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ANALYSIS OF FLAVONOID COMPOUNDS FROM ETHANOL EXTRACT OF MORINGA LEAVES (MORINGA OLEIFERA L.) USING FTIR AND HPLC

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Abstract: Moringa leaves contain flavonoids, phenols, tannins, alkaloids, saponins, terpenoids, and steroids. The flavonoid and phenol content has antioxidant activity. This research aimed to determine compounds in the ethanol extract of Moringa leaves To determine the type of flavonoid content using FTIR and HPLC. FTIR analysis shows that Moringa leaves possibly contain compounds from quercetin derivatives, which is supported by HPLC analysis. This quercetin compound is a group of flavonols that are characterized by the presence of a hydroxyl group and a carbonyl group. HPLC analysis was carried out by making a quercetin standard with a concentration of 200 ppm. The resulting wavelength is 254 nm. The results of the quercetin standard chromatogram which contains one main peak obtained from the quercetin standard. This peak shows the retention time at 3 minutes. The results of the standard chromatogram, quercetin has an outer area of 44763473 and a sample area of 17455781 with 2 times dilution, so the % purity of fraction 9 for quercetin is 77.99 ppm. This HPLC analysis shows that Moringa leaves probably contain quercetin derivatives which are characterized by the same retention time between the sample and the quercetin standard, namely at a retention time of 3 minutes, and have a peak pattern that is almost the same as the quercetin standard.

Keywords: *Moringa Oleifera* L., Total Phenolic Content, Total Flavonoid Content, Antioxidant Activity

INTRODUCTION

The Moringa plant (Moringa oleifera L.) is a plant commonly used in spices, cosmetic oils, and various treatments (Abdul Razis et al., 2014). The Moringa plant is known as the "miracle tree" because almost all parts of the plant can be used as medicine. One of the contents of Moringa leaves is flavonoids (Farooq, 2012). Flavonoid compounds are compounds contained in Moringa leaves. Moringa plants contain 17 flavonoid compounds (Makita et al., 2016). The flavonoid compound content in Moringa leaves is known to be used in medicine because of its pharmacological activity, one of which is as an antioxidant (Widiyarti et al., 2018).

Antioxidants are compounds that can inhibit the oxidation process caused by free radicals (Makky et al., 2021). Free radicals are very dangerous because they can react with important components of cells, for

example, DNA and cell membranes. These free radicals can cause protein dysfunction, DNA damage, and lipid peroxidation which can cause cell death (Sun et al., 2018). Based on research by Xu et al. (2019), Moringa leaves extract has antioxidant activity with an IC50 value of 1.02 ± 0.13 mg/mL. IC50 (inhibitory concentration) is a sample concentration that can inhibit half the maximum (50%) in the free radical oxidation process (Julizan, 2019). Other research also states that Moringa leaf extract has antioxidant activity with an IC50 value of 0.02 ± 0.06 mg/mL (Gothai et al., 2017). Research by Vats and Gupta (2017) states that Moringa leaves extract has antioxidant activity with an IC50 value of 0.12 mg/mL. Confirmed by research conducted by Wright et al. (2017), the IC50 value of Moringa leaves extract ranges from 0.02 to 4.46 mg/ml. Several studies have examined the flavonoids in Moringa leaves, however, the characteristics of flavonoid compounds which act as antioxidants, especially in the ethanol extract of Moringa leaves, have not been studied. Apart from that, determining the effect of total phenol and total flavonoid content on antioxidant activity has not been widely studied. Based on this background, it is necessary to determine the characteristics of flavonoid compounds from the ethanol extract of Moringa leaves to determine the antioxidant activity and also determine the effect of the total phenol and total flavonoid content of the ethanol extract of Moringa leaves on antioxidant activity.

METHODS

1. Tools and Materials

The tools used in this research are glassware commonly used in laboratories, dropper pipettes, vials, stir sticks, watch glasses, test tubes, measuring glasses, measuring pipettes, measuring flasks, sprayers, porcelain cups, filter paper, and Millipore membranes. 0.22 µm, syringe, black cloth, container, stirrer, crucible (Thermolyne), furnace (Thermolyne), analytical balance (Pioneer), maceration vessel, clamp and stand, chromatography column, blender, vortex, centrifuge, moisture analyzer, rotary evaporator, water bath, oven (Memmert), incubator (Memmert), HPLC (Thermos scientific), and FTIR.

The materials used are Moringa leaves and the chemicals used are: Moringa leaves taken in the Banyumanik area of Semarang, 96% ethanol (C_2H_5OH), chloroform ($CHCl_3$), ammonia (NH_3), concentrated sulfuric acid (H_2SO_4), Mayer's reagent, wagner, dragendorf, magnesium powder (Mg), concentrated hydrochloric acid (HCl), distilled water (H_2O), anhydrous acetic acid ($(CH_3CO)_2O$), hexane (C_6H_{14}), ethyl acetate ($CH_3CH_2OCCH_3$), GF_{254} TLC plate, aluminum chloride ($AlCl_3$), quarcetin, Folin–Ciocalteu, ferric chloride ($FeCl_3$), potassium ferricyanide ($K_3Fe(CN)_6$), potassium dihydrogen phosphate (KH_2PO_4), sodium hydroxide (NaOH), trichloroacetic acid ($C_2HCl_3O_2$), vitamin C ($C_6H_8O_6$), potassium acetate (KCH_3COO), Liebermann Burchard, boric acid (H_3BO_3), oxalic acid ($H_2C_2O_4$), and n-butanol ($C_4H_{10}O$).

2. Sample Collection

Moringa leaves are collected and then wet sorted. After that, the leaves are washed with running water and dried by drying them in the sun, covered with a black cloth for one week. In the next process, the dried Moringa leaves are sorted dry, then quenched using a grinder, and then sifted to separate the fine stalks, so that a fine powder is obtained (Robby et al., 2022). Moringa leaves that have been reduced in size to powder are weighed and prepared for drying shrinkage testing using a moisture analyzer. To use this tool, turn on the moisture analyzer, then wait 30 minutes and ensure that the tool has been calibrated before use. When the tool is ready to be used, set the time, temperature, and heating mode. Drying losses obtained from Simplicia must comply with quality requirements, namely ≤ 10% (Farmakope Herbal Indonesia, 2017).

3. Moringa leaves ethanol extract process

The extraction process is carried out using the maceration and remaceration method once. 500 grams of Moringa leaves powder was put into a maceration vessel, and then 1500 mL of 96% ethanol solvent was added, the solvent was added little by little until all the samples were wetted. Then the sample was left for 3x24 hours at room temperature (25oC) and stirred. After 3x24 hours of soaking, filter it to get the filtrate. The powder resulting from the previous soaking was macerated again by adding 1500 mL of 96% ethanol solvent, then the sample was left for 3x24 hours and stirred. The soaking results are then filtered to obtain a second filtrate. The extract obtained was collected then filtered and evaporated using a rotary evaporator at a temperature of 40oC to produce a concentrated extract, then

evaporated again using a water bath until a thick extract was obtained (Robby et al., 2022). Next, the yield was calculated from the ethanol extract of Moringa leaves.

4. Isolation of Flavonoid Compounds

The mobile phase used was the result of the mobile phase in the confirmation test using TLC to determine the chemical content of flavonoids and the stationary phase used GF_{254} silica gel. The chromatography column is filled with cotton, the stationary phase has been moistened with the mobile phase, the sample, and the mobile phase. The hatch of the chromatography column was set at a flow rate of 1 mL/minute. The fractions obtained from the results of column chromatography separation were then identified using TLC to detect fractions containing flavonoids (Etika and Iryani, 2019)

5. Analysis of Flavonoid Compounds using FTIR

Determination of flavonoid compounds from isolated compounds was carried out using FTIR. Fractions resulting from column chromatography separation were characterized using FTIR spectroscopy. Interpretation of functional groups can be seen from the group strain at certain wave numbers.

6. Data Analysis

Data analysis using univariate, bivariate, and multivariate analysis. Univariate analysis is used to analyze the chemical compound groups contained in extracts from the phytochemical screening results. Apart from that, we also carried out results analysis to determine the purity of the isolate results by analyzing the peak area and number of peaks produced. If the number of peaks is more than one, it can be said that the resulting isolate is not pure. Meanwhile, the peak area is used to determine the purity of the sample. Next, functional group analysis was carried out from the isolates containing the highest total flavonoids which were analyzed using FTIR.

Bivariate analysis was used to analyze the effect of total flavonoid content on antioxidant activity in extracts and isolates containing flavonoids. In this data analysis, a normality test was carried out with Shapiro Wilk, if the data was normally distributed (>0.05) then the Pearson correlation test was used and if the data was not normally distributed (<0.05) the Kendall Tau correlation test was used. Multivariate analysis was used to analyze the effect of total phenol content and total flavonoids on the antioxidant activity of extracts and isolates containing flavonoids. If the data is normally distributed (>0.05) then the Pearson correlation test is used and if the data is not normally distributed (<0.05) the Kendall-Tau correlation test is used. This analysis was carried out using IBM SPSS Statistics 22.

RESULT AND DISCUSSION

1. Simplicia Preparation

In this research, Moringa plants were taken from Banyumanik, Semarang, which is a moringa cultivation site in Central Java. The location for collecting Moringa leaves was chosen because Moringa can grow in the lowlands and highlands up to an altitude of ± 1000 meters above sea level. The Banyumanik area is at an average altitude of 300 meters above sea level with an average air temperature of 20 -22^{o}C, so Moringa plants can grow well in this area. Moringa leaves can be harvested after the plants are 6 to 12 months old. This harvesting is done by picking the leaves stalks from the branches, in the morning at 09.00 so that the plant wounds dry quickly and to minimize the loss of volatile compounds (Sudewo,2004)

2.5 kg of Moringa leaves were collected, then washed and wet sorted. The purpose of wet sorting is to separate the leaves from dirt or gravel that is still attached and wash them with running water until clean, then dry them. Drying is done by spreading it evenly on a container then drying it in the sun and covering it with a black cloth. According to research from Green (2004), drying with a black cloth has the highest flavonoid levels compared to the oven or without covering with a black cloth. Drying in direct sunlight has lower levels of flavonoids because sunlight directly hits the simplicial, it is thought that sunlight damages the flavonoids, drying in an oven also has lower levels because drying in ovens has poor air circulation and this is one of the factors that can influence the drying process (Green, 2004) (sahin et al, 2018).

Drying aims to reduce the water content still contained in the leaves. Water content that is too high can affect the storage of simply because it can cause the leaves to rot quickly, becoming a source of growth for microorganisms such as fungi. The next step is dry sorting which aims to separate foreign objects such as unwanted plant parts and other impurities that are still present and

left behind in the dried simplicia. The dried Moringa leaves are then reduced in size using a blender until they become powder. A small or fine sample size can facilitate contact of the sample with a larger solvent to obtain better yield results (Ali et al., 2018). The Simplicia powder was then tested for water content using a moisture analyzer. The working principle of this tool is to measure the water content of a sample directly using the loss on drying (LOD) technique. This LOD technique measures the weight of the sample before and after drying and uses delta weight to determine the percentage of water content as weight removed by the drying process compared to the initial weight of the sample (Ohaus,2016).

Determining the water content of simplicia is very important to provide a maximum limit for the water content in simplicia because high amounts of water can become a medium for the growth of microorganisms which can damage the compounds contained in simplicia. Testing the water content of Moringa simplicia leaves obtained a result of 7.31%. The same results were obtained from research by Augustyn et al., (2017); Handayani et al,. (2018); and Listiani et al., (2023) which stated that the resulting water content was between 7.80-9.57% (Handayani et al., 2018) (Augustyn et al., 2017) (Listiani et al., 2023). The results obtained are by the standards of the Indonesian Herbal Pharmacopoeia Edition 2 (2017), namely the requirement for water content in Simplicia ≤10%

2. Extracts Processing

Extraction is the separation of plant parts that have medicinal properties using selective solvents through standard procedures (Handa et al., 2008). The purpose of extraction is to separate soluble plant metabolites or leave insoluble metabolites (residues) (Azwanida,2015). The extraction method used is the cold extraction method because there is a group of flavonoid compounds that are not heat resistant and are easily oxidized (Ramayani et al.,2021) According to research from Septiani et al., (2021), the total phenol content using the maceration method is greater than the soxhletation method which is a hot method, namely 13.93 mgQE/g (1.39%) and 8.12 mgQE/g (0.81%) (Septiani e al., 2021). The cold extraction method chosen is the maceration method. The choice of this method was based on the effect of the differences in maceration and percolation extraction methods on higher antioxidant activity with the maceration method compared to the percolation method, namely IC_{50} 33.49 µg/mL and 83.89 µg/mL (Fatmawati,2019) . The advantages of the maceration method are that it is effective for compounds that are not heat resistant (degraded due to heat), and the equipment used is relatively simple, cheap, and easy to obtain (Naviglio,2019).

The maceration method is most often used to extract active compounds in Moringa leaves. The amount of raw material, solvent selection, and extraction time will affect the effectiveness of this method. The extraction process using the maceration method is carried out by immersing the sample in the extraction solvent (Patel, 2019). In the extraction process, 96% ethanol solvent was used, because ethanol is polar so it can extract polar compounds in the sample (Hidayah et al, 2016). Phenolic and flavonoid compounds are polar compounds so they will dissolve in polar solvents, by the principle like dissolves like (Kemit et al, 2016). According to research from Zullaikah et al., (2019) and Do et al., (2014), conducting research using various ethanol concentrations to determine extract yield, total phenolic compounds, antioxidant activity, and the highest flavonoid content was obtained with an ethanol solution concentration of 96%. The extract yield increased to 60.50% with an increase in the ethanol solution concentration from 0% (solvent was water) to an ethanol solution concentration of 96%.

The extraction process was carried out for 3 days and was stirred once every day so that the contact between the simplicial and the solvent increased until it reached the saturation point of the solution. The stirring process can increase the contact between simplicia and the solvent (Chairunnisa et al., 2019). The liquid extract obtained is dark green because the ethanol solvent not only extracts flavonoids and phenolic compounds but can also extract chlorophyll in Moringa leaves samples (Kiorewoa et al, 2012). In the next process, to remove the remaining solvent in the liquid extract, a rotary evaporator is used at a temperature of 40^{o}C. The function of the rotary evaporator is to evaporate the solvent in the macerate and only leave the extracted compound. To get a thick extract, the extract is evaporated in a water bath at a temperature of 37^{o}C until a thick extract is obtained. The thick extract obtained during the extraction process was 52.59 grams with a yield of 10.52%. In research conducted by Mahdi et al., (2016) and Kiswandono (2011), using the same method and solvent an extract yield of 9.98% - 25.02% was obtained.

3. Isolation of Flavonoid Compounds

The eluent or mobile phase used is the result of the mobile phase in the confirmation test using TLC to determine the chemical content of flavonoids, namely n-butanol: acetic acid: water (4:1:5) and the stationary phase used is silica gel GF254. The resulting fractions were 23 fractions. The fractions obtained were then identified using TLC to detect fractions containing flavonoids. From the TLC results, 5 fractions were obtained that had the same stain. The TLC results can be seen in Table 1.

Table 1. Results of Thin Layer Chromatography of Flavonoid Compounds Fraction 5 and Fraction 6

Spot	Retention factor	Spraying*					
		Before			After		
		Visual	UV_{254nm}	UV_{366nm}	Visual	UV_{254nm}	UV_{366nm}
1	0,56	-	Blue	-	-	Blue	-
2	0,93	Greenish yellow	Greenish yellow	Magenta purple	Greenish yellow	Greenish yellow	Magenta purple

The results of the flavonoid test on fraction 5, fraction 6, fraction 7, fraction 8, and fraction 9 showed positive results for the presence of flavonoid compounds. The five fractions have a blackish-yellow color. In fraction 5 and fraction 6, there is a color that glows blue under UV366 nm light with the stain code (1). In fraction 7, fraction 8, and fraction 9 there is a color that glows blue under UV254 nm light with the stain code (1). The results of the flavonoid test in the research are by Hanani (2017) who stated that the fluorescent light blue color is suspected to be flavonoids. Flavonoids are thought to form bonds with a mixture of boric acid and citric acid on heating and are better known as cytoroborate reagents (Sjahid,2016). Apart from that, looking at the Retention factor value, stain code (1) has an Retention factor value of 0.56, which according to research conducted by Lim et al., (2019), states that the Retention factor value of flavonoids is around 0.543

4. Analysis of Flavonoid Compounds using FTIR

FTIR absorption analysis was carried out to determine the functional groups contained in the fraction containing the highest antioxidants. The sample transmission spectrum can be seen in Figure 1.

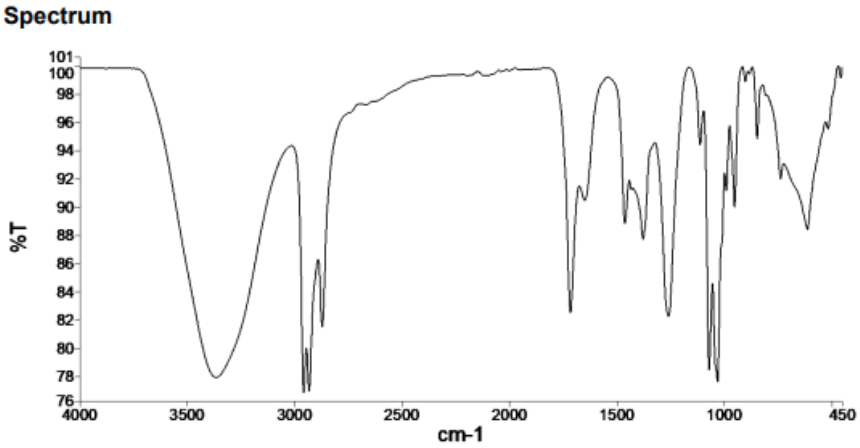

Figure 1. Fraction 9 FTIR Spectrum

Table 10. IR Spectrum Analysis

Absorption (cm^{-1})	Group	Class	Referential
3368,05	O-H *stretching*	Hidroksil	3550-3200
2959,78	C-H *stretching*	Alkana	3000-2840
1716,36	C=O *bending*	Karbonil	1725-1705
1650,05	C=C *stretching*	Aromatics	1650-1475
1462,53	C-H *bending*	Alkane	1500-1375
1258,82	C-O *stretching*	alkyl aryl ether	1300-1100
951,12	C=C *bending*	Alkene	980-960

In the FTIR spectrum of fraction 9 in Figure 1, the max peak at absorbance 3368.05 is the O-H bond which is the hydroxyl group, and at absorbance 1716.36 is the C=O bond which is the carbonyl group. FTIR analysis shows that Moringa leaves possibly contain compounds from quercetin derivatives, which is supported by HPLC analysis. This quercetin compound is a group of flavonols that are characterized by the presence of a hydroxyl group and a carbonyl group. According to research conducted by Vergara-Jimenez (2017), Moringa leaves contain flavonoid compounds such as quercetin. Supported by research conducted by Lin et al, (2018), Moringa leaves contain flavonoid compounds such as quercetin.

5. HPLC analysis

The working principle of this instrument is the separation of analytes in a chromatographic column based on the polarity of the mobile phase flow which carries the analyte mixture through the stationary phase where the separation of the components occurs due to differences in the strength of interaction between the solutes and the stationary phase resulting in differences in the transfer time of each component in the mixture (Weston and Brown, 1997). HPLC analysis was carried out by making a quercetin standard with a concentration of 200 ppm. The resulting wavelength is 254 nm. The results of the quercetin standard chromatogram can be seen in Figure 2 which contains one main peak obtained from the quercetin standard. This peak shows the retention time at 3 minutes. Fraction 9 is then injected and measured at the same wavelength. The results of the chromatogram of fraction 9 can be seen in Figure 3 which contains one main peak obtained from sample fraction 9. The peak of this chromatogram also shows the retention time at 3 minutes. Based on the results of the standard chromatogram, quercetin has an outer area of 44763473 and a sample area of 17455781 with 2 times dilution, so the % purity of fraction 9 for quercetin is 77.99 ppm. This HPLC analysis shows that Moringa leaves probably contain quercetin derivatives which are characterized by the same retention time between the sample and the quercetin standard, namely at a retention time of 3 minutes, and have a peak pattern that is almost the same as the quercetin standard.

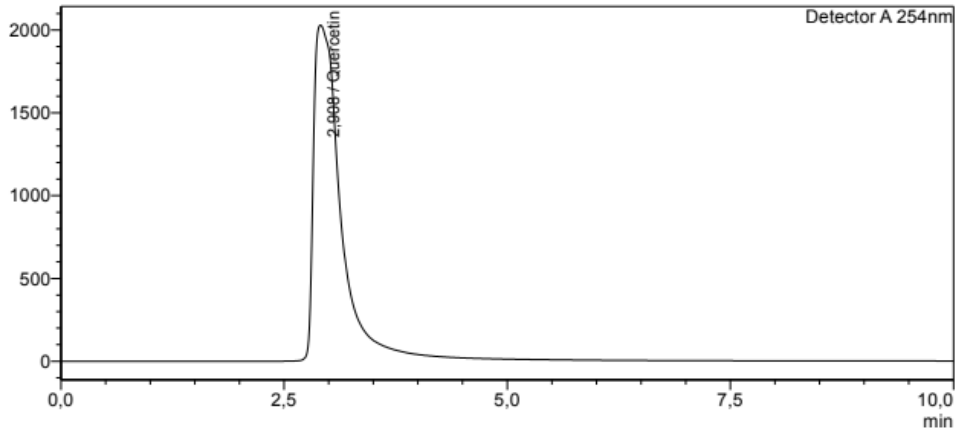

Figure 2. Chromatogram Standart Quercetin

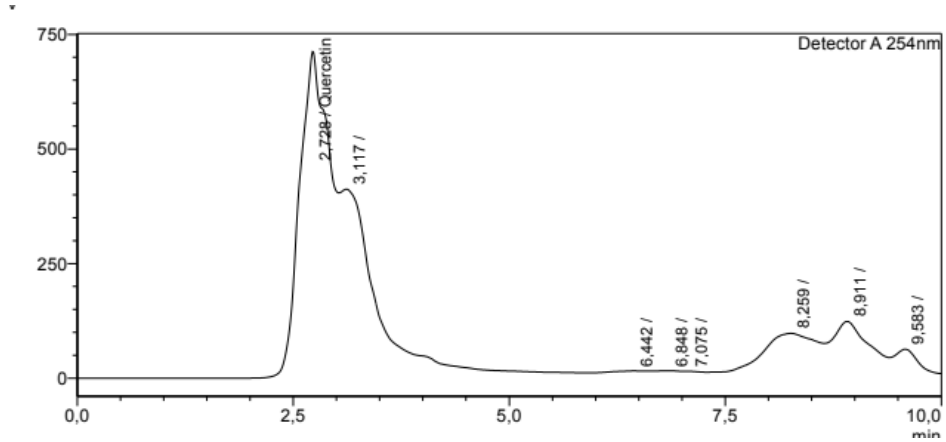

Figure 3. Chromatogram Fraction 9

CONCLUSION

The ethanol extract of Moringa leaves used in this research is *Moringa oleifera* L. which is positive for containing flavonoids. Moringa leaves ethanol extract has the characteristics of specific parameters and non-specific parameters drying shrinkage of 5.65%, water content of 7.31%, total ash content of 6.20%, and acid insoluble ash content of 0.47%. The flavonoid compound has a blue stain that fluoresces with TLC under UV254 nm light, has an Retention factor value of 0.56, contains hydroxyl (-OH) and carbonyl (C=O) groups which are thought to be derivatives of the quercetin compound.

CONFLIC OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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