical, *Vitex negundo*

INTRODUCTION

Stable molecules naturally have several paired electrons, but free radical molecules have a single electron in their valence position. Free radicals can be generated from organic and inorganic chemical reactions (Wang et al. 2021). Free radicals have a dual role in the cell, i.e., part of a biochemical reaction or cause cell damage. The presence of unpaired electrons causes free radicals to be very reactive in obtaining electron pairs from other molecules, such as reactive oxygen species (ROS). These molecules react very easily by oxidizing other molecules, such as lipids (Shadyroa et al. 2019). The reaction of free radicals can damage the cell membrane and even cause cell death. Cell or tissue damage due to free radicals contribute to several diseases such as cancer, premature aging, and other degenerative diseases. These diseases are triggered by the uncontrolled number of free radicals (Halliwell 2020).

However, free radicals (O_2^-) can also indicate infection caused by bacteria or viruses. Hosts infected by bacteria produce O_2^- as a defense system with an antibacterial effect. Free radicals generated from viral infections may cause pathological consequences, such as in mice infected with the influenza virus and COVID-19 patients (Camini et al. 2017; Wu 2020). However, free radicals in cells or tissues do not always result in detrimental effects because free radicals also help in tissue homeostasis, signal regulation, and control of cell death (Harris and DeNicola 2020). Therefore, controlling free radical levels is very important in the process of cell life.

Antioxidants are molecules that can neutralize free radicals by donating electrons to radical molecules so that the free radicals become relatively stable molecules. Based on the source, antioxidants can be categorized as endogenous and exogenous antioxidants. Endogenous antioxidants are antioxidants produced by cells as enzymes or cofactors that can eliminate ROS, while exogenous antioxidants can be metabolites from plants, such as vitamins, flavonoids, alkaloids, and other phenolic compounds (Chan and Chan 2015; Fleming and Luo 2021).

Lagundi (*Vitex negundo*) belongs to the Verbenaceae family that can be used as an antioxidant source. It grows mostly in low to moderate lands and is widespread from China to Southeast Asia, such as Indonesia and the Philippines (Ahuja et al. 2015; Boy et al. 2018). Boiling leaves of *V. negundo* have been used as a traditional medicine in the Philippines to treat coughs, indigestion, and skin diseases. The Philippine Department of Health has also promoted this plant as an antitussive and anti-inflammatory (Bautista et al. 2015). According to Gill et al. (2018), *V. negundo* contains polyphenols such as flavonoids, which have potent antioxidant activity. These activities can be related to alternative medicine methods in

cardiovascular disease, cancer, aging, diabetes mellitus, and neuro-degenerative diseases.

Because this plant also grows in Indonesia, such as Jakarta, it is interesting to study the properties and contents of *V. negundo*. Differences in weather and soil conditions affect the metabolite produced by the plant, so it can affect bioactivity. This research aimed to assess the antioxidant activity and determine the metabolite content of *V. negundo* grow in Jakarta, Indonesia. The results of this study could be used as scientific information to develop *V. negundo* as a natural antioxidant.

MATERIALS AND METHODS

Collection of plant material and extraction

Leaves and stems of *V. negundo* were collected from Jakarta, Indonesia (6°15'43.4"S 106°52'39.9"E) and identified at Herbarium Bogoriense, Research Center for Biology, LIPI, Indonesia. The samples used for extraction were 125 g of trifoliate leaves (TF), 125 g of pentafoliate leaves (PF), and 250 g of fresh stem (S). Each sample was extracted using the maceration technique with 90% ethanol as a solvent at room temperature. After 72 h, the filtrate was filtered with Whatman filter paper and evaporated at 60°C with a rotary evaporator (Buchi R-100). The extracts of leaves and stems were analyzed for their antioxidant activity and chemical content by the GC-MS method.

Antioxidant activity assay

Determination of antioxidant activity was performed using DPPH (2,2-diphenyl-1-picrylhydrazyl) (Sigma) assay (Yuningtyas et al. 2021). One mL of sample solutions (10, 50, 100, 150, and 200 ppm) were mixed with 1 mL of 0.1 mM DPPH. Furthermore, the solutions were incubated for 30 min at room temperature. The absorbance of solutions was measured at 517 nm. The DPPH without samples solution was used as a negative control, and the positive control was ascorbic acid (Sigma). The percentage of DPPH reduction was calculated as follows:

$$\%\ inhibition = \frac{absorbance\ negative\ control - absorbance\ samples}{absorbance\ negative\ control} \times 100\%$$

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The chemical compounds of leaves and stem extract were analyzed using Gas Chromatography-Mass Spectrometry (GC-MS) at Laboratory for Jakarta Regional Health, Jakarta, Indonesia. Five µl extracts were injected in a GC-MS (Model 7890, Agilent Technologies, Palo Alto, CA, USA) equipped with an autosampler (Model 7693, Agilent Technologies, Palo Alto, CA, USA). This instrument was connected to a Mass Selective Detector and a Chemstation Data System (Model 5975C inert MSD with Triple-Axis Detector, Agilent Technologies, Palo Alto, CA, USA). It is also equipped with an HP ultra 2 capillary column (0.11 µm). The temperature of the injector, ion source, interface, and quadrupole were set up respectively as follows 250°C, 230°C, 280°C, and 140°C. Helium with a 1.2 mL/min flow rate was used as the gas carrier. The mass spectrum was detected in a mass-to-charge range of 20-500 m/z. Metabolite identification was performed based on the Wiley W8N08.L database.

Data analysis

Metabolites were analyzed using Venny 2.1.0 online web-based program (https://bioinfogp.cnb.csic.es/tools/venny/). In addition, the metaboAnalyst 5.0 online web-based program (https://www.metaboanalyst.ca/) was used for biplot and heatmap-clustering analysis. Finally, the correlation between antioxidant activity and the detected metabolites in the extract was analyzed using Pearson's correlation analysis with SPSS 15.0 program.

RESULTS AND DISCUSSION

Antioxidant activity

The results showed that all extracts had antioxidant activity indicated by the extract's ability to inhibit the DPPH molecule. The sample concentration was directly proportional to the increase in antioxidant activity. The highest inhibitory activity (around 80%) of each sample was obtained at the concentration of 200 ppm, while the lowest inhibitory (around 40%) was obtained at the concentration of 10 ppm. The S extract has the highest antioxidant activity, with the highest inhibitory of 84.94%. The lowest antioxidant activity was obtained on PF extract, with an inhibitory of 41.74% (Figure 1). The IC_{50} value for the TF, PF, and S extract was 7.3 ppm, 31.20 ppm, and 8.44 ppm, respectively. The IC50 of ascorbic acid was 2.16 ppm.

Metabolite profile

The results of GC-MS analysis of three extracts showed 8 identified metabolites consisting of aldehyde, phenol, phytosterol, aromatic, benzene, terpenoid, and fatty acids groups. There were 6 identified metabolites in the TF extract, 5 identified metabolites in the PF extract, and 4 identified metabolites in the S extract. The percentage of each metabolite was varied. The highest metabolite content in TF and PF extracts was phytol, while the highest content in the S extract was sitosterol. Several metabolites were detected in more than 1 extract (Table 1). Phytol and hexadecenoic acid were detected in all extracts. Three metabolites were detected in the TF and PF extracts, i.e., naphthalene, pyrogallol, and squalene. Acrolein was only detected in the TF extract, While hydroquinone and sitosterol were only detected in the S extract (Figure 2).

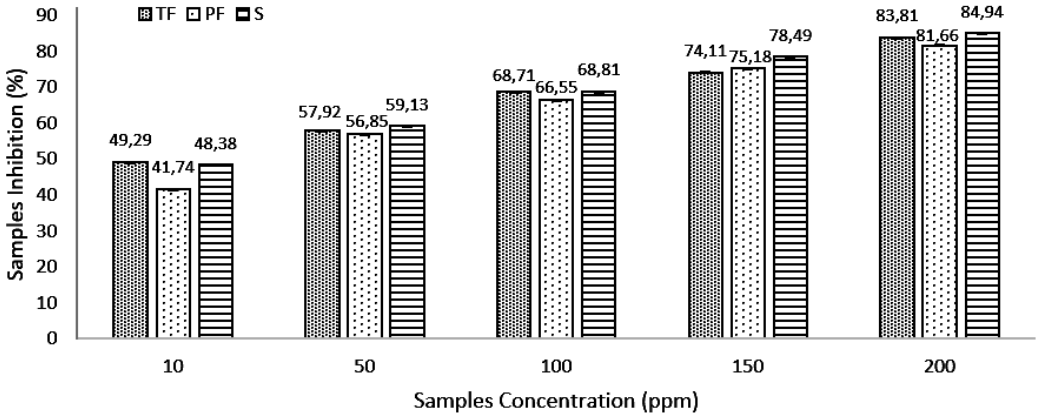


Figure 1. Antioxidant activity of leaves and stem of *Vitex negundo* extracts at different concentrations. Different scores on the top of the bar chart showed an inhibition percentage of each sample. TF: trifoliate leaves extract, PF: pentafoliate leaves extract, S: stem extract

Table 1. Identified metabolites in plant part of *Vitex negundo* extract

Compounds	Group compounds	Concentration (%)		
		TF	PF	S
Acrolein	Aldehyde	15.24		
Hydroquinone	Phenolic	-	-	4.76
Sitosterol	Phytosterol	-	-	19.04
Naphthalene	Aromatic	2.19	1.83	-
Pyrogallol	Benzene	1.95	2.06	-
Squalene	Terpenoid	2.59	2.76	-
Phytol	Terpenoid	25.33	23.14	6.77
Hexadecenoic acid	Fatty acid	6.03	7.26	13.47

Note: TF: trifoliate leaves extract, PF: pentafoliate leaves extract, S: stem extract, (-): not detected

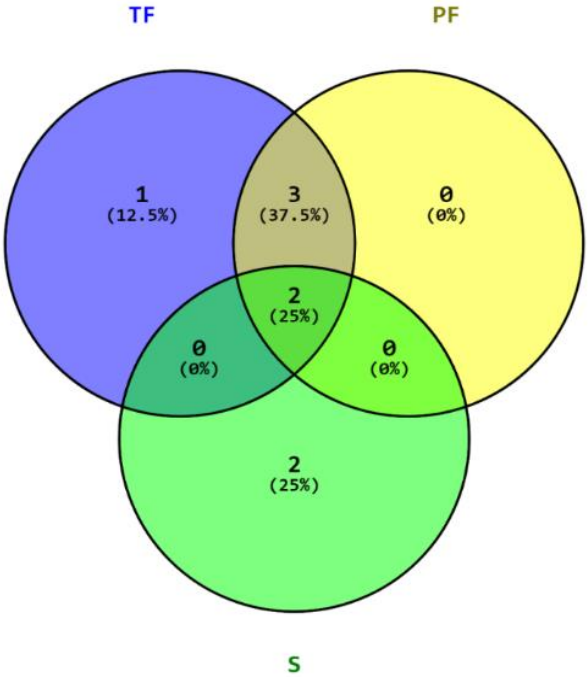
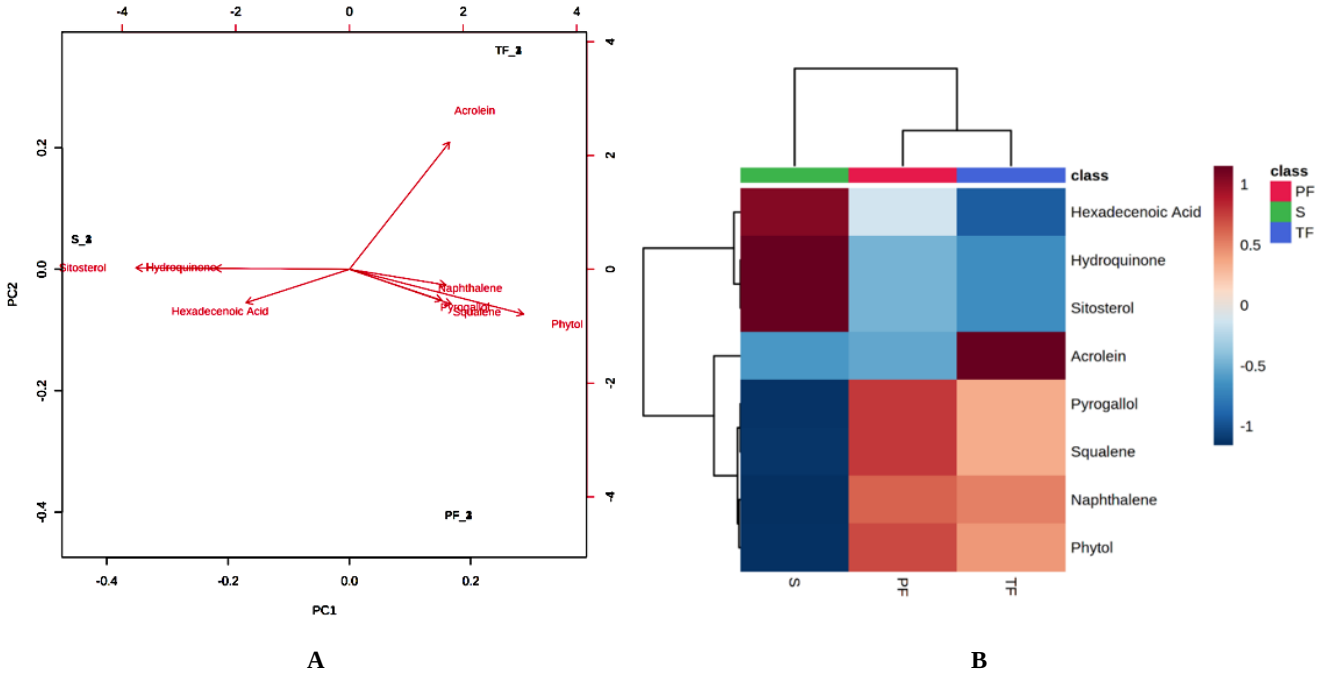


Figure 2. The comparison of identified metabolites in each *Vitex negundo* extract. TF: trifoliate leaves extract, PF: pentafoliate leaves extract, S: stem extract

The compositions of the detected metabolites in each extract were analyzed using biplot analysis to determine the relationship between the detected metabolites and extract sources in *V. negundo*. Biplot results showed that the three extracts had different metabolite compositions. It was indicated by the position of the three extracts, which are separated from each other. In addition, these results also indicated the presence of several metabolites that can be used as markers of *V. negundo* extract. Acrolein was indicated as a metabolite marker in the TF extract, while sitosterol can be used as a metabolite marker in the S extract (Figure 3A). Some of the metabolites contained in the TF and PF extracts formed into one cluster, but this analysis could not properly explain the differences between the TF and PF extracts. Therefore, a heatmap-clustering analysis was carried out to investigate the characteristics of metabolites in *V. negundo* extracts. The results showed two main clusters of the three extracts based on their metabolite profiles. The TF and PF extracts were in the same cluster, while the S extract was separated from two other extracts. The TF and PF extract had similar characteristics based on the naphthalene content (Figure 3B). In the TF extract, acrolein was suggested as a metabolite marker, and it was in accordance with biplot analysis. Hydroquinone and sitosterol were suggested as metabolite markers in S extract, and these two metabolites were absent in both TF and PF extracts.

The correlation between the identified metabolites in each extract and its antioxidant activity was analyzed by Pearson's correlation. The results showed that each metabolite had a moderate correlation with antioxidant activity. Five metabolites, including acrolein, hydroquinone, sitosterol, naphthalene, and hexadecenoic acid, showed a negative correlation, so the antioxidant activity increased by decreasing the concentration of these metabolites. Meanwhile, three metabolites, i.e., pyrogallol, squalene, and phytol, showed a positive correlation. Therefore, increasing the concentration of these metabolites increases the antioxidant activity of the *V. negundo* extract (Table 2).



A **B**

Figure 3. A. Biplot analysis. B. Heatmaps clustering analysis for metabolites in each *Vitex negundo* extract. TF: trifoliate leaves extract, PF: pentafoliate leaves extract, S: stem extract

Table 2. Pearson's correlation coefficient between identified metabolites of *Vitex negundo* extracts and its antioxidant activity

Metabolites	Pearson's correlation coefficient
Acrolein	-0.64
Hydroquinone	-0.56
Sitosterol	-0.56
Naphthalene	-0.49
Pyrogallol	0.58
Squalene	0.58
Phytol	0.51
Hexadecenoic acid	-0.49

Discussion

The antioxidant activity of V. negundo extract was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method. The DPPH molecule is a free radical molecule that is relatively stable at room temperature. Therefore, the purple color of the DPPH molecule will turn yellow in the presence of antioxidant compounds. There are two mechanisms of the antioxidant reaction using DPPH. The first mechanism is the electron or H atoms donation from the antioxidant molecules to the DPPH molecule so that the molecule becomes neutral. As a result, the antioxidant molecule loses electrons and possibly becomes a new radical molecule. Secondly, the antioxidant molecules and DPPH share electrons (Blois 1958).

The low antioxidant activity of the samples compared to ascorbic acid could be caused by the samples as crude extracts contained a lot of metabolites, and not all of these metabolites had antioxidant activity. It was even possible that other metabolites inhibited the antioxidant reaction between the extract and the DPPH molecule. Therefore, it might be influenced by the physical-chemical properties of these metabolites (Gan et al. 2017; Dillak et al. 2019). Nevertheless, all samples had IC_{50} values of less than 50 ppm. So, the extract could inhibit 50% of DPPH free radicals at a concentration of less than 50 ppm. The IC_{50} in this study was higher than the IC_{50} of methanol and hexane extracts of *V. negundo*. The IC_{50} values of methanol and hexane extracts of *V. negundo* from Panchtahr district, Nepal, were 38-73 ppm (Koirala et al. 2020). However, the antioxidant activity in this study was lower than the water extract of *V. negundo* leaves from Aurangabad, India, with an IC_{50} value of 5.77 ppm (Fatema et al. 2019). The difference in results might be due to the influence of the growing environment and the solvent used in the extraction process. Each organic solvent has different characteristics, especially its polarity. Polar metabolites are extracted with polar solvents, while non-polar metabolites are extracted with non-polar solvents. Therefore, the different solvents in extraction can affect the metabolites obtained in the extraction process and the bioactivity of these metabolites. We used ethanol 90% as a solvent to adapt the traditional extraction for *V. negundo*'s leaves, using boiled water as a polar solvent.

The variation in antioxidant activity of the three extracts could be due to the different parts of the plant used (Figure 1). Each plant's parts, such as roots, stems, and leaves, had different physiological functions, so the metabolites' composition was also different (Table 1, Figure 3). For example, leaves are organs that have a major function in plant growth and development. Photosynthesis is the main process in producing energy that occurs in the

leaves, while the stems are an organ that has the main function of supporting plant structures and transporting nutrients (Heldt and Piechulla 2011).

Multivariate analysis showed that the three extracts had different metabolite content. The analysis results showed the presence of 2 main clusters consisting of stem extract and leaves extract (Figure 3). The differences in metabolite content might be due to differences in the part of the plant used as the extracted source. There are differences between the TF and PF extract clusters, even though many of the same metabolites are present in both extracts. *V. negundo* used in this study has trifoliate and pentafoliate leaves. Differences in leaf character allow for variations in leaf phytochemicals so that they might have different bioactivity. It was proven in the biplot results that acrolein is a marker metabolite in the TF extract. The study of leaf character is very important to determine a plant's identity and the use of leaves related to their phytochemical content (Salvaña et al. 2019).

Correlation analysis showed a moderate correlation between antioxidant activity and the identified metabolites in each extract, although there were metabolite markers in extracts based on biplot results (Table 2). It indicates that the antioxidant activity of the leaf and stem extracts of *V. negundo* was influenced by the interaction of the metabolites contained in the extracts. Therefore, this result suggested that no dominant metabolite affects the antioxidant activity in the extract. The results indicated the variation of antioxidant activity. The differences in metabolite composition in extract result in different characteristics. It was suggested that metabolite differences cause differences in antioxidant activity. However, the antioxidant activity of the extract was the result of the interaction of the metabolites contained in the extract without any dominant metabolite affecting antioxidant activity.

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Metabolites were analyzed using Venny 2.1.0 online web-based program (https://bioinfogp.cnb.csic.es/tools/venny/). In addition, the metaboAnalyst 5.0 online web-based program (https://www.metaboanalyst.ca/) was used for biplot and heatmap-clustering analysis. Finally, the correlation between antioxidant activity and the detected metabolites in the extract was analyzed using Pearson's correlation analysis with SPSS 15.0 program.

RESULTS AND DISCUSSION

Antioxidant activity

The results showed that all extracts had antioxidant activity indicated by the extract's ability to inhibit the DPPH molecule. The sample concentration was directly proportional to the increase in antioxidant activity. The highest inhibitory activity (around 80%) of each sample was obtained at the concentration of 200 ppm, while the lowest inhibitory (around 40%) was obtained at the concentration of 10 ppm. The S extract has the highest antioxidant activity, with the highest inhibitory of 84.94%. The lowest antioxidant activity was obtained on PF extract, with an inhibitory of 41.74% (Figure 1). The IC_{50} value for the TF, PF, and S extract was 7.3 ppm, 31.20 ppm, and 8.44 ppm, respectively. The IC50 of ascorbic acid was 2.16 ppm.

Metabolite profile

The results of GC-MS analysis of three extracts showed 8 identified metabolites consisting of aldehyde, phenol, phytosterol, aromatic, benzene, terpenoid, and fatty acids groups. There were 6 identified metabolites in the TF extract, 5 identified metabolites in the PF extract, and 4 identified metabolites in the S extract. The percentage of each metabolite was varied. The highest metabolite content in TF and PF extracts was phytol, while the highest content in the S extract was sitosterol. Several metabolites were detected in more than 1 extract (Table 1). Phytol and hexadecenoic acid were detected in all extracts. Three metabolites were detected in the TF and PF extracts, i.e., naphthalene, pyrogallol, and squalene. Acrolein was only detected in the TF extract, While hydroquinone and sitosterol were only detected in the S extract (Figure 2).

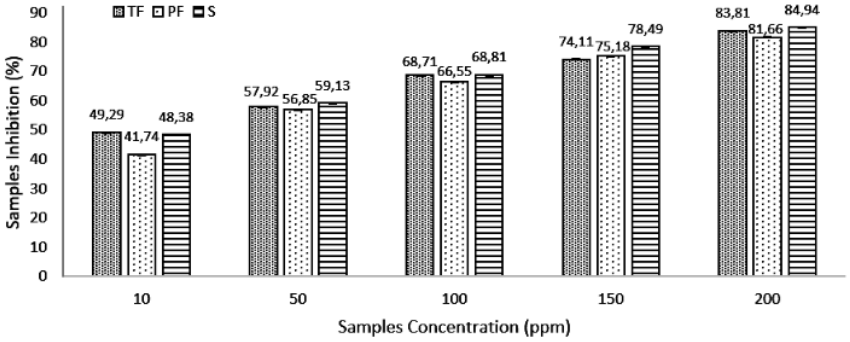


Figure 1. Antioxidant activity of leaves and stem of *Vitex negundo* extracts at different concentrations. Different scores on the top of the bar chart showed an inhibition percentage of each sample. TF: trifoliate leaves extract, PF: pentafoliate leaves extract, S: stem extract

Table 1. Identified metabolites in plant part of *Vitex negundo* extract

Compounds	Group compounds	Concentration (%)		
		TF	PF	S
Acrolein	Aldehyde	15.24		
Hydroquinone	Phenolic	-	-	4.76
Sitosterol	Phytosterol	-	-	19.04
Naphthalene	Aromatic	2.19	1.83	-
Pyrogallol	Benzene	1.95	2.06	-
Squalene	Terpenoid	2.59	2.76	-
Phytol	Terpenoid	25.33	23.14	6.77
Hexadecenoic acid	Fatty acid	6.03	7.26	13.47

Note: TF: trifoliate leaves extract, PF: pentafoliate leaves extract, S: stem extract, (-): not detected

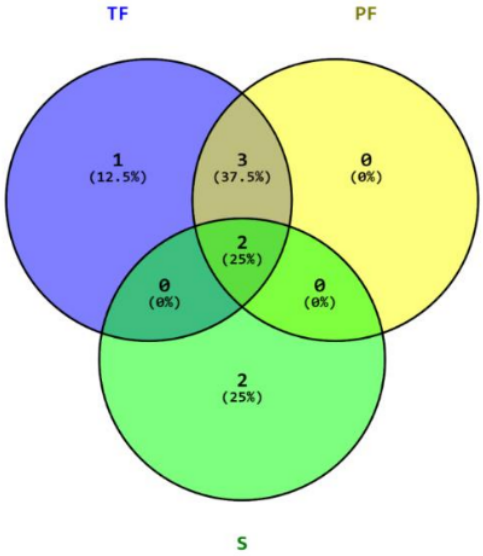
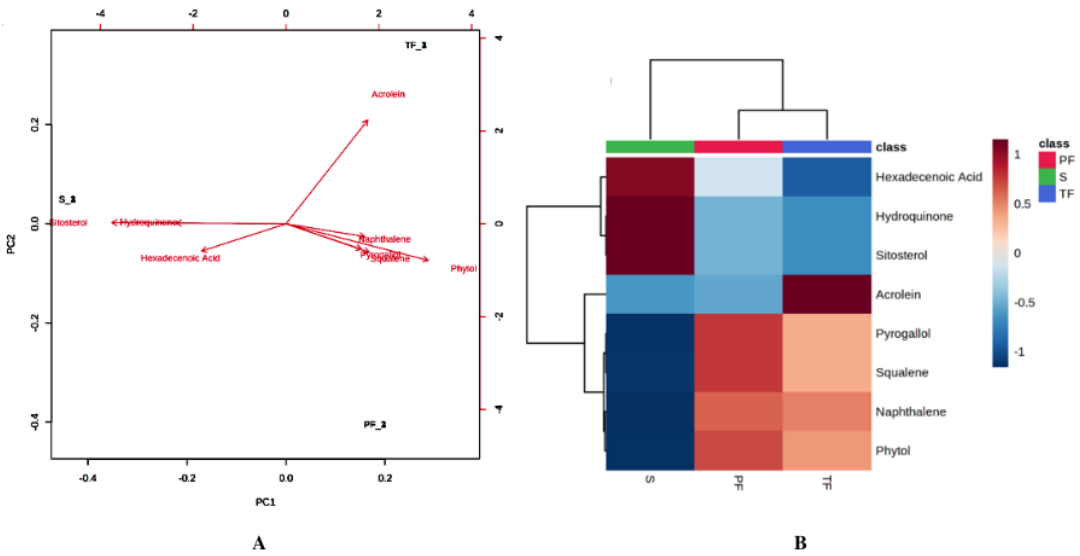


Figure 2. The comparison of identified metabolites in each *Vitex negundo* extract. TF: trifoliate leaves extract, PF: pentafoliate leaves extract, S: stem extract

The compositions of the detected metabolites in each extract were analyzed using biplot analysis to determine the relationship between the detected metabolites and extract sources in *V. negundo*. Biplot results showed that the three extracts had different metabolite compositions. It was indicated by the position of the three extracts, which are separated from each other. In addition, these results also indicated the presence of several metabolites that can be used as markers of *V. negundo* extract. Acrolein was indicated as a metabolite marker in the TF extract, while sitosterol can be used as a metabolite marker in the S extract (Figure 3A). Some of the metabolites contained in the TF and PF extracts formed into one cluster, but this analysis could not properly explain the differences between the TF and PF extracts. Therefore, a heatmap-clustering analysis was carried out to investigate the characteristics of metabolites in *V. negundo* extracts. The results showed two main clusters of the three extracts based on their metabolite profiles. The TF and PF extracts were in the same cluster, while the S extract was separated from two other extracts. The TF and PF extract had similar characteristics based on the naphthalene content (Figure 3B). In the TF extract, acrolein was suggested as a metabolite marker, and it was in accordance with biplot analysis. Hydroquinone and sitosterol were suggested as metabolite markers in S extract, and these two metabolites were absent in both TF and PF extracts.

The correlation between the identified metabolites in each extract and its antioxidant activity was analyzed by Pearson's correlation. The results showed that each metabolite had a moderate correlation with antioxidant activity. Five metabolites, including acrolein, hydroquinone, sitosterol, naphthalene, and hexadecenoic acid, showed a negative correlation, so the antioxidant activity increased by decreasing the concentration of these metabolites. Meanwhile, three metabolites, i.e., pyrogallol, squalene, and phytol, showed a positive correlation. Therefore, increasing the concentration of these metabolites increases the antioxidant activity of the *V. negundo* extract (Table 2).



A B

Figure 3. A. Biplot analysis. B. Heatmaps clustering analysis for metabolites in each *Vitex negundo* extract. TF: trifoliate leaves extract, PF: pentafoliate leaves extract, S: stem extract

Table 2. Pearson's correlation coefficient between identified metabolites of *Vitex negundo* extracts and its antioxidant activity

Metabolites	Pearson's correlation coefficient
Acrolein	-0.64
Hydroquinone	-0.56
Sitosterol	-0.56
Naphthalene	-0.49
Pyrogallol	0.58
Squalene	0.58
Phytol	0.51
Hexadecenoic acid	-0.49

Discussion

The antioxidant activity of V. negundo extract was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method. The DPPH molecule is a free radical molecule that is relatively stable at room temperature. Therefore, the purple color of the DPPH molecule will turn yellow in the presence of antioxidant compounds. There are two mechanisms of the antioxidant reaction using DPPH. The first mechanism is the electron or H atoms donation from the antioxidant molecules to the DPPH molecule so that the molecule becomes neutral. As a result, the antioxidant molecule loses electrons and possibly becomes a new radical molecule. Secondly, the antioxidant molecules and DPPH share electrons (Blois 1958).

The low antioxidant activity of the samples compared to ascorbic acid could be caused by the samples as crude extracts contained a lot of metabolites, and not all of these metabolites had antioxidant activity. It was even possible that other metabolites inhibited the antioxidant reaction between the extract and the DPPH molecule. Therefore, it might be influenced by the physical-chemical properties of these metabolites (Gan et al. 2017; Dillak et al. 2019). Nevertheless, all samples had IC_{50} values of less than 50 ppm. So, the extract could inhibit 50% of DPPH free radicals at a concentration of less than 50 ppm. The IC_{50} in this study was higher than the IC_{50} of methanol and hexane extracts of *V. negundo*. The IC_{50} values of methanol and hexane extracts of *V. negundo* from Panchtahr district, Nepal, were 38-73 ppm (Koirala et al. 2020). However, the antioxidant activity in this study was lower than the water extract of *V. negundo* leaves from Aurangabad, India, with an IC_{50} value of 5.77 ppm (Fatema et al. 2019). The difference in results might be due to the influence of the growing environment and the solvent used in the extraction process. Each organic solvent has different characteristics, especially its polarity. Polar metabolites are extracted with polar solvents, while non-polar metabolites are extracted with non-polar solvents. Therefore, the different solvents in extraction can affect the metabolites obtained in the extraction process and the bioactivity of these metabolites. We used ethanol 90% as a solvent to adapt the traditional extraction for *V. negundo*'s leaves, using boiled water as a polar solvent.

The variation in antioxidant activity of the three extracts could be due to the different parts of the plant used (Figure 1). Each plant's parts, such as roots, stems, and leaves, had different physiological functions, so the metabolites' composition was also different (Table 1, Figure 3). For example, leaves are organs that have a major function in plant growth and development. Photosynthesis is the main process in producing energy that occurs in the

leaves, while the stems are an organ that has the main function of supporting plant structures and transporting nutrients (Heldt and Piechulla 2011).

Multivariate analysis showed that the three extracts had different metabolite content. The analysis results showed the presence of 2 main clusters consisting of stem extract and leaves extract (Figure 3). The differences in metabolite content might be due to differences in the part of the plant used as the extracted source. There are differences between the TF and PF extract clusters, even though many of the same metabolites are present in both extracts. *V. negundo* used in this study has trifoliate and pentafoliate leaves. Differences in leaf character allow for variations in leaf phytochemicals so that they might have different bioactivity. It was proven in the biplot results that acrolein is a marker metabolite in the TF extract. The study of leaf character is very important to determine a plant's identity and the use of leaves related to their phytochemical content (Salvaña et al. 2019).

Correlation analysis showed a moderate correlation between antioxidant activity and the identified metabolites in each extract, although there were metabolite markers in extracts based on biplot results (Table 2). It indicates that the antioxidant activity of the leaf and stem extracts of *V. negundo* was influenced by the interaction of the metabolites contained in the extracts. Therefore, this result suggested that no dominant metabolite affects the antioxidant activity in the extract. The results indicated the variation of antioxidant activity. The differences in metabolite composition in extract result in different characteristics. It was suggested that metabolite differences cause differences in antioxidant activity. However, the antioxidant activity of the extract was the result of the interaction of the metabolites contained in the extract without any dominant metabolite affecting antioxidant activity.

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Short Communication: Antioxidant activity and metabolite profiles of leaves and stem extracts of Vitex negundo

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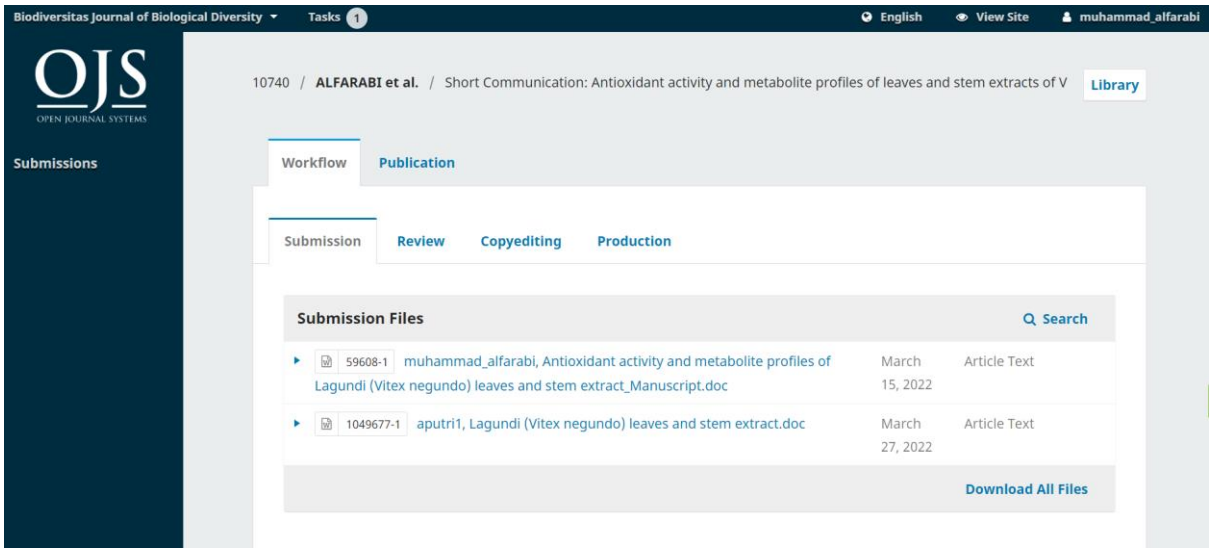

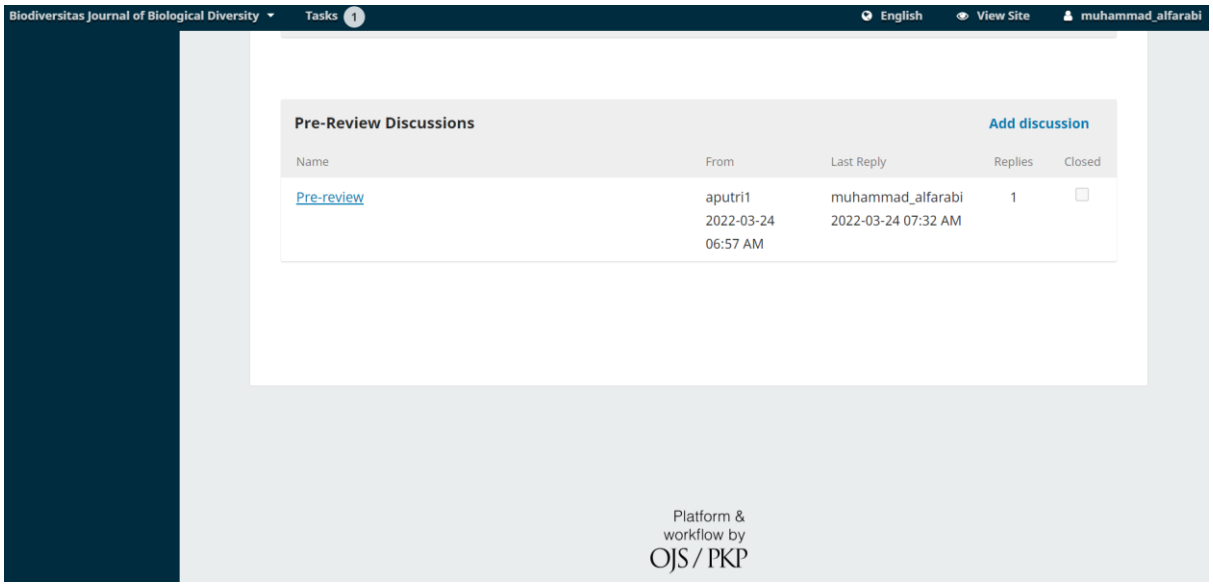

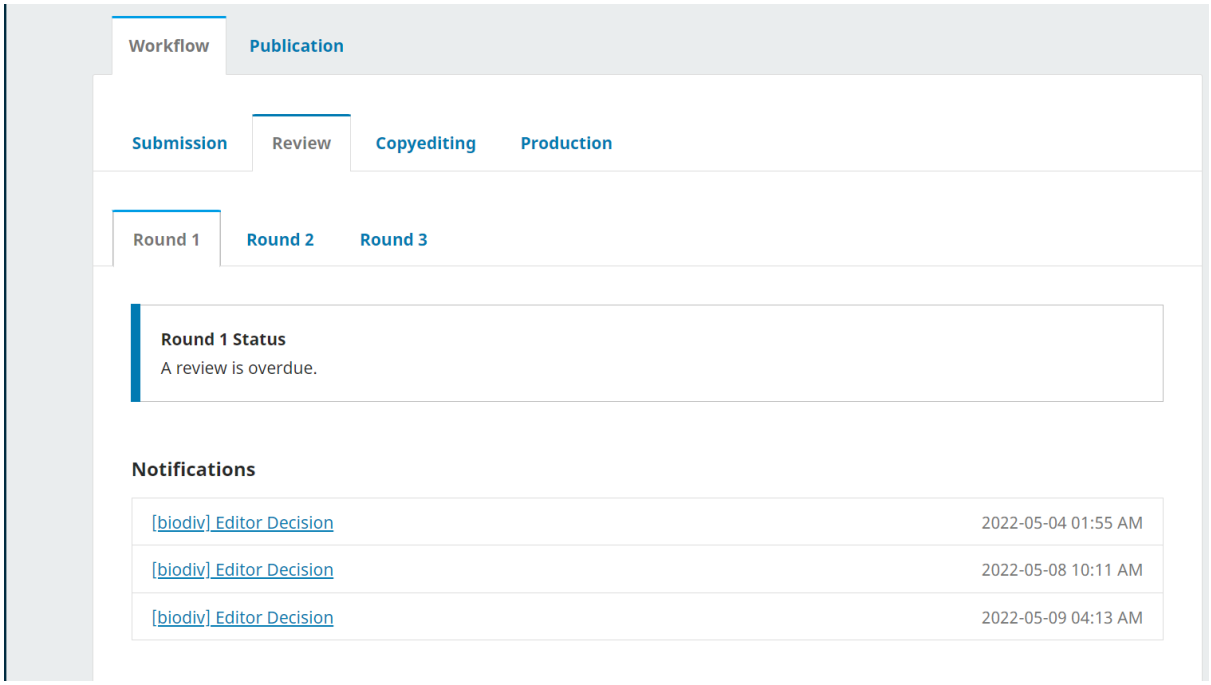
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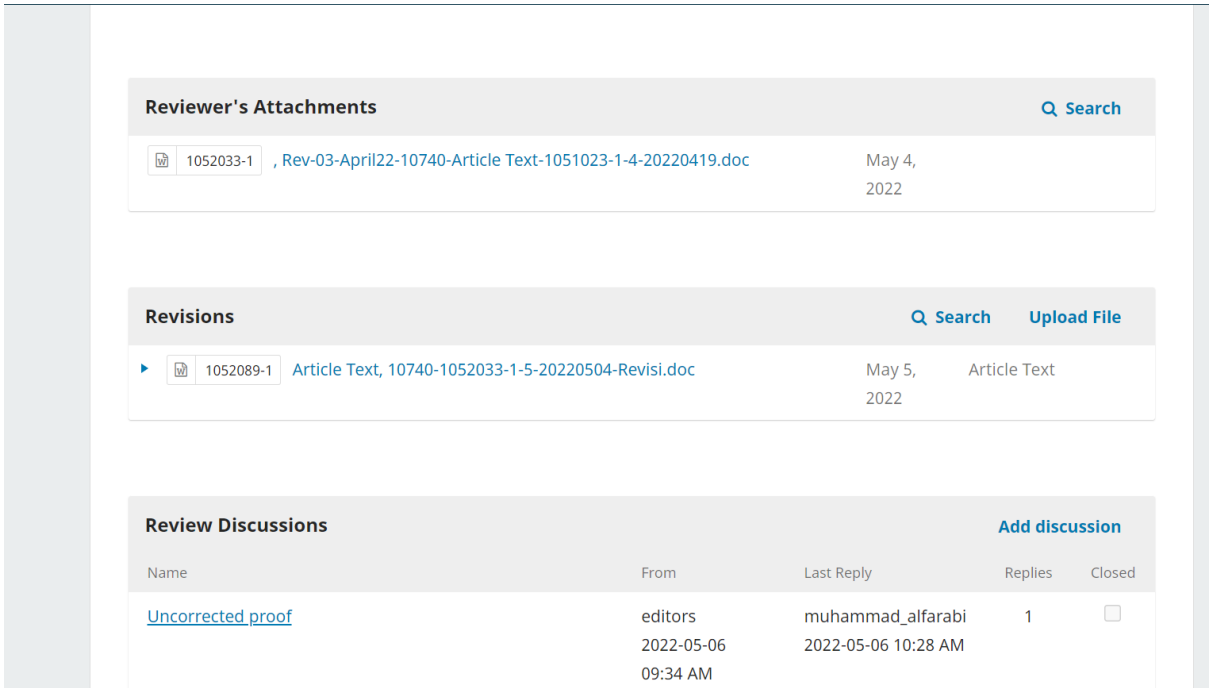
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Short Communication:
Antioxidant activity and metabolite profiles of leaves and stem extracts of Lagundi (*Vitex negundo*)

Abstract. Many plant species could be used as natural sources of antioxidants, one of which is *Vitex negundo* which grows widely in Southeast and South Asia. Due to the wide distribution of growth areas, so different habitats could be a factor causing variations in *V. negundo* bioactivity, especially antioxidant activity. In addition to environmental factors, extract derived from different plant parts could also contribute to variations in the content of metabolites that affect bioactivity. The objective of this study was to determine the antioxidant activity of leaf and stem extracts of *V. negundo* and evaluate the metabolites contained in the extracts. Antioxidant activity analysis was performed using the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), and GC-MS analysis was used to identify bioactive metabolites. Leaf and stem extract of *V. negundo* showed antioxidant activity directly proportional to the increase in extract concentration. A total of 8 metabolites were identified from the three extracts, i.e., acrolein, hydroquinone, sitosterol, naphthalene, pyrogallol, squalene, phytol, and hexadecenoic acid. There were correlations between antioxidant activity and the metabolites contained in the three extracts. It is suggested that the bioactivity of the extract is due to the interaction of these metabolites.

Keywords: *Vitex negundo*, phytochemical, bioactivity, metabolite profiling, natural product

Running title: Antioxidant activity from *Vitex negundo*

INTRODUCTION

Stable molecules naturally have several paired electrons, but free radical molecules have a single electron in their valence position. Free radicals can be generated from organic and inorganic chemical reactions (Wang et al., 2021). Free radicals have a dual role in the cell, i.e., part of a biochemical reaction or cause cell damage. The presence of unpaired electrons causes free radicals to be very reactive in obtaining electron pairs from other molecules such as reactive oxygen species (ROS). These molecules react very easily by oxidizing other molecules such as lipids (Shadyroa et al., 2019). The reaction of free radicals can damage the cell membrane and even cause cell death. Cell or tissue damage due to free radicals contribute to several diseases such as cancer, premature aging, and other degenerative diseases. These diseases are triggered by the uncontrolled number of free radicals (Halliwell 2020).

However, free radicals (O_2^-) can also indicate infection caused by bacteria or viruses. Hosts infected by bacteria produce O_2^- as a defense system with an antibacterial effect. Free radicals generated from viral infections may cause pathological consequences, such as in mice infected with the influenza virus and COVID-19 patients (Camini et al. 2017; Wu 2020). However, free radicals in cells or tissues do not always result in detrimental effects because free radicals also help in tissue homeostasis, signal regulation, and control of cell death (Harris and DeNicola 2020). Therefore, controlling free radical levels is very important in the process of cell life.

Antioxidants are molecules that can neutralize free radicals by donating electrons to radical molecules so that the free radicals become relatively stable molecules. Based on the source, antioxidants can be categorized as endogenous and exogenous antioxidants. Endogenous antioxidants are antioxidants produced by cells as enzymes or cofactors that can eliminate ROS, while exogenous antioxidants can be metabolites from plants such as vitamins, flavonoids, alkaloids, and other phenolic compounds (Chan and Chan 2015; Fleming and Luo 2021).

Lagundi (*Vitex negundo*) belongs to the Verbenaceae family that can be used as an antioxidant source. It grows mostly in low to moderate lands and is widespread from China to Southeast Asia, such as Indonesia and the Philippines (Ahuja et al., 2015; Boy et al., 2018). Boiling leaves of Lagundi have been used as a traditional medicine in the Philippines to treat coughs, indigestion, and skin diseases. The Philippine Department of Health has also promoted this plant as an antitussive and anti-inflammatory (Bautista et al., 2015). According to Gill et al. (2018), *V. negundo* contains polyphenols such as flavonoids that have potential as a medicinal raw material for cardiovascular disease, cancer, aging, diabetes mellitus, and neurodegenerative diseases.

Because this plant also grows in Indonesia, such as Jakarta, it is interesting to study the properties and contents of *V. negundo*. Differences in weather and soil conditions affect the metabolite produced by the plant so that it can affect bioactivity. This research aimed to assess the antioxidant activity and determine the metabolite content of *V. negundo* grow in Jakarta, Indonesia. The results of this study could be used as the scientific information to develop Lagundi as a natural antioxidant.

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MATERIALS AND METHODS

Collection of plant material and extraction

Leaves and stems of *Vitex negundo* were collected from Jakarta, Indonesia (6°15'43.4"S 106°52'39.9"E) and identified at Herbarium Bogoriense, Research Center for Biology, LIPI, Indonesia. The samples used for extraction were 125 g of trifoliate leaves (TF), 125 g of pentafoliate leaves (PF), and 250 g of the fresh stem (S). Each sample was extracted using the maceration technique with 90% ethanol as a solvent at room temperature. After 72 h, the filtrate was filtered with Whatman filter paper and evaporated at 60°C with a rotary evaporator (Buchi R-100). The extracts of leaves and stems were analyzed for their antioxidant activity and chemical content by the GC-MS method.

Antioxidant activity assay

Determination of antioxidant activity was performed using DPPH (2,2-diphenyl-1-picrylhydrazyl) (Sigma) assay (Yuningtyas et al., 2021). One ml of sample solutions (10, 50, 100, 150, and 200 ppm) were mixed with 1 ml of 0.1 mM DPPH. Furthermore, the solutions were incubated for 30 min at room temperature. The absorbance of solutions was measured at 517 nm. The DPPH without samples solution was used as a negative control, and the positive control was ascorbic acid (Sigma). The percentage of DPPH reduction was calculated as follows:

$$\%\ inhibition = \frac{absorbance\ negative\ control - absorbance\ samples}{absorbance\ negative\ control} \times 100\%$$

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The chemical compounds of leaves and stem extract were analyzed using Gas Chromatography-Mass Spectrometry (GC-MS) at Laboratory for Jakarta Regional Health, Jakarta, Indonesia. Five µl extracts were injected in a GC-MS (Model 7890, Agilent Technologies, Palo Alto, CA, USA) equipped with an autosampler (Model 7693, Agilent Technologies, Palo Alto, CA, USA). This instrument was connected to a Mass Selective Detector and a Chemstation Data System (Model 5975C inert MSD with Triple-Axis Detector, Agilent Technologies, Palo Alto, CA, USA). It is also equipped with an HP ultra 2 capillary column (0.11 µm). The temperature of the injector, ion source, interface, and quadrupole was set up respectively as follows 250°C), 230°C, 280°C, and 140°C. Helium with a 1.2 ml/min flow rate was used as the gas carrier. The mass spectrum was detected in a mass-to-charge range of 20–500 m/z. Metabolite identification was performed based on the Wiley W8N08.L database.

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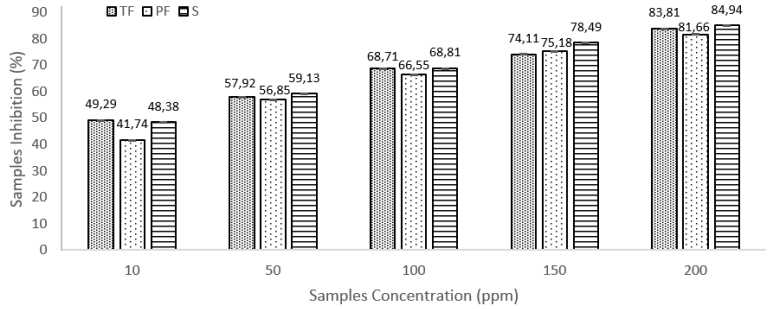

Figure 1. Antioxidant activity of leaves and stem of *Vitex negundo* extracts at different concentrations. Different scores on the top of the bar chart showed an inhibition percentage of each sample. TF (trifoliate leaves extract), PF (pentafoliate leaves extract), S (stem extract).

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The results of GC-MS analysis of three extracts showed 8 identified metabolites consisting of aldehyde, phenol, phytosterol, aromatic, benzene, terpenoid, and fatty acids groups. There were 6 identified metabolites in the TF extract, 5 identified metabolites in the PF extract, and 4 identified metabolites in the S extract. The percentage of each metabolite was varied. The highest metabolite content in TF and PF extracts was phytol, while the highest content in the S extract was sitosterol. Several metabolites were detected in more than 1 extract (Table 1). Phytol and hexadecenoic acid were detected in all extracts. Three metabolites were detected in the TF and PF extracts, i.e., naphthalene, pyrogallol, and squalene. Acrolein was only detected in the TF extract, While hydroquinone and sitosterol were only detected in the S extract (Fig. 2).

Table 1. Identified metabolites in plant part of *Vitex negundo* extract

Compounds	Group compounds	Concentration (%)		
		TF	PF	S
Acrolein	Aldehyde	15.24		
Hydroquinone	Phenolic	-	-	4.76
Sitosterol	Phytosterol	-	-	19.04
Naphthalene	Aromatic	2.19	1.83	-
Pyrogallol	Benzene	1.95	2.06	-
Squalene	Terpenoid	2.59	2.76	-
Phytol	Terpenoid	25.33	23.14	6.77
Hexadecenoic acid	Fatty acid	6.03	7.26	13.47

Note: TF (trifoliate leaves extract), PF (pentafoliate leaves extract), S (stem extract) (-), not detected

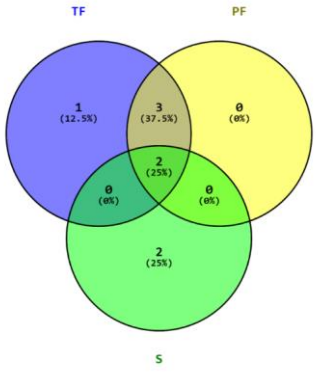

Figure 2. The comparison of identied metabolites in each *Vitex negundo* extract. TF (trifoliate leaves extract), PF (pentafoliate leaves extract), S (stem extract)

The compositions of the detected metabolites in each extract were analyzed using biplot analysis to determine the relationship between the detected metabolites and extract sources in *Vitex negundo*. Biplot results showed that the three extracts had different metabolite compositions. It was indicated by the position of the three extracts, which are separated from each other. In addition, these results also indicated the presence of several metabolites that can be used as markers of *Vitex negundo* extract. Acrolein was indicated as a metabolite marker in the TF extract, while sitosterol can be used as a metabolite marker in the S extract (Fig. 3A). Some of the metabolites contained in the TF and PF extracts formed into one cluster, but this analysis could not properly explain the differences between the TF and PF extracts. Therefore, a heatmap-clustering analysis was carried out to investigate the characteristics of metabolites in *Vitex negundo* extracts. The results showed two main clusters of the three extracts based on their metabolite profiles. The TF and PF extracts were in the same cluster, while the S extract was separated from two other extracts. The TF and PF extract had similar characteristics based on the naphthalene content (Fig. 3B). In the TF extract, acrolein was suggested as a metabolite marker, and it was in accordance with biplot analysis. Hydroquinone and sitosterol were suggested as metabolite markers in S extract, and these two metabolites were absent in both TF and PF extracts.

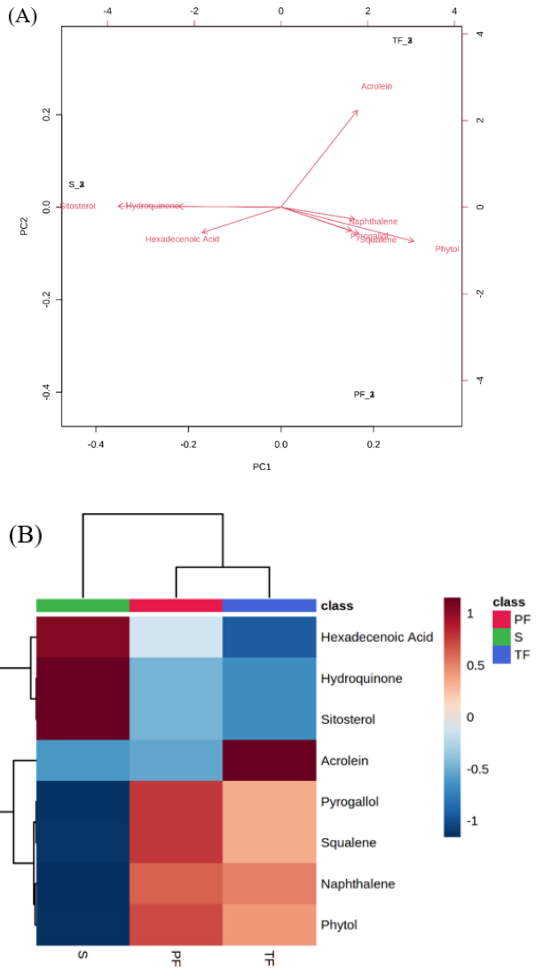

Figure 3. Biplot analysis (A) and heatmaps clustering analysis (B) for metabolites in each *Vitex negundo* extract. TF (trifoliate leaves extract), PF (pentafoliate leaves extract), S (stem extract).

The correlation between the identified metabolites in each extract and its antioxidant activity was analyzed by Pearson's correlation. The results showed that each metabolite had a moderate correlation with antioxidant activity. Five metabolites, including acrolein, hydroquinone, sitosterol, naphthalene, and hexadecenoic acid, showed a negative correlation, so the antioxidant activity increased by decreasing the concentration of these metabolites. Meanwhile, three metabolites, i.e., pyrogallol, squalene, and phytol, showed a positive correlation. Therefore, increasing the concentration of these metabolites increases the antioxidant activity of the *V. negundo* extract (Table 2).

Table 2. Pearson's correlation coefficient between identified metabolites of *Vitex negundo* extracts and its antioxidant activity

Metabolites	Pearson's correlation coefficient
Acrolein	-0.64
Hydroquinone	-0.56
Sitosterol	-0.56
Naphthalene	-0.49
Pyrogallol	0.58
Squalene	0.58
Phytol	0.51
Hexadecenoic acid	-0.49

Discussion

The antioxidant activity of V. negundo extract was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method. The DPPH molecule is a free radical molecule that is relatively stable at room temperature. Therefore, the purple color of the DPPH molecule will turn yellow in the presence of antioxidant compounds. There are two mechanisms of the antioxidant reaction using DPPH. The first mechanism is the electron or H atoms donation from the antioxidant molecules to the DPPH molecule so that the molecule becomes neutral. As a result, the antioxidant molecule loses electrons and possibly becomes a new radical molecule. Secondly, the antioxidant molecules and DPPH share electrons (Blois, 1958).

The low antioxidant activity of the samples compared to ascorbic acid could be because the samples still being crude extracts that contained a lot of metabolites, and not all of these metabolites had antioxidant activity. It was even possible that other metabolites inhibited the antioxidant reaction between the extract and the DPPH molecule. It might be influenced by the physical-chemical properties of these metabolites (Dillak et al., 2019; Gan et al., 2017). Nevertheless, all samples had IC_{50} values of less than 50 ppm. Therefore, the extract could inhibit 50% of DPPH free radicals at a concentration of less than 50 ppm. When reviewed with other extracts of *V. negundo* leaves and branches (using methanol and hexane for extraction), the antioxidant activity of the extract in this study was higher than in the previous study. The samples have an IC_{50} value of 38-73 ppm and used fresh *V. negundo* from Panchtahr district, Nepal (Koirala et al. 2020). However the antioxidant activity of the samples in this study was lower than the water extract of *V. negundo* leaves from Aurangabad, India, with an IC_{50} value of 5.77 ppm (Fatema et al., 2019). The difference in results might be due to the influence of the growing environment and the solvent used in the extraction process

The variation in antioxidant activity of the three extracts could be due to the different parts of the plant used (Fig. 1). Each plant's parts, such as roots, stems, and leaves, had different physiological functions, so the metabolites' composition was also different (Table 1 and Fig. 3). For example, leaves are organs that have a major function in plant growth and development. Photosynthesis is the main process in producing energy that occurs in the leaves, while the stems are an organ that has the main function of supporting plant structures and transporting nutrients (Heldt and Piechulla, 2011).

Multivariate analysis showed that the three extracts had different metabolite content. The analysis results showed the presence of 2 main clusters consisting of stem extract and leaves extract (Fig. 3). The differences in metabolite content might be due to differences in the part of the plant used as the extracted source. There are differences between the TF and PF extract clusters, even though many of the same metabolites are present in both extracts. *Vitex negundo* used in this study has trifoliate and pentafoliate leaves. That differences in leaf character allow for variations in leaf phytochemicals so that they might have different bioactivity. It was proven in the biplot results that acrolein is a marker metabolite in the TF extract. The study of leaf character is very important to determine a plant's identity and the use of leaves related to their phytochemical content (Salvaña et al., 2019).

Correlation analysis showed a moderate correlation between antioxidant activity and the identified metabolites in each extract, although there were metabolite markers in extracts based on biplot results (Table 2). It indicates that the antioxidant activity of the leaf and stem extracts of *V. negundo* was influenced by the interaction of the metabolites contained in the extracts. Therefore, this result suggested that no dominant metabolite in the extract affects the antioxidant activity. The results indicated the variation of antioxidant activity. The differences in metabolite composition in extract result in different characteristics. It was suggested that metabolite differences cause differences in the antioxidant activity. However, the antioxidant activity of the extract was the result of the interaction of the metabolites contained in the extract without any dominant metabolite affecting antioxidant activity.

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Commented [u3]: Why the type of solvent used in the extraction is important in extracting antioxidant compounds?? Why the extraction in this study used ethanol 90%?? Please, add discussion

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