

SCOPOLETIN AS A MARKER COMPOUND FROM FERMENTED *Morinda citrifolia* FRUITS EXTRACT: POTENTIAL α -GLUCOSIDASE INHIBITOR AND ANTIOXIDANT COMPOUNDS

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ABSTRACT

Morinda citrifolia juice has long been used as a traditional herbal medicine in Indonesia to treat various diseases, including lowering blood sugar levels. However, the compounds responsible for this activity have not been widely studied. This research aimed to isolate the marker compounds from fermented *M. citrifolia* juice extract based on bioassay guidelines of antidiabetic activity and reduction of DPPH. Separation and purification of the ethyl acetate fraction yielded two, namely compounds 1 and 2. The chemical structures of these marker compounds, identified as scopoletin (1) and quercetin (2), were elucidated by LCMS Spectrometry and NMR analyses. The IC₅₀ values of compounds 1 and 2 were respectively 61.93 and 21.65 μ M for α -glucosidase, with 844.06 and 35.23 μ M for DPPH free radical scavenger effect. Based on these results, scopoletin and quercetin can be used as quality control biomarkers of products produced from fermented *M. citrifolia* juice extract.

Keywords: *Morinda citrifolia*, α -glucosidase Inhibitor, Antioxidant, Scopoletin.

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INTRODUCTION

Morinda citrifolia Linn is a tropical plant that can be cultivated throughout the Pacific. In Indonesia, this plant is usually known as "noni" or "pace". This plant is broadly cultivated in many regions, especially in tropical countries. Literature has shown that *M. citrifolia* juice has antidiabetic activity because it can reduce postprandial blood glucose levels. Currently, only a few studies have studied *M. citrifolia* juice's antidiabetic activity. It has been reported that *M. citrifolia* fruit juice given to female rats given diabetes-inducing steroids at a dosage of 3.6 ml/kg and 1.8 ml/kg could decrease their glucose levels compared to control mice induced with dexamethasone.¹ Another study found that the fermented juice of *M. citrifolia* could control the blood sugar levels of streptozotocin-induced rats.² In addition, *M. citrifolia* methanol extract also exhibits a stimulatory of glucose uptake in adipose cells.³ It was also reported that *M. citrifolia* juice decreased body heaviness and improved glucose tolerance in rats. Thus, it is intriguing to deepen our of various aspects of the anti-diabetes properties of fermented *M. citrifolia* juice.⁴ More than 100 chemical compounds have been elucidated from the *M. citrifolia* plant. The content of phytochemicals in *M. citrifolia* were alkaloids, organic acids, and phenolic compounds with phenolic compounds and flavonoids being the main phytochemicals groups. Examples are alizarin, damnacanthal, nordamnacanthal, scopoletin, rubiadin, aucubin, rubiadin 1-methyl ether, morindone, and other anthraquinone glycosides.⁵⁻⁷ The presence of phenolic compounds influences the total antioxidant activity of this plant.⁸ *M. citrifolia* leaf, fruit, and root extracts showed significant DPPH activity and total phenolic content.^{9,10} A report has revealed that neolignans and americanin A were found to have significant antioxidant activity from the partitions of butanol and methanol.^{11,12} The antioxidant properties of *M. citrifolia* fruit extract are proven to be a potent inhibitor of lipid oxidation. Another report found that the superoxide radical anion activity in *M. citrifolia* juice was particularly robust.¹³

However, the potential of the active compounds as an antidiabetes and antioxidants from *M. citrifolia* fruit have not been widely identified. Therefore, this research is aimed to isolate and characterize active compounds from fermented *M. citrifolia* juice. The antidiabetic activity was defined as the inhibiting action

of the α -glucosidase enzyme. At the same time, the antioxidant properties were determined based on the number of total phenols and total flavonoid levels, as well as the value of DPPH free radical activity.

EXPERIMENTAL

Material and Methods

Fresh, ripe *M. citrifolia* fruits (white-colored fruit with firm flesh) were obtained from the Center for Research, Science and Technology (PUSPIPTEK). The reagents used in this study include DPPH free radical (2,2-Di-Phenyl-1-Picryl-Hydrazyl), ABTS (2,2'-Azino-Bis(3-ethylbenzoThiazoline-6-Sulfonic acid)), standard gallic acid and quercetin, Folin-Ciocalteu, and *p*-NitroPhenyl- α -D-Glucopyranoside (*p*-NPG) obtained from Sigma Aldrich (St. Louis, USA). α -Glucosidase (this enzyme comes from yeast *S. cerevisiae*; EC 3.2.1.20) was purchased from WAKO-Japan. Silica gel 60 (0.040-0.063) was used in column chromatography, and TLC aluminum plates with silica gel GF254 were purchased from Merck. All extraction solvents, ethyl acetate, hexane, and methanol with the analytical grade, were obtained from Full Time. The JEOL JNM ECZR500 was used to measure the NMR spectra of the isolated compound and as the internal standard was used trimethylchlorosilane (TMS). LCMS-MS, Xevo G2-XS QToF Waters MS Technologies (USA) was used to measure the atomic mass of isolated compounds. An Agilent Cary 60 UV-Vis was used for measuring and recording absorbance in bioassay.

General Procedure

Preparation of Fermented Noni Juice Extract and Fractionation

The ripe *M. citrifolia* fruits (40 kg) were washed and air-dried, before being placed in a clean, closed container at room temperature and incubated for 6 days. The clear liquid formed from the incubation process was filtered and then extracted successively with ethyl acetate and butanol to obtain ethyl acetate (F.EtOAc₁) (1.8 g), butanol (6.2 g), and residual water fractions (7.5 g). To compare the activities of fermented and dried *M. citrifolia* extracts, the ripened fresh fruits were sliced and then placed in a drying oven at a temperature of 45°C for two days, before being ground into powder. The dried fruit powder (100 g) was macerated three times with ethyl acetate (1:10). This proceeds provided crude ethyl acetate extract (Ex.EtOAc₂) (3.2 g).

Isolation and Characterization of Active Compounds

The EtOAc₁ (3 g) extract was then entered into a silica gel column (3x20 cm), and mobile phase solvent was used *n*-hexane and ethyl acetate to obtain six fractions (F1-F6) (Fig.-1). Further purification from F4 by recrystallization with CHCl₃ and methanol, produced white crystals called "compound 1" (22 mg) and a yellow powder called "compound 2" (8.2 mg).

Isolate 1: White crystalline. ¹H NMR (500 MHz, CDCl₃) δ H: 7.60(1H, *d*, J=9.5 Hz, H4), 6.89 (1H, *s*, H9), 6.84(1H, *s*, H5), 6.26(1H, *d*, J= 9.5Hz, H3), 3.93(3H, *s*, O-CH₃). ¹³C-NMR (125 MHz, CDCl₃) δ C: 161.7(C=O), 150.4(C9), 149.9(C6), 144.2(C7), 143.5(C4), 113.6(C3), 111.7(C10), 107.7(C5), 103.4(C8), 56.4(O-CH₃). The measurement results of the LCMS analysis showed a single peak C₁₀H₈O₄ [M+Na] at *m/z* 215.02.

Isolate 2: Yellow crystalline, ¹H-NMR (500 MHz, CDCl₃) δ H: 7.82(1H, *d*, J= 2.6Hz, H2'), 7.71(1H, *dd*, J= 1.9Hz, 8. Hz, H6'), 6.99(1H, *d*, J= 8.5Hz, H3'), 6.52(1H, *d*, J= 2.6Hz, H8), 6.26(1H, *d*, J= 1.9Hz, H6)). ¹³C-NMR (125 MHz, CDCl₃) δ C: 176.6 (C1), 165.1 (C7), 161.8(C5), 157.8(C9), 148.4(C4'), 146.9(C3), 145.9(C5'), 136.8(2), 123.8(C1'), 121.5 (C2'), 116.2(C3'), 115.8(C6'), 104.1(C4), 99.2(C6), 94.5(C8). The measurement results of the LCMS analysis showed a single peak C₁₅H₁₀O₇ [M+H] at *m/z* 303.2372.

Determination of Scopoletin Concentrations with TLC-Densitometry

Preparation of Standard Solutions

Scopoletin levels measurement in fermented noni juice extract were carried out using TLC-Densitometry (CAMAG set: TLC-Scanner 4, Linomat 5, twin trough glass chamber, UV cabinet) with the steps of making scopoletin standard solution and then testing the solution. The mother liquor was made with a concentration of 1000 μ g/ml. Then, dilution was created so that the concentration of the standard scopoletin solutions was as follows: 10, 50, 100, and 1000 μ g/ml. The sample solution was made by liquefying 5 mg of the sample in 1 ml of methanol p.a (5000 μ g/ml).

Measurement of Scopoletin Levels

Scopoletin standard and test solutions (2 μ l each) were spotted on the Kieselgel 60 F₂₅₄ TLC sheet (10x10 cm) plates using the Linomat V-CAMAG semiautomatic sampler. The plates were eluted in ethyl acetate: methanol (20:1). After the solvent had risen to the boundary line, the TLC plate was taken and dried. The chromatogram was observed under a UVL₃₆₆, and measured with a densitometer at a wavelength of λ_{366} nm.

α -Glucosidase Inhibition Assay

The alpha-glucosidase inhibition assay of extracts *M. citrifolia*, fractions, and purified compounds was performed according to our previous report.¹⁴ In brief, 5 μ l of samples solution (dissolved in DMSO), 495 μ l of 0.1 M Potassium Phosphate buffer (pH 7), and 250 μ l of p-NPG (5 mM) as the substrate were combined and warmed at 37 °C for five minutes. α -Glucosidase in the buffer (250 μ L of 0.6 U/ml) was then added and the mixture was kept warm for another fifteen minutes. The mixture was finally ended by adding 1000 μ L of Na₂CO₃ (0.2M). The wavelength in this measurement was recorded at 410 nm. The positive control was used quercetin.

Antioxidant Activities

Determination of Phenolic Contents

Folin-Ciocalteu reagents were used to determine the total phenolic content of *M. citrifolia* extract. Still, with minor modifications. Appropriately 0.5 ml of the test sample (1000 μ g/ml) or gallic acid standard solution (50, 100, 150, 200, and 250 μ l) was mixed with 0.5 N of Folin–Ciocalteu reagents (0.5 ml). After it had settled for eight minutes, 1.5 ml of 20 % saturated Na₂CO₃ solution was added to the mixture, and the final volume was increased to 3 ml by adding distilled water accordingly. The mixture was then stored at room temperature in the dark for two hours. Samples that produce color are measured at a wavelength of 765 nm. The gallic acid was used to calculate the phenolic content. The gallic acid equivalents was equivalent to the phenolic content based on the regression equation of the calibration curve ($y = 0.0903 + 0.039x$, $R^2 = 0.99$).

Total Flavonoids Content

Determination of the total flavonoid content of the extract *M. citrifolia* was used with the AlCl₃ colorimetric method.¹⁵ In a 5 ml test tube, 0.5 ml of extract, 2 ml distilled water, 0.15 ml of aluminum chloride (5%), and 0.15 ml of AlCl₃ (10%) were added. Six minutes later, 2 ml of NaOH (1M) and distilled water were added to reach a total volume of 5 ml. The absorbance of the reaction mixtures was measured against a blank at 420 nm. Standard calibration curves were made using the standard quercetin compound. The concentration of total flavonoid content was calculated using the calibration plot ($y = 0.0108x - 0.004$, $R^2 = 0.99$).

DPPH Radical Scavenging Activities

In this study, the DPPH radical scavenging activities of the extracts of the *M. citrifolia* and isolated compounds were evaluated by the previously reported method with a minor modification.^{14,16} Ethanol was used as a solvent to prepare stock solutions of DPPH (1mM). Briefly, 4 ml of extract solution (1–100 μ g/ml) in methanol was added to 1 ml of DPPH (0.1 mM) solution. The mixture was homogenized and kept aside in the dark area for 30 minutes. The mixture recorded the absorbance at a wavelength of 517 nm.

ABTS-Free Radical Reduction Activity

The antioxidant measurements were based on the sample's ability to reduce equivalent ABTS radicals compared to that of Trolox according to the method of Wang.¹⁷ The ABTS solution (7mM) was mixed with potassium persulfate (140 mM) with a ratio of 62.5: 1. Afterward, the solution was stored for sixteen hours in a dark room, so that the ABTS radical solution was produced as the mother solution. For the measurement, an ABTS radical solution is prepared with an absorbance of 0.7 at λ_{734} nm. The test was carried out by mixing 5 μ l sample solution (concentration sample 1 mg/ml) with 200 μ l of the ABTS radical solution. The mixture was then left for 6 minutes in a dark room before the absorption was read at λ_{734} nm. Trolox was used to making the standard calibration curve.

RESULTS AND DISCUSSION

In this study, 40 kg of white *M. citrifolia* fruits with firm flesh were chosen for fermentation, and 1 kg of those was extracted with ethyl acetate. Ethyl acetate and butanol solvents were used to partition the clear liquids produced from the fermented noni fruits during the curing process (2.5 L). EtOAc, BuOH, and H₂O fractions were obtained after all the extracts had been evaporated and dried (Fig.-1). All fractions obtained were analyzed for scopoletin levels and their bioactivity (Table-1, Fig.-2).

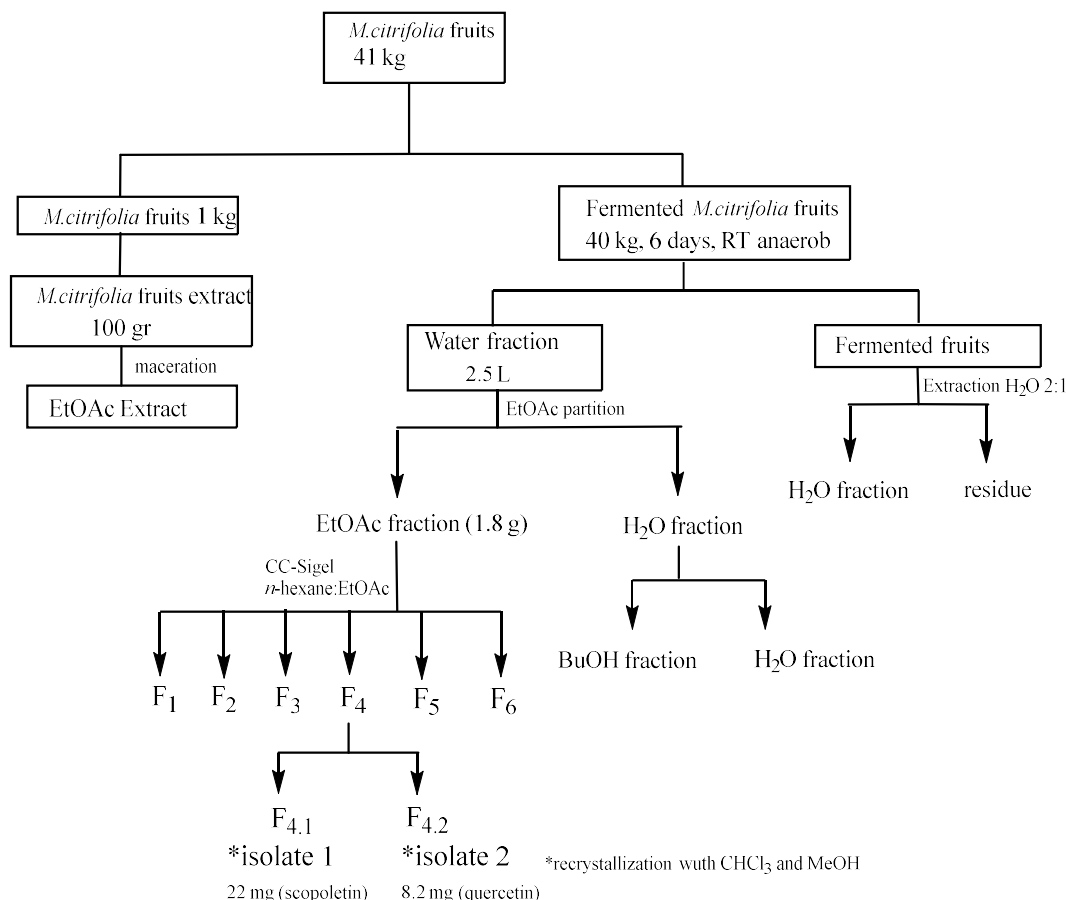


Fig.-1: Schematic Fractionation and Separating Active Compounds from Noni Fruits

One of the *M. citrifolia* fruit content is scopoletin or 7-hydroxy-6-methoxycumarin, a marker compound that determines the quality of noni fruit.¹⁸ In this study, scopoletin levels were determined by TLC-densitometer. This method is more economical, faster, easier to operate, and more reproducible (Fried and Sherma, 1994). Based on the measurement results, the levels of scopoletin in the ethyl acetate fraction of fermented *M. citrifolia* fruits were higher than that of fruit juices and other fractions like the EtOAc extract non-fermented *M. citrifolia* fruits. The total phenol content of the fermented *M. citrifolia* fruits EtOAc extract was much higher than that of the non-fermented EtOAc extract. In contrast, the total flavonoid content of the fermented *M. citrifolia* fruit EtOAc extract was lower than the non-fermented EtOAc extract (Table-1). The bioactivity assay results showed a decrease in antioxidant activity after the fermentation process. It was described that the fermentation process significantly reduced the anti-radical activities. It was reported that the fermentation process of *M. citrifolia* could decrease its anti-radical activities. The antiradical activities reduction could reach up to 90 %. Thus, the fermentation process could be responsible for the differences in the activities that were observed.¹⁹

Here we could show the phytochemical changes of *M. citrifolia* fruits due to the fermentation process. The fermentation process in noni fruit changed the content of its chemical compounds. Only the fermented extracts contained 2 α , 3 β , 4 α -trihydroxy nortropane, heterodendrin, evodiamine, cirsiomaldehyde, fabriatin, 5-hydroxymethyl furoic acid, and 2 α , 3 β , 4 α -Trihydroxy nortropane, according to the

identification results using the QTOF LCMS-MS chromatogram (Table-2 and Fig.-3). Furthermore, the most significant finding was the change observed in the levels of anti-diabetic activities marker in fermented *M. citrifolia* EtOAc extract, which was better than the non-fermented counterpart.

Table-1: Scopoletin Levels and Activity of *M. citrifolia* Fruits Extracts and Fermented *M. citrifolia* Fruits

Sampel	Extract/ Fraction	Scopoletin mg/g extract	TPC (%) GA equivalent	TFC (%) Quercetin equivalent	% Inhibition (200 μg/ml)	
					DPPH	AGIs
<i>M. citrifolia</i> fruits	EtOAc	2.548	3.4	12.8	27.58	53.21
Fermented <i>M. citrifolia</i> fruits	<i>M. citrifolia</i> juice extract	2.15	5.10	3.72	18.73	34.28
	EtOAc	17.26	4.63	6.52	58.80	72.59
	BuOH	3.34	4.67	4.88	33.07	48.96
	H ₂ O	nd	3.35	1.08	0.79	-

nd: not detected

α-GIs : α-Glucosidase inhibitor

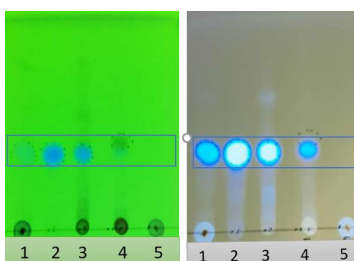


Fig.-2: TLC Profile of Fraction 1-5 (1: *M. citrifolia* Juice Extracts, 2: Scopoletin, 3: Ethyl Acetate Fraction, 4: Butanol Fraction, 5: Water Fraction) Developed With Hexane: Ethyl Acetate (3:1) as Mobile Phases and Visualized Under Ultraviolet Light UVL₂₅₄ (A) and UVL₃₆₆ (B).

Table-2: Chemical Composition of Fermented Liquid, Fraction, and Extract of *M. citrifolia* Fruit

Sample	Components name	Observed m/z	Observed RT (min)
Fermented liquid extract	2α,3β,4α-Trihydroxy nortropane	160.0968	1.18
	Heterodendrin	262.1286	1.37
	Evodiamine	326.1235	1.71
	Cirsiumaldehyde	235.0601	4.28
	Fabiatrin	509.1261	4.70
	Quercetin	303.0499	8.47
	Scopoletin	193.0498	6.64
	Quercetin-3-O-α-L-rhamnosa-7-O-β-D-glucoside	611.1613	5.86
Ethyl acetate fraction	5-Hydroxymethyl furoic acid	143.0337	1.94
	Cirsiumaldehyde	235.0601	4.28
	Methyl gallate	185.0441	4.74
	Achillin	247.1329	8.50
	Quercetin	303.0505	8.57
	Scopoletin	193.0494	6.79
	Quercetin-3-O-α-L-rhamnosa-7-O-β-D-glucoside	611.1616	5.92
Butanol fraction	Quercetin-3-O-α-L-rhamnosa-7-O-β-D-glucoside	611.1615	5.87
	Scopoletin	193.0497	6.64
	Quercetin	303.0503	8.45
Water fraction	2α,3β,4α-Trihydroxy nortropane	160.0970	1.17
	Evodiamine	326.1235	1.75
	Heterodendrin	262.1285	1.30

Ethyl acetate extract	Cirsiumaldehyde	235.0602	4.28
	Fabiatrin	509.1261	4.70
	Scopoletin	193.0493	6.69
	Stigmastan-3,6-dione	429.3722	12.06

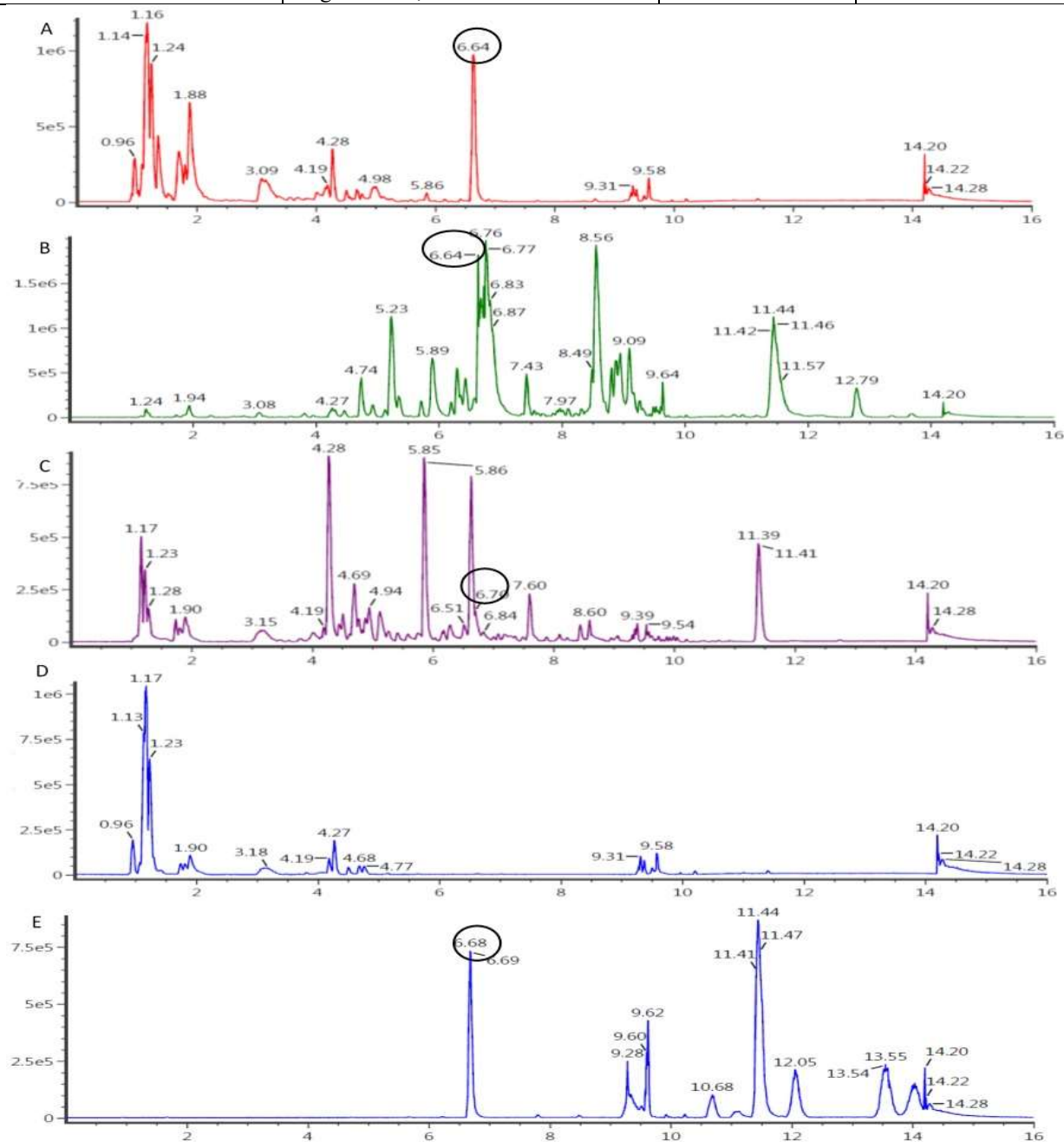


Fig.-3: LC-MS-MS Chromatogram of the Chemical Constituents of Fermented Liquid (A) EtOAc Fraction (B) Butanol Fraction (C) Water Fraction (D) and Ethyl Acetate Extract (E) Scopoletin Rt; 6.64 Or 6.68

Isolation and Characterization of Active Compounds

Column chromatography was used to separate chemical compounds in *M. citrifolia* extract by using hexane and ethyl acetate as mobile phases with a 1:1 ratio. It produced six fractions (F1-F6) based on the stain spot pattern on TLC. After testing the activity of each fraction obtained, the F4 showed a strong activity as both antioxidants and α -glucosidase inhibitors (Table-3). Further purification by recrystallization with the CHCl_3 solvent and methanol yielded white needle crystal (compound **1**) from F4, and bright yellow precipitate (compound **2**) from F4, as shown in Fig.-1.

Structure Elucidation

The results of LCMS and NMR measurements for isolate **1** and isolate **2** showed that the chemical structure could be. Isolate **1** was identified as a scopoletin compound while isolate **2** was identified as a quercetin compound. The structures of compounds **1** and **2** are shown in Fig.-4.

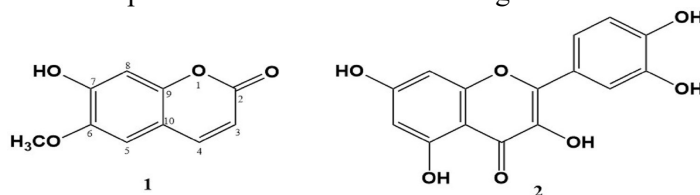


Fig.-4: Chemical Structure of Scopoletin Compounds (**1**) and Quercetin (**2**)

The measurement results of the LCMS analysis of isolate **1** showed a retention time of 2.9 minutes with a single peak. The results of the LCMS spectrum indicated a single peak at $[M+H]^+$ at m/z 193.03. This result was the same as that shown in theory for $C_{10}H_8O_4$ $[M+Na]^+$ at m/z 215.02. The m/z value of isolate **1** was identical to the scopoletin compound which was the main compound of *M. citrifolia* fruit. Further characterization of isolate **1** with 1H showed a singlet chemical shift for methyl at and ^{13}C NMR, further affirming that isolate **1** is a scopoletin compound by the appearance of a chemical shift identical to scopoletin. Scopoletin (**1**) showed a weaker antidiabetic activity than quercetin (**2**) with values of IC_{50} 61.93 and 21.65 μM , respectively. Quercetin compounds also showed that the DPPH free radical scavenging activities were far more active than scopoletin compounds, with IC_{50} of 35.23 and 844.06 μM . Based on the results of this study, scopoletin compound was found to be responsible for α -glucosidase inhibition. Antioxidants from *M. citrifolia* juice, and quercetin, which has been widely reported as a compound that can inhibit the increase in post-meal blood sugar levels (postprandial hyperglycemia), were also acting as antioxidants.²⁰ Nurul has also reported a scopoletin isolated from the herbal *Artemisia capillaris* showing antidiabetic activities with an IC_{50} of 159.16 μM , equivalent to 30.56 $\mu g/ml$.²¹ It has been reported that there is a change in the anti-radical activity of the *M. citrifolia* due to fermentation. The fermentation process reduces anti-radical activity. The α -glucosidase inhibitor activity of noni juice is possible due to the presence of phenolic compounds including flavonoids which were known to provide a significant antidiabetic activity.²² Thus, fermentation is responsible for the differences observed in the activities that occur.²³ This is consistent with the report by Lohani showing that noni has an anti-diabetic activity because it can stimulate the pancreas to release more insulin, owing to the stimulation of the extract on Langerhans β -cells.²⁴ The increase in insulin levels can be affected due to the phytochemical content of the extract. It can also modulate transcription factors and control the gluconeogenesis process. *M. Citrifolia* can inhibit the gluconeogenic genes, phosphoenolpyruvate C kinase, and glucose-6-phosphatase, which are naturally regulated by insulin.²⁴

CONCLUSION

Based on these results, scopoletin and quercetin can be used not only as biomarkers for quality control but also contributed to the pharmacologic activities of products produced from fermented *M. citrifolia* juice extract.

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