Identification of Antioxidant Active Compounds from Watercress (*Nasturtium officinale* R.Br)

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Abstract. Watercress (*Nasturtium officinale* R.Br) is a highlands plant that has been eaten as a vegetable by many people. Watercress contains protein, calcium, phosphorus, iron, vitamin A, vitamin E, vitamin C, flavonoids and phenols. Antioxidants are components that can delay or prevent the oxidation of lipids, nucleic acids, or other molecules by inhibiting the initiation or propagation of oxidation chain reactions. The function of antioxidants is to neutralize free radicals, so that the body is protected from various degenerative diseases and helps suppress the aging process. The purpose of this study was to isolate and identify antioxidant compounds in watercress. The studies included maceration and reflux extraction with 96% ethanol, partitioning (with n-hexane and ethyl acetate), antioxidant activity determination, and compound determination. Antioxidant activity test by 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenger method showed that the watercress ethyl acetate extract had the highest antioxidant activity with IC_{50} of 67.77 ppm. Fractionation by first column chromatography [SiO_2; n-hexane: ethyl acetate (10:1–1:1), $CHCl_3$: MeOH (15:1–1:1)] obtained 5 combined fractions (F1–F5) where the results of the antioxidant activity test of fraction 5 (F5) had a great inhibition value compared to other fractions of 61.04%. Furthermore, F5 fractionated second column chromatography [SiO_2; n-hexane: ethyl acetate (1:1), $CHCl_3$: MeOH (5:1)] obtained 2 combined fractions (F5.1 and F5.2). After two purifications using column chromatography and testing for antioxidant activity, fraction 5.2 had the highest activity with an IC_{50} value of 75.65 ppm. Fraction 5.2 contained phenols and/or flavonoids according to phytochemical screening, while UV-Vis, Fourier transform infrared (FTIR) spectrophotometry and gas chromatography-mass spectrometry (GC-MS) spectroscopic results indicated that, the active ingredient in fraction 5.2 is expected to be 2-methoxy-4-vinylphenol.

Keywords: antioxidant · DPPH · *Nasturtium officinale* R.Br · phenol

1 Introduction

Watercress (*Nasturtium officinale* R.Br) contains large amounts of iron, calcium, folic acid, vitamins A and C. Many benefits of eating watercress for our bodies, such as

I. Nurlaila et al. (Eds.): ICHR 2022, AHSR 56, pp. 374–384, 2023.
https://doi.org/10.2991/978-94-6463-112-8_35

diuretic drugs, expectorants, aid digestion, and protect the body against lung cancer [1]. In a previous study, the antioxidant activity and bioactive components of watercress (*Nasturtium officinale* R.Br) stated that the crude extract of watercress had weak antioxidant activity when compared to the antioxidant activity of BHT. The IC_{50} value for the whole watercress extract was 337.32 ppm using the free radical scavenging method with DPPH and containing secondary metabolites such as alkaloids, steroids, and phenol hydroquinone [1].

Pharmacopoeia Indonesia [2] stated that vitamins A, C, E contained in watercress tend to be soluble in ethanol solvent, while compounds in the polyphenol group, such as flavonoids, according to Rahman *et al.*[3] can be macerated using 70% ethanol which tends to be semipolar in water-ethanol combination [2, 4]. Watercress contains isothiocynate compounds, kaempferol glycosides and l-tryptophan which functions to ward off free radicals, help repair damage and DNA synthesis [5].

The strength of antioxidant compounds, such as flavonoids in watercress extract can be identified using IC_{50}. The IC_{50} value is the smallest concentration capable of inhibiting 50% of free radicals using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent. The lower the IC_{50} value, the higher the antioxidant activity of the substance, meaning that it only requires a small concentration to inhibit free radicals by 50% [5], the antioxidant activity test of 70% ethanolic watercress extract is by produces an IC_{50} value of 102.26 ppm [3].

Solvent extraction using cold method (maceration), the extraction process technique is carried out with several times of shaking or stirring at room temperature. The advantage of this method is that it is easy and does not require heating so it is less likely that natural materials will be damaged or decomposed. The choice of solvent based on its solubility and polarity facilitates the separation of natural substances in the sample. The long operation of the maceration method and the stationary state during maceration allows many compounds to be extracted. Other extraction processes are carried out by heating, reflux, which is extraction with the solvent at its boiling point temperature, for a certain time with a relatively constant amount of limited solvent and the presence of reverse cooling. Extraction can take place efficiently and the compounds in the sample can be more effectively withdrawn by the solvent.

In this study, two extraction methods, (i.e.: maceration and reflux) were applied to determine whether the active compounds contained in watercress were heat resistant or not. Watercress has many benefits for health, but information about the chemical composition in watercress is still lacking. Research on chemical compounds in this plant, especially the content of antioxidants, needs to be carried out so that it is expected to provide the information needed for its use in the pharmaceutical, food, and other industries.

2 Materials and Methods

2.1 Materials

Watercress (*Nasturtium officinale* R.Br) was obtained from the Research Institute for Medicinal and Aromatic Plants (BALITTRO), Bogor, Indonesia. All reagents were used in the analytical grade including silica gel 60 (Merck, Germany), ethyl acetate (Bratachem, Indonesia), n-hexane (Bratachem, Indonesia), dichloromethane (Bratachem,

Indonesia), methanol (Bratachem, Indonesia), DPPH (Sigma, USA), vitamin C (Sigma, USA). Cerium sulfate (Merck, Germany), methanol (Merck, Germany).

2.2 Extraction and Partition

Watercress extraction was carried out by 2 methods, maceration and reflux. In the maceration method, dry lettuce that has been cut into small pieces is macerated with 96% ethanol for 24 h at room temperature. Meanwhile, for the reflux method, the dried watercress sample that has been cut into small pieces is refluxed with 96% ethanol for 3 h at a temperature of ±70 °C. Each solvent was filtered and the filtrate was collected, the extraction process was repeated until the filtrate was colorless. The filtrate obtained was evaporated using a rotary evaporator at a temperature of 50 °C to obtain crude extract.

The crude extract was dissolved in distilled water, sonicated and then partitioned using a separating funnel with n-hexane and ethyl acetate as solvents. Thereafter, the partition was stiffened by adding n-hexane in the ratio of water and n-hexane is 1:1. The aqueous extract was separated and re-partitioned with ethyl acetate at a 1:2 ratio. The aqueous extract was separated and combined with the previous one. Last, the extract was evaporated using a rotary vacuum evaporator to obtain a partitioned extract.

2.3 Antioxidant Test

Samples and ascorbic acid as a positive reference were prepared in dry methanol at various stock concentrations. 40 μL of DPPH solution (1 mM) was added to a 96-well microplate, then sample and ascorbic acid into the well, and finally added up to 200 μL of methanol per test to obtain different final concentrations. As a blank, 40 L of DPPH solution containing anhydrous methanol was added to the well to 200 μL. All test solution samples, positive controls and blanks were incubated at room temperature for 30 min. The absorbance of all samples and blanks was then measured at a wavelength of 515 nm. Antioxidant activity was obtained using the following equation and IC_{50} values by constructing a linear curve between test solution concentration (x-axis) and percent antioxidant activity (y-axis).

$$\%Inhibition = \frac{A - B}{A} \times 100\%$$

A is blank absorbance
B is the sample absorbance.

2.4 Fractionation with Column Chromatography

The phase with the greatest antioxidant activity was first fractionated by gradient column chromatography, with SiO_2 as stationary phase and with mobile phase is n-hexane: ethyl acetate (10:1–1:1); chloroform: methanol (15:1–1:1). Second fractionation with SiO_2 as stationary phase, isocratic mobile phase with n-hexane: ethyl acetate (1:1); chloroform: methanol (5:1). Each sub fractions was collected in TLC, the sub fractions with the similar TLC pattern were combined to obtain a simpler fraction.

2.5 Identification of Active Compounds

Compounds of active components were identified using FTIR spectrophotometry and gas chromatography-mass spectrometry (GCMS). In this regard, the extract was injected into a GC-MS Agilent Technologies 7890 equipped with an RHP Ultra 2 capillary column (30 m × 0.20 mm ID × 0.11 μm) and electron impact (EI) for the ionization method under the following conditions: Oven temperature program initial temperature at 80 °C hold for 0 min, rising at 3 °C/min to 150 °C hold for 1 min and finally rising 20 °C/min to 280 °C hold for 26 min; Injector port temperature 250 °C; Ion Source Temperature 230 °C; Interface temperature 280 °C; Quadrupole 140 °C; Gas sources Helium; Colum mode constant flow; Column flow 1.2 mL/min; Injection volume 5 μL; Split ratio 8: 1.

3 Results

3.1 Extraction, Partitioning and Screening Antioxidant Activity

The viscous extract from the rotavapor has the characteristics of a green thick extract. The yields obtained from the thick extract of n-hexane, ethyl acetate, and water from watercress can be seen in Table 1.

Preliminary testing of antioxidant activity aims to select one of the extracts of n-hexane, ethyl acetate, and water from watercress which has the highest antioxidant activity using the free radical reduction method with positive control of vitamin C. The results of the antioxidant activity test results of positive control of Vitamin C and preliminary antioxidant activity can be seen in Fig. 1

3.2 Column Chromatography Fractionation

From the results of the first column chromatography, 5 fractions (F1–F5) were combined, the results of testing the antioxidant activity of the 5 combined fractions can be seen in Fig. 2.

The antioxidant test results showed that the fraction that had the highest antioxidant activity compared to the other fractions was fraction 5 with free radical scavenging activity of 61.04% at a test concentration of 100 ppm.

Table 1. Extract Yield Results

Extraction method	Phase	Extract weight (g)	Yield (%)	Consistency /color
Reflux	n-hexane	8.47	4.24	green
	Ethyl acetate	2.34	1.17	green
	Water	35.79	17.89	green
Maceration	n-hexane	5.96	1.49	green
	Ethyl acetate	1.65	0.41	green
	Water	79.16	19.75	green

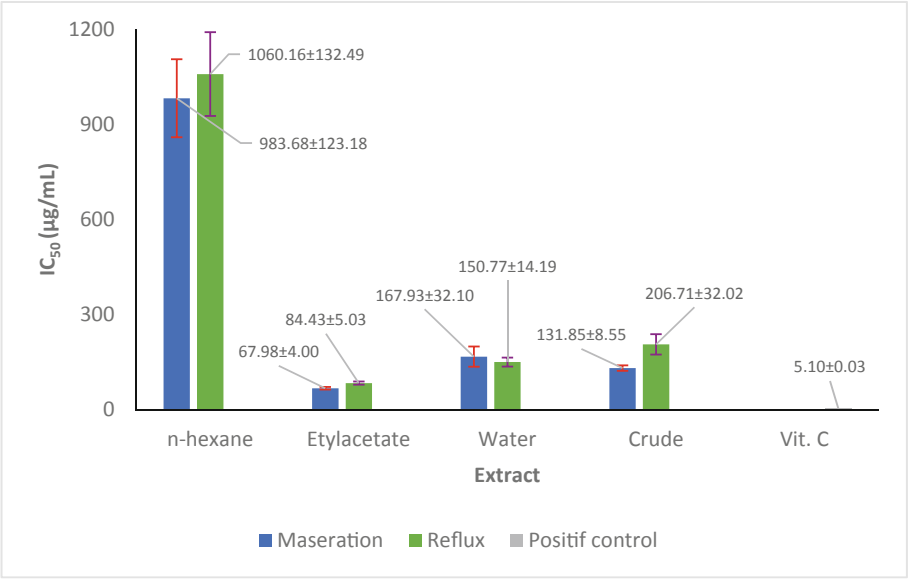


Fig. 1. Preliminary Test Results of Antioxidant Activity Using DPPH

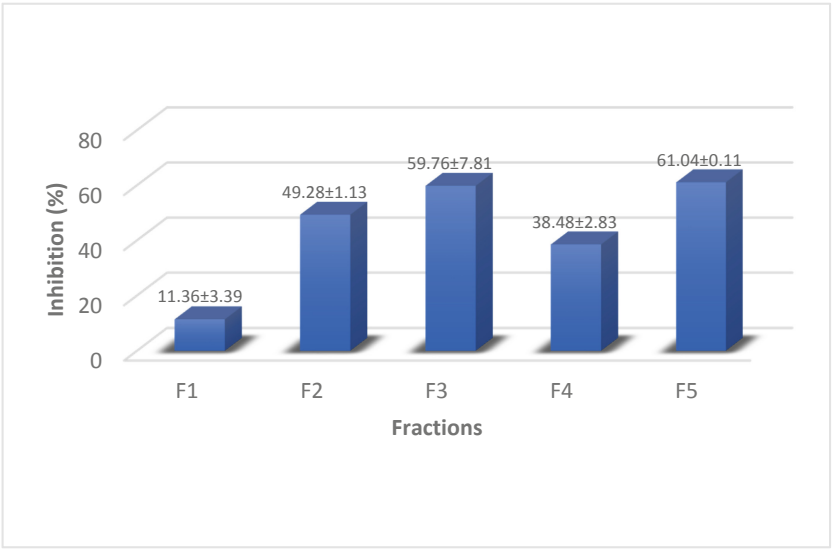


Fig. 2. Antioxidant Activity of the First Column Chromatography Fraction

Furthermore, fraction 5 was carried out by second column chromatography, in which 2 fractions (F5.1–F5.2) were combined. The results of the antioxidant activity test for the two fractions can be seen in Fig. 3.

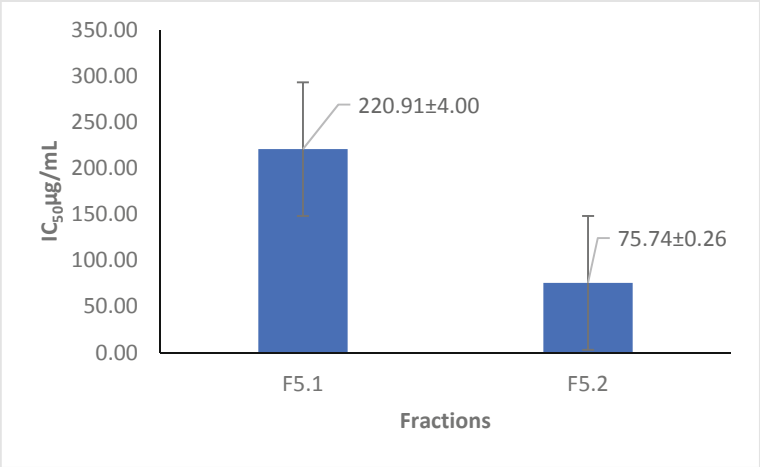


Fig. 3. Antioxidant Activity of the Second Column Chromatography Fraction

Table 2. Results of Phytochemical Screening of Watercress Extract

No.	Group of compounds	Result
1	Alkaloids	−
2	Flavonoids	+
3	Tannins	−
4	Phenol	+

Description: + (positive); − (negative).

3.3 Phytochemical Screening

The results of phytochemical screening of fraction 5.2 aimed to determine and ensure that the fraction contains secondary metabolites that are efficacious as antioxidants which can be seen in Table 2.

From the results of the phytochemical screening test, it is known that fraction 5.2 contains active antioxidant compounds from the flavonoid and phenol groups.

3.4 GC-MS Analysis

The secondary metabolites of fraction 5.2 were analyzed using FTIR and GCMS instruments to obtain information on their organic compounds. FTIR spectrophotometer was used for the preliminary identification of the functional groups of the compounds. At the same time, GCMS was used to identify compound names based on the percent similarity of each fragment peak in the instrument's Wiley library.

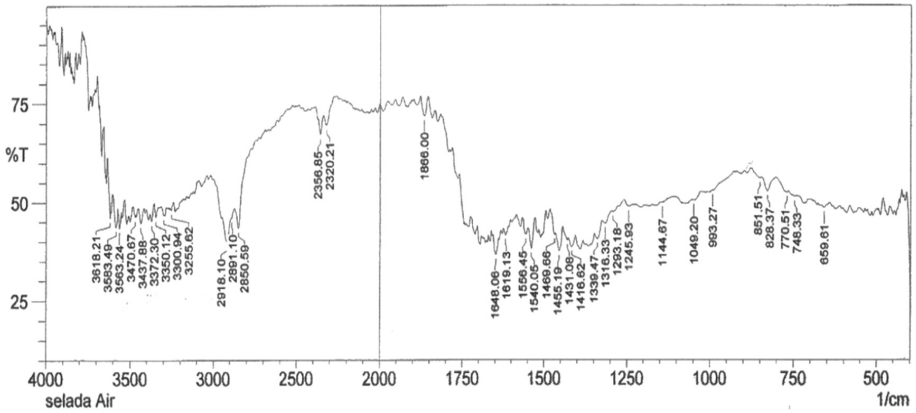


Fig. 4. Results of the 5.2 FTIR Spectrum

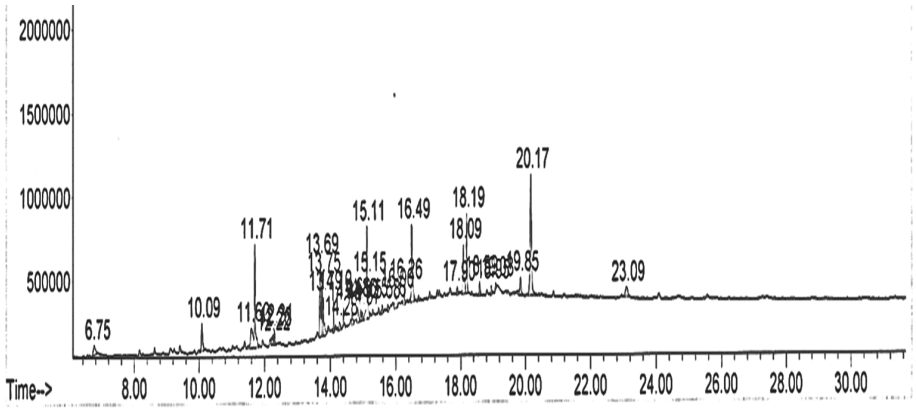
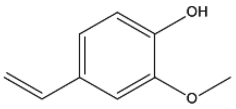
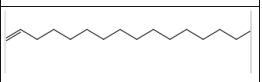
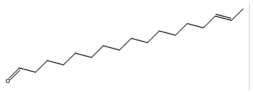
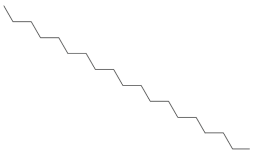
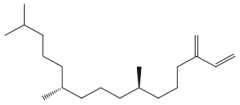
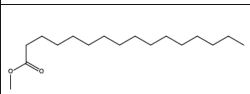
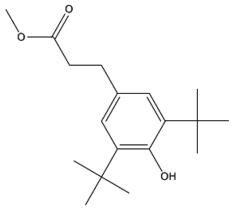
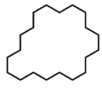


Fig. 5. Gas Chromatography Chromatogram Mass Spectrometry of 5.2 Fraction

The FTIR spectrum (Fig. 4) shown that some typical functional groups were detected at wavenumber 3255.62; 3300.94 cm^{-1} (-OH group of hydroxyl group), 1144.67; 1245.93; 1293.18 cm^{-1} (C-O group of alcohol, ether, carboxylic acid, ester), 828.37; 851.51; 993.27 cm^{-1} (=CH group alkene) and 748.33; 770.51 cm^{-1} (C=C aromatic hydrocarbon group) [6].

The chromatogram of the GC-SM analysis for fraction 5.2 (Fig. 5) gave several chromatogram peaks indicating the presence of several compounds with a retention time (Rt) of 10.09; 12.26; 13.75; 13.79; 14.10; 14.66; 14.92; and 15.11 min have a percentage of similarity (Qual) above 95% according to the Willey09th.L database (Table 3).

Table 3. Estimated Compounds Contained in the 5.2 Fraction

No.	Retention time (minute)	Qual (%)	Compound	Approximate chemical structure
1	10.09	96	2-Methoxy-4-vinylphenol	
2	12.26	97	1-Hexadecane	
3	13.75	99	E-15-Heptadecane	
4	13.79	97	Nonadecane	
5	14.10	99	7,11,15-Trimetil,3-Metilen-1-Hexadecane	
6	14.66	96	Methyl palmitate	
7	14.92	96	Methyl-3-(3,5-Ditersierbutyl-4-Hydroxyphenyl) Propionate	
8	15.11	96	Propionate Cycloeicosane	

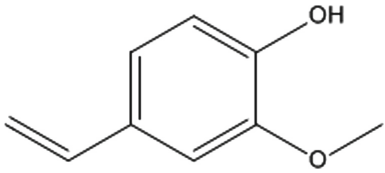


Fig. 6. 2-Methoxy-4-Vinylphenol

4 Discussion

Yield is the percentage ratio between the weight of the part of the material that can be utilized and the total weight of the material [7]. The yield of the reflux process is higher than the maceration process. The reflux process uses heat so that it is easier to dissolve and attract substances of watercress. Other factors that can affect the amount of extract obtained such as the extraction method, the solvent used, and the type of active compound in the simplicial. One study that shows the extraction process can attract active compounds that watercress leaf extract can fight and reduce lipid peroxidation in the liver, brain and kidneys [8].

From the results of the antioxidant activity test (Fig. 1), it is known that some of the partition phases have antioxidant activity because they have an IC_{50} value of <200 g/mL, and some other fractions have an IC_{50} value of >200 g/mL, where the results showed that the ethyl acetate phase by maceration method had the highest antioxidant activity compared to the other phases with an IC_{50} value of 67.77 g/mL.

The first process of the column chromatography resulted 5 fractions (F1–F5). The antioxidant activity test to those fractions revealed that fraction 5 (F5) had the highest inhibitory power (61.04%) than the other fractions. The second column chromatography to the F5 obtained 2 fractions (F5.1 and F5.2), based on the antioxidant test F5.2 had an IC_{50} value of 75.65 g/mL smaller than F5.1. The parameter that is commonly used to interpret the results of the DPPH test is the IC_{50} value. The smaller the IC_{50} value, the higher the antioxidant activity [9, 10].

Based on the results of phytochemical screening, the compounds containing flavonoid and phenolic compounds were obtained and analysis by UV-VIS spectrophotometry showed that the active compounds had double bonds, then the FTIR spectra showed the appearance of aromatic rings, alkene, ether and hydroxyl groups and on the GC-MS chromatogram using the Willey09th database. It was found that the compounds in fraction 5.2 were 2-methoxy-4-vinylphenol and methyl-3-(3,5-ditersierbutyl-4-hydroxyphenyl) propionate. Of those two mentioned phenolic compounds, the most likely compound is the 2-methoxy-4-vinyl phenol due to having 96% similarity level (qual) and in accordance with the group shown by FTIR Spectrophotometry (Fig. 6).

Antioxidant-enriched bioactive compounds of plants are particularly useful for precluding cancer by inducing the apoptosis of cancer cells [11–13]. Ethanol and acetone extracts from *Dendrobium crepidatum* scavenged 94.69 ± 0.10% and 93.41 ± 0.86% of DPPH free radicals, respectively. They showed 50% inhibition of DPPH free radicals (IC_{50}) at concentrations of 73.90 μg/mL and 99.44 μg/mL, which were found to be

statistically similar to that of ascorbic acid (control). The above extracts showed antioxidant and cytotoxic properties, potentially due to the presence of tetracosane, triacontane, stigmasterol, and some phenol derivatives (2-methoxy-4-vinylphenol, 2-methoxy-5-(1-propenyl)-phenol, p-mesyloxyphenol, and 2,6-dimethoxy-4-(2-propenyl)-phenol [14]. 2-Methoxy-4-vinylphenol (2M4VP), a member of the phenolic class, has been shown to possess anti-inflammatory properties and induce cell cycle arrest, making it an attractive drug candidate for the treatment of pancreatic cancer. 2M4VP had anticancer effects on pancreatic cancer cell lines Panc-1 and SNU-213. 2M4VP reduces Panc-1 cell viability by inhibiting the expression of cell nuclear antigen (PCNA) protein. 2M4VP also inhibited the migratory activity of both cell lines. Furthermore, 2M4VP treatment effectively decreased the phosphorylation of focal adhesion kinase (FAK) and AKT. 2M4VP can be used as adjuvant therapy for pancreatic cancer [15]. Contains Buckwheat, Buckwheat flavorings such as 2,5-dimethyl-4-Hydroxy-3(2H)-furanone, (E-)-2,4-decadienal and 2-Methoxy-4-vinylphenol (2M4VP). Especially using 2M4VP. As a fragrance, it is also present in apples and peanuts. 2M4VP is also known to induce cell cycle arrest by blocking it. Retinoblastoma protein hyperphosphorylation NIH3T3 cells were treated with benzopyrene and anti-inflammation by inhibiting mitogen-activated protein kinase (MAPK) activation [16, 17].

5 Conclusion

The macerated ethyl acetate extract had the highest antioxidant activity with an IC_{50} value of 67.77 g/mL, after being purified twice by column chromatography, fraction 5.2 had the highest antioxidant activity with an IC_{50} value of 75.65 g/mL. The results of the identification of phytochemical screening, UV-VIS spectrophotometry, FTIR and GC-MS fraction 5.2 are predicted to be 2-Methoxy-4-vinylphenol compounds belonging to a group of phenolic compounds that have antioxidant and anticancer activity based on several literatures.

Acknowledgement. We thank Galih Putra Pratama for his kind assistance throughout the study.

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