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Identification of Active Compounds and Testing the Antioxidant Properties of Neem Leaf Extract

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Abstract. Due to its important role, the use of antioxidant compounds has recently grown rapidly. Antioxidant compounds are known to be able to inhibit auto oxidation through radical scavenging mechanism, by donating one unpaired electron in free radical which leads to a reduced number of free radicals. In Indonesia there are many of medicinal plants that have active chemical compounds which are likely to be potential sources of antioxidants, which is neem (*Azadirachta indica* A.Juss). This plant has been widely used in various fields including natural pesticides (biopesticide) and natural medicine. The purpose of this research was to identify the active chemical compounds in neem leaves extracts and determine the antioxidant activity of neem leaves and the effective solvents in extracting active compounds of neem leaves. The materials used in this study were Neem leaves (*Azadirachta indica* A.Juss) from Yogyakarta. There were 5 types of solvents used, including: Ethyl acetate, Ethyl acetate-ethanol, Ethanol, Ethanol-water and Water. Neem leaves is a potential resources for natural antioxidant. In addition to obtaining highest yield using Ethanol-Water as a solvent during the extraction process, this solvent also results better characteristic of extract than 4 other solvents. The properties of the extract that produced by extraction using Ethanol-Water is 2.067 g GAE/g extract, yield 1.39%, flavonoid 0.326 mg QE/g extract, antioxidant activity 226.118 ppm and reducing power 0.778 g BHT/g extract. Therefore, it is recommended that Ethanol-Water as a solvent for the extraction of active compound on neem leaves.

INTRODUCTION

Many research on antioxidant has been performed currently both in the field of food and health. Due to its important role, the use of antioxidant compounds has recently grown rapidly. The antioxidant can be obtained naturally or synthetically. Synthesis antioxidants have high effectiveness but are unsafe for health. Therefore, its users are closely monitored in various countries. Awareness of food security also supports the search and use of natural food sources. Therefore, it is necessary to look for safer natural antioxidant sources than synthetic antioxidant to be developed, for example, antioxidants derived from spices, fruits, or plants such as vitamin C, vitamin E, carotene, phenol acids, polyphenol, and flavonoid. These compounds are known to be able to inhibit auto oxidation through radical scavenging mechanism, by donating one unpaired electron in free radical which leads to a reduced number of free radicals [1]. Based on the studies conducted, some medicinal plants in Indonesia have active chemical compounds which are likely to be potential sources of antioxidants, which is neem (*Azadirachta indica*

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A.Juss). This plant has been widely used in various fields including natural pesticides (biopesticide) and natural medicine.

Neem extracts, both in seeds, stems, flowers, and leaves are known to contain active compounds such as azadirachtin, nimbin, nimbinena, desacetyl nimbinase, nimbandial, and salanin which belong to the terpenoid group. Additionally, there are also quercetin, rutin, and gallic acied which are part of the phenolic group. The compound is known to be spermicidal, antiviral, antibacterial, antiprotozoal, insecticidal, insect repellent, antifungal, and antioxidative [2]. The use of neem plants is currently developing in the field of food and health. In Thailand, neem's potential as an antioxidant has been studied, where neem leaves are cooked and made into daily foods mixed with sauce as healthy food. Research conducted by [3] showed that Siamese neem leaves (*Azadirachta indica* A.Juss var. *siamensis* Valeton), neem (*Azadiratcha indica* A.Juss), and marrango trees (*Azadirachta excelsa*) has relatively high content of total phenol and has free radical scavenging power tested by the Fremy's salt reduction and ESR detection method. Based on the existing research, further research on the antioxidant potential of neem leaves in the field of food and its application is necessary considering that food products are a system with greatly varied conditions influenced by pH, ionic strength, temperature, light, and processing, therefore the added antioxidant should be stable against such condition [3].

In the current study, the antioxidant potential of neem leaves will be tested. Antioxidant activity of neem leaves is influenced by the composition of active compounds in it, therefore extraction with several solvents such as ethyl acetate, alcohol, and water and its combinations are performed to find out which solvent is effective to extract the active compounds in neem leaves.

From the results of this research, it is expected to find out the active extraction of neem leaves and provide information of antioxidant activity in neem leaves so that it can be used further as functional foods or natural antioxidants used in processing food products. The research problems studied are how antioxidant activities in neem leaves are so that it is necessary to identify the active chemical compounds and solvent selections in the extraction process. Therefore, the purpose of this research was to identify the active chemical compounds in neem leaves extracts and determine the antioxidant activity of neem leaves and the effective solvents in extracting active compounds of neem leaves.

MATERIALS AND METHODS

Research Materials

The materials used in this study were Neem leaves (*Azadirachta indica* A.Juss) in Yogyakarta. The leaves were picked in the morning and were collected from several branches (10 leaves from the tip of the branch). Fresh picked neem leaves were cleaned from dust and dirt and then dried in the cabinet dryer in ± 50◦C until the water content reached ± 5% (approximately 8 hours of drying). The dried leaved were then made into powder until all of them passed through 40 mesh. Neem powder was then packaged in aluminium foil and stored at room temperature until it is used for extraction or as material stock for research.

Research Methods

The stages of research performed including preparation of neem leave extract and chemical analysis, as well as antioxidant activity testing are as follow: Fresh neem leaves were dried using Cabinet Dryer (± 50˚C). The dried sample was grinded into powder and sieved (40 mesh). Extraction was conducted at ±90˚C using the 5 different solvents, including: Ethyl acetate, Ethyl acetate-ethanol, Ethanol, Ethanol-water and Water. After the extraction process, the solvents were evaporated by rotary vacuum evaporator (40^{o} C), therefore, extract obtained from each treatment. Variables evaluated were yield of each treatment, content of phenol and flavonoid, antioxidant activities, and reduction power test. The data was analyzed by One way ANOVA and followed by DMRT.

RESULTS AND DISCUSSION

Neem Leaves Extract

Extraction of powdered neem leaves was performed with the soxhlet method. There are some advantages of using the soxhlet method, including not requiring large quantities of solvents and able to extract ingredients continuously. Neem leaves powder was extracted with three solvents of different polarity levels, namely ethyl acetate, ethanol, and water and its combination (ethyl acetate-ethanol and ethanol-water) so that all active components in neem leaves, both non-polar, semi-polar and polar, can be extracted. Organic compounds from plant parts have different affinity to the polarity nature of the solvent used to extract. To extract mainly antioxidant compounds contained in plant tissues, some solvents need to have different levels of polarity. The level of polarity will determine the results of extraction and antioxidant activity contained in the extract

Neem leaf extract obtained from soxhlet extraction showed different colors for each solvent used. Water extract and water-ethanol extract were brownish yellows in color, while the ethanol extract was blackish-green brown and ethyl acetate extract was blackish green and ethyl acetate extract was deep green. This brownish yellow to blackish green brown is thought to be the contribution of extraction of natural dyes (brownish yellow), especially from phenol or polyphenol polymers such as tannin, melanin, lignin and or quinone and a small number of colored alkaloids. Quinone pigments in plants are known to have colors ranging from yellow to dark brown. According to [4], ethyl acetate dissolves components from alkaloid and glycosides groups, whereas ethanol generally can extract components and a little essential oil and water generally dissolve components from sugars, amino acids, and glycosides groups.

Yield and Water content of Neem Leaves Extract

Preparation of fresh neem leaves into dry form or paste is to reduce the water content so that samples can be stores longer, last longer, and avoid microbe contamination so that its active components remain stable during testing. The yield and water content of neem leaves from fresh to powder from are showed in Table 1 while the yield of neem extract is calculated from fresh neem leaves such as in the Table 2

TABLE 1. The Yield and Water Content of Fresh Neem Leaves, Dried Neem Leaves and Powder Neem Leaves

No	Sample	Yield (%w/w)	Water content (%)
1	Fresh neem leaves	100	70.52 ± 0.44
2	Dried neem leaves	39.02	8.44 ± 0.05
3	Powder neem leaves	37.77	9.25 ± 0.25

The moisture content of fresh neem leaves, dry neem leaves, and neem leaves powder are significantly different in the statistical test with (α=0.05). Dry neem leaves have the lowest water content. After turned into powder form, the water content is slightly increased because it becomes hygroscopic.

The yield of ethyl acetate – ethanol extract was not significantly different from other extracts, while ethanol – water extract was significantly different with ethyl acetate extract, ethanol extract, and water extract but not significantly different from ethyl acetate – ethanol extract.

The use of ethyl acetate, ethanol, water, and its combination in this research was to obtain active components from neem leaves, both polar, semi polar, and non-polar which have the potential to be antioxidant. The compounds containing antioxidants tend to dissolve in semi-polar solvents, while terpenoid compounds including azadirachtin have functional groups that are polar and non-polar in nature so they may be extracted in polar or non-polar solvents.

TABLE 2. Yield of Neem Leaves Extract

No	Sample	Yield(%w/w)
1	Ethyl acetate extract	$0.93^{a} \pm 0.17$
2	Ethyl acetate-etanol extract	$1.11^{ab} \pm 0.02$
3	Etanol extract	$0.92^{a} \pm 0.03$
4	Etanol-water extract	$1.39^{b} \pm 0.38$
5	Water extract	$0.90^{a} \pm 0.02$

Based on data from Table 2 it is seen that neem leaves extracted using water solvents provide the lowest yield compared to other solvent extracts, while the ethanol-water mixture extract provide the highest yield. The yield of ethyl acetate extract, ethyl acetate – ethanol extract, ethanol extract, and water extract does not provide significant differences in statistic test with (α=0.05)

The difference in yield of extraction results is related to the suitability of the polarity range of the extracted compounds and their solvents. The greater the dielectric constant of a solvent, the more polar the solvent is. Most of the components contained in neem leaves are in the range of the polarity index of ethanol-water mixtures which tend to be semi-polar. However, the yield obtained has not been able to show both quantitatively or qualitatively the active compounds of neem leaves that have the potential as antioxidants because the solvents that produce the largest yield are not necessarily the ones that have the greatest antioxidant activity, therefore further testing of antioxidant activities are performed.

Ethyl acetate solvents which tend to be non-polar can dissolve non-polar compounds such as chlorophyll, fat, and wax in neem leaves. In addition, compounds can also be dissolved such as essential oils, steroids, triterpenoids, carotenoids, and high molecular weight fatty acids. Ethyl acetate solvents can dissolve chlorophyll, therefore the sample extract obtained is still green because a lot of chlorophyll extracted inside. Ethanol solvents will dissolve phenol compounds, flavonoids, and polar alkaloids [4]. Ethanol has a polarity that is relatively suitable for most of the compounds contained in plants, so ethanol is a very commonly used solvent in the extraction process. In the results of the yield above, it can be seen that ethanol can dissolve relatively a lot of components in neem leaves from the yield compared to ethyl acetate and water. If ethanol is mixed with water in a ratio of 1: 1, the yield obtained is higher which means that many chemical compounds dissolve in the water and ethanol polarity index.

In this study, it was shown that the use of mixed solvents, both a mixture of ethanol and water as well as a mixture of ethyl acetate and ethanol can increase yield in the extraction process compared to a single solvent use.

Research on the comparison of extraction processes and types of solvents on neem leaves had also been carried out by [5]. The results showed that water extract had a yield of 10.789% with reflux method and 6.243% with maceration method, while ethanol extract had a yield of 6.008% with reflux method and 2.431% with maceration method.

Determination of the Total Phenol Content in Neem Leaves Extract

Determination of total phenol content aims to find out the content of phenolic compounds in neem leaf extract and see the correlation between antioxidant activity and the total phenolic content. In the current research, the total phenolic compounds content was expressed with gram equivalent gallic acid because the chemical structure of phenolic compounds contained in ethyl acetate extract, ethyl acetate-ethanol extract, ethanol extract, ethanol-water extract, and water extract was known. Gallic acid is an acid with 3 phenolic hydroxy groups, therefore, it was used to determine the content of phenolic compounds. In addition, gallic acid is available in high purity, stable, and relatively cheaper prices [6]. The results of total phenol content determination of each neem leaf extract can be seen in Table 3.

TABLE 3. Total Phenol Content in Neem Leaves Extract

No	Sample	Total Phenol content (g GAE/g extract)
1	Ethyl acetate extract	$0.874^{a} \pm 0.056$
2	Ethyl acetate-etanol extract	$0.926^{a} \pm 0.046$
3	Etanol extract	$1.538^{b} \pm 0.081$
4	Etanol-water extract	$2.067^{c} \pm 0.032$
5	Water extract	$0.932^{a} \pm 0.042$

Table 3 shows that the total phenol as gallic acid in the ethanol-water extract is the highest compared to the other extracts of 2.067 g EAG/g extract. Ethyl acetate extract had the lowest total phenol which of 0.874 g EAG/g extract and was not significantly different from ethyl acetate-ethanol extract and water extract based on statistical tests with ($\alpha = 0.05$).

It is seen that ethanol solvents and combinations tend to extract phenol compounds in neem leaves, especially in semi-polar-polar ethanol-water mixture extracts which can extract relatively high phenol compounds from single solvents on ethanol extract. The mixture of ethyl acetate-ethanol was able to extract more phenol compounds than a single solvent on ethyl acetate extract or water extract even though it was smaller than the ethanol-water extract because of its non-polar-semi-polar polarity.

The total phenol content increases when the polarity of the solvent increases, especially in ethanol-water mixture solvents indicating that non-polar solvents are less effective for the extraction of phenol compounds . Phenol compounds are the most effective types of antioxidants and have high antioxidant activity [7].

Determination of Total Content of Flavonoid in Neem Leaves Extract

Determination of total content of flavonoid aims to determine the total content of flavonoids and the relationship between antioxidant activity and its flavonoids content. In this research, the total content of flavonoids was expressed with mg equivalent quercetin/gram samples. The basis for determining the flavonoid content by spectrophotometry is the ability of flavonoids to form complexes with $AlCl_3$ forming a yellow color measured by its absorbance at λ 415 nm. The results of the determination of the content of ethyl acetate extract, ethyl acetate-ethanol extract, ethanol extract, ethanol-water extract, and water extract can be seen in Table 4.

TABLE 4. Total Content of Flavonoid in Neem Leaves Extract

No	Sample	Flavonoid content (mg QE/g extract)
1	Ethyl acetate extract	$1.331^{e} \pm 0.020$
2	Ethyl acetate-etanol extract	$0.648^{c} \pm 0.020$
3	Etanol extract	$1.076^{d} \pm 0.007$
4	Etanol-water extract	$0.326^{b} \pm 0.007$
5	Water extract	$0.073^{a} \pm 0,004$

Table 4 shows that the highest flavonoid content is found in the ethyl acetate extract and the lowest content is in water extract. This is in contrast to the total content of phenol which is relatively lower in ethyl acetate extract than

in ethanol or water. This test is supported by the results of identification of flavonoids with the TLC method which shows that the ethyl acetate extract has the highest flavonoid content compared to the other extracts of 26.909 ppm. This result is accordance with previous research that conducted by [8].

Antioxidant Activities of Neem Leaves Extract with DPPH methods

The parameters used for antioxidant activity with the DPPH radical scavenging method are IC_{50} values, namely the concentration of the compound (extract) of test needed to reduce the intensity of the radical DPPH color by 50% [9]. Neem leaf extract activity can be seen in Table 5.

TABLE 5. Antioxidant Activities of Neem Leaves Extract

No	Sample	Antioxidant activities (%) in the different extract concentration				IC_{50} (ppm)
		50 ppm	100 ppm	200 ppm	500 ppm	
1	Ethyl acetate extract	7.24	9.84	11.28	35.41	748.123
2	Ethyl acetate-etanol extract	10.20	22.22	14.77	36.91	776.399
3	Etanol extract	6.24	9.54	21.79	34.57	726.368
4	Etanol-water extract	13.75	36.13	70.11	78.21	216.118
5	Water extract	5.10	6.54	15.13	20.59	504.883

Based on the antioxidant activity testing with the DPPH method, it is found that ethanol-water extract which has the highest total content of phenol turned out to also have the highest antioxidant activity compared to other extracts expressed with IC_{50} values of 216,118 ppm. The total sequence of phenols from the highest to the lowest is ethanol-water extract, ethanol extract, ethyl acetate-ethanol extract, water extract, and ethyl acetate extract. The research conducted by [10] also showed that extracts derived from plants (fruit, leaves, and vegetables) have a direct correlation between the total content of phenols and their antioxidant activity. This is supported by [11] which stated that neem plants contain a lot of secondary metabolites in the form of phenolic compounds. In his research on young and old neem leaves, gallic acid and ferulic acid was detected but the older leaves were three times larger in number than young leaves.

Reduction Power Testing

Reduction power of bioactive compounds can be associated with antioxidant activity. Reduction power in samples containing antioxidants is a reductant. In this study, the reduction power of the sample extract was equated as a BHT compound (gram unit of BHT equivalent per gram of extract) and BHT was made as a standard. Reduction power of ethyl acetate extract, ethyl acetate-ethanol extract, ethanol extract, ethanol-water extract, and water extract is shown in Table 6.

TABLE 6. Reducing Power of Neem Leaves Extract

No	Sample	Reduction power (g BHT/g extract)
1	Ethyl acetate extract	$0.575^{a} \pm 0.016$
2	Ethyl acetate-etanol extract	$0.727^{ab} \pm 0.011$
3	Etanol extract	$0.771^{b} \pm 0.011$
4	Etanol-water extract	$0.778^{b} \pm 0.048$
5	Water extract	$0.856^{b} \pm 0.017$

Based on Table 6, it is found that water extract has the highest reduction power compared to other extracts, but not significantly different from ethyl acetate-ethanol extract, ethanol extract and ethanol-water extract in statistical tests (α=0.05). Meanwhile, the ethyl acetate extract has the lowest reduction power. From these results, it can be concluded that extracts with non-polar solvents tend to have a relatively lower reduction power than

extracts with polar-semi polar solvents, although ethyl acetate-ethanol mixtures that are non-polar-semi-polar have higher reduction power than extract with single ethyl acetate solvent.

Water extract, ethanol-water extract, and ethanol extract have a relatively high ability to donate electrons and can react with free radicals. High reduction extracts are good electron donors that have the ability to stop free radical chain reactions by turning free radicals into more stable products[12]. The results of this reduction power test generally provide reinforcement that ethyl acetate extract, ethyl acetate-ethanol extract, ethanol extract, ethanol-water extract, and neem leaf water extract have antioxidant potential.

CONCLUSION

Neem leaves is a potential resources for natural antioxidant. In addition to obtaining highest yield using Ethanol-Water as a solvent during the extraction process, this solvent also results better characteristic of extract than 4 other solvents. The properties of the extract that produced by extraction using Ethanol-Water is 2.067 g GAE/g extract, yield 1.39%, flavonoid 0.326 mg QE/g extract, antioxidant activity 226.118 ppm and reducing power 0.778 g BHT/g extract. Therefore, it is recommended that Ethanol-Water as a solvent for the extraction of active compound on neem leaves.

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