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A Study on the Antioxidant and Active Compounds of *Gardenia jasminoides* Ellis (GJE) Leaves Extract

I.B.K.W. Yoga[1*], D.N. Suprapta[2], I.M. Jawi[3] and I.D.G.M. Permana[4]

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ABSTRACT

Purpose: *The aim of this study was to isolate and identify the active compound antioxidant fractions from Gardenia jasminoides Ellis leaves.*

Research Method: *The antioxidant activity was determined using DPPH 0.1 mM (1:1), and was analyzed spectrophotometrically. In addition, separation was performed using column chromatography. The most active isolate was identified by UV-Vis, FT-IR, and LC-MS/MS. The quantitative analysis was done in three replicated.*

Findings: *It was discovered that the antioxidant activity in the semi-polar fraction (IC_{50} = 62,50 ppm) was higher than both the polar and non-polar fractions. Combining 295 isolates via column chromatography, 11 combined isolates were obtained (fA-fK). The fK isolate had the highest antioxidant activity of 90.38% and IC_{50} 15.74 ppm. It also obtained 2 maximum wavelengths (336 and 275 nm) and could be categorised as flavonoid (flavonol with 3-OH substituted). The FTIR analysis also showed that the fK isolate had functional groups found in flavonoid. Finally, the identification of the fK isolate by LC-MS/MS showed that the compound at retention times of 5,91 and 5,06 minutes were kaempferol 3-O-rutinoside and rutin, respectively.*

Originality/ Value: *Based on the obtained results, it was concluded that GJE leaves have the potential to be developed as a new and inexpensive source of flavonoids.*

Keywords: *antioxidant, flavonoid, Gardenia jasminoides Ellis, kaempferol, rutin*

INTRODUCTION

Bioactive compounds found in plants have physiologic effects, similar to herb medicine (Vandana *et al.,* 2017). There are many plants that are yet to be developed as functional food or pharmaceutical products from the complex of secondary metabolic yield. One such plant is *Gardenia jasminoides* Ellis (GJE), which has the potential to be developed as a source of antioxidant, especially using its leaves. GJE grows well throughout the year in Indonesia, and it has many regional names including *kacapiring, cepiring, peciring, ceplok piring, raja patih, jempiring, meulu bruek*, and *sangklapa*. In Indonesia, the plant is unable to produce any fruits; however, its flower is used as the symbol of Denpasar. In China, the GJE fruit is used as a food coloring, and it also functions as an anti-oxidant, anti-hyperglycemic, anti-inflammatory, anti-atherosclerotic, anti-cancer, anti-arthritis anti-apoptotic, anti-thrombotic, anti-angiogenic, anti-microbial and in several miscellaneous activities (Phatak, 2015). The chemical compounds of the fruit have been detected as genipin, geniposide,

[1] *Faculty of Mathematic and Natural Science, Udayana University, Bali, Indonesia*
anargya696@gmail.com
[2] *Faculty of Agriculture, Udayana University, Bali, Indonesia*
[3] *Faculty of Medicines, Udayana University, Bali, Indonesia*
[4] *Faculty of Agricultural Technology, Udayana University, Bali, Indonesia*
https://orcid.org/0000-0002-4598-7238

crocin, and crocetin (Xiao *et al.,* 2016). Meanwhile, the phytochemical of GJE leaves contains flavonoid, alkaloid, saponin, tannin, steroid, and terpenoid (Kesavan *et al.,* 2018). Some of them are of phenolic compound with antioxidant properties, in which the antioxidant activity is related to the hydroxyl group in ring B (Airoldi *et al.,* 2018). In a previous study, flavonoids (e.g., quercetin), glycosides (e.g., rutin), and simple phenols (e.g., gallic acid), which were isolated from *Capparis spinosa*. L leaves ethanol extract, showed antidiabetic active ingredients (Hussain *et al.,* 2017). According to Kim (2020), the high content of flavonoid in GJE has beneficial effects on inflammation, metabolic diseases, and cancers. In addition, it does not affect the viability of normal astrocytes. On the other hand, gallic acid is able to protect against oxidative damage and chemo-preventive agents (Mutalib, 2016). *Morus alba* L. leaves extract which was used as tea contained five phenolic compounds, namely 4-O-Caffeoylquinic acid, 5-O-Caffeoylquinic acid, gastrodin, iso quercetin, and rutin, showed antioxidant activity against 2,2 diphenyl-1-picrylhydrazyl radical (Ganzon *et al.,* 2017). Meanwhile, rutin is a flavone derivative acting as an antioxidant by strengthening blood cell walls (Sun and Ho, 2005). Kaempferol-3-rutinoside has been known to have anti-adipogenic potential and α-glucosidase inhibitor (Jang *et al.,* 2016).

Bioactive plant components can be detected through extraction, isolated by fractionation, and identified to obtain active fraction potency. In the existing literature, there are not many reports on the isolation and identification of the bioactive compounds in GJE leaves, especially in the active fraction. A review by Shan *et al.* (2017) on the phytochemical, pharmacologic, pharmacokinetics, and toxicology of the geniposide ($C_{17}H_{24}O_{10}$) specific in *rubiacea* showed iridoid glycoside, which has the biological activity to function as an antiinflamatory, antioxidant, antidiabetic, and neuroprotective. Accordingly, given that GJE has geniposide in its roots, leaves, flowers, and fruits. The flavonoid group was isolated on *Gardenia sessiliflora* leaves and twigs extract (Thanasansurapong *et al.,* 2020), was detected to have two new flavanones; 5,3′,5′-trihydroxy-6,7,4′-trimethoxyflavanone and 5-hydroxy-6,7,3′,4′,5′-pentamethoxyflavanone, where they both have anti-HIV-1 activity. It was studied, to isolate and identify its antioxidant active compound in fractions, particularly using the crude extract of GJE leaves.

MATERIAL AND METHODS

Materials and chemicals

The materials and chemicals used in this study included ethanol absolute 99,9% (Merck), hydrochloric acid 37% (Merck), sodium hydroxide (Merck), sulfuric acid 96% (Merck), methanol (Merck), gallic acid (Sigma Aldrich), Folin-Ciocalteu (Merck), sodium carbonate (Merck), chloroform (Merck), silica gel (Merck), eisen (III) chloride hexahydrate (Merck), and aluminum chloride hexahydrate (Merck). In addition, petroleum ether, 2-propanol from JT Baker, and quercetin from Sigma Aldrich were also utilized. The instruments used were spectrophotometer (Biochrome S26), FTIR (Shimadzu), and LC-MS/MS (Shimadzu).

Preparation of extracts

Dried GJE leaves were collected from the Abiansemal district of Bali province, and were subsequently ground into powder 40 mesh sieve. The powder (100 g) was then extracted with ethanol 96% (1:10) at room temperature (RT) for 24 h and filtered (Jamei and Anvari, 2018) with slight modifications. The residue was extracted two more times in the same manner. The mixture was then further filtered, before it was evaporated at 40 ^{o}C, 50 mBar, 100 rpm in a vacuum rotary evaporator, and was finally weighed to obtain its total crude extract. The crude extract (% w/w) was calculated by [total extracted sample weight (g)/total dry sampel weight (g)] x 100 and the water content before it was stored in a cool storage at 4^{o}C until it was used.

Determination of total phenolic content (TPC)

Total phenolic content (TPC) of the extract was obtained using a spectrophotometer (Chiu *et al.,* 2013) with some minor modifications using the FC (Folin-Ciocalteu) reagent. Briefly, 10 mg of each crude extract was diluted in methanol and various concentrations of gallic acid (0-100 ppm), as a standard, in distilled water. A sample or standard (200 µL) was mixed with 200 µL of the FC reagent. The mixture was firstly incubated (5 min) at RT, followed by the addition of 800 µL of 5% sodium carbonate solution and was then incubated again (30 min). The absorbance value was measured with UV-Vis spectrophotometer λ 760 nm. Finally, TPC was calculated from the standard as a percentage of gallic acid equivalents (GAE) per 100 g.

Determination of total flavonoid content (TFC)

Total flavonoid content (TFC) was calculated using aluminum chloride colorimetric assay (Chiu *et al.,* 2013). The crude extract (10 mg) was dissolved in 5 ml ethanol, while the quercetin was set in a range of 0-20 µg/mL, as a standard. A sample and standard (1,0 ml) was mixed with 1.0 ml of 2% $AlCl_3 6H_2O$. The absorbance was measured at λ 415 nm after it was incubated at RT (10 min). The TFC was calculated as a percentage of quercetin equivalents (QE) per 100 g sample.

Determination of activity and antioxidant capacity

The effect of crude ethanol extract and the positive control to DPPH radical 0.1 mM (1:1) were determined by a spectrophotometer (Huang *et al.,* 2010). 500 uL of different concentration samples were mixed with 500 µl DPPH 0.1 mM solution, and was then incubated at RT for 30 min. The absorbance value was measured at λ 517 nm. The control methanol was prepared using the same procedure. DPPH radical-scavenging activity (%) was calculated using the following equation:

=[(absorbance control-absorbance sample)/ absorbance control] x 100. The value IC_{50} was obtained by interpolation using linear regression analysis. The antioxidant capacity in the sample was calculated using gallic acid (0.1;0.4;0.8 and 1.2 µg/mL), respectively, with the unit (mg) of gallic acid equivalent to the antioxidant capacity (GAEAC)/100 g crude extract. The antioxidant activity index (AAI) was calculated by comparing the DPPH concentration (µg/mL) and IC_{50} (µg/mL) (Vasic *et al.,* 2012).

Fractionation and isolation of crude ethanol extract

The crude extract was fractionated liquid-liquid using n-hexane, ethyl acetate, and distilled water, with a separating funnel to obtain non-polar, semi-polar, and polar fractions (Abu *et al.,* 2017). The fractions were evaporated and both their flavonoids and antioxidant activity were determined. The fractions with the highest levels of flavonoids and antioxidants were separated by a thin layer chromatography (TLC) using 10 types of combined solvents, column chromatography, and combined TLC to obtain fraction isolates with the same RF value (Georgeta *et al.,* 2016). The combined isolates were subsequently analyzed for their antioxidant activity. The highest antioxidant activity was screened and identified for its active compounds using UV-Vis, FTIR, and LC-MS/MS.

Column chromatography

Approximately 60 g of silica gel was dried in an oven at 105 °C, which was added with a little mobile phase until it was slurry, and was put into the column. The mobile phase was eluted until complete compression of the stationary phase occurred. The fraction with the highest antioxidant activity and flavonoid levels was dissolved with the best mobile phase, entered in the column, and the flow of the mobile phase was adjusted to 1 mL/minute. The mobile phase was then added continuously until separation occurred. Eluate was collected in a container from the fractions after every 4 mL. The eluate obtained was observed by its stain pattern on

the TLC. Eluate with the same stain pattern was combined and then evaporated (Xiao *et al.,* 2016).

Identification of fraction with a high value of TFC and antioxidant activity by color test, UV-Vis by chemical reagent, FTIR, and LC-MS/MS

Active isolates were color tested to determine the class of the flavonoid compounds through Wilstater reagent, Bate Smith-Metcalfe, 10% NaOH. The active isolate (1.0 mg) was diluted in 5 mL of methanol, and the color absorption was measured using a UV-Vis spectrophotometer (250-500 nm). The position of the hydroxyl groups on the flavonoid core can be determined by adding a shear reagent, 10% NaOH, $AlCl_3$5%, NaOAc, NaOAc + H_3BO_3. If decomposition occurs, the spectral measurement is done by adding 5 drops of boric acid powder, which is then saturated with NaOAc + H_3BO_3, before measuring the wavelength.

The wave number of the active isolate in methanol was measured using a FTIR spectrophotometer and was identified using LC-MS/MS (Garcia *et al.,*2018), with the UPLC-MS-TOF method (Xevo G2-S QT Model, Waters, USA). 5,0 µL of the sample was injected into the ACQUITY UPLS @BEH C_{18} column (1.7 µM; 2.1 X50 MM). The mobile phase was used, in which acetonitrile and 0.05% formic acid functioned as solvent A, while solvent B was consisted of water and 0.05% formic acid, with a flow rate of 0.2 mL/minute. A mass range of 50-3000 m/z positive ions were recorded. The data was then processed with the Masslynx software to obtain the molecular formula from the positive ions and their fragmentation. The structure of the compounds was based on readily available online database websites (www. chemspider.com or www.massbank.go/QuickSearch.html).

Statistical analysis

The analysis of each sample was run in three replicates. All data are presented as means ± standard error means (SEM).

RESULTS AND DISCUSSION

Phytochemical crude extract of the kacapiring leaf powder

Water content is one of the important parameters when discussing simplicia powder requirements, which should be less than 10%. From drying 500 g of fresh GJE leaves at 45 ^oC for 24 hours, the powder was passed through a 40 mesh sieve, and was found to contain 7.60% water content. Extraction by maceration resulted in a thick extract of 9.39%. Phytochemical results (Table 01), showed that the total phenolic content (5,225.78 mg/100 g as gallic acid) was higher than flavonoid (1,622.66 mg /100 g as quercetin). The antioxidant capacity to DPPH 0,1 mM radicals (1:1) was 15,160.64 mg GAEAC/L, and the IC_{50} analysis was 74.54 ppm, with a moderate antioxidant activity criteria. The TFC and TPC of the ethanol extract of the gardenia fruit were 20.7 mg catechin/100 g and 44.8 mg GAE/100 g, respectively (Debnath *et al.,* 2011). Therefore, under the conditions of 70 °C at 28.6 min, the extracted ethanol from 70% GJE fruit managed to yield TPC (2497 mg) (Yang *et al.,* 2009), which was lower than the leaf extract. Likewise, the antioxidant activity against DPPH radicals (1:1) obtained a IC_{50} value of 360 ppm. This demonstrated that the GJE leaves had more potential as a source of antioxidant.

Liquid-liquid fractionation

Liquid-liquid extraction is one of the several methods applied to enrich and separate flavonoids in leaves with a mechanism of "like dissolves like" to solvent. The results of the liquid-liquid fractionation (Table 2), showed that the highest yield of 36.46% was obtained in the semi-polar fraction (ethyl acetate solvent). The semi-polar fraction also attained the highest levels of flavonoids, total phenolic and antioxidant capacity, and therefore, the ethyl acetate fraction was chosen as the best fraction to be identified and fractionated by column chromatography. According to Rowe *et al.* (2009), ethyl acetate is a good solvent for extraction because it can easily evaporate, not hygroscopic, and has low toxicity. Furthermore, in a study by Hussain *et*

al. (2017), it was effectively used as an organic solvent for glycosides extraction in *Capparis spinosa*. L leaves.

Huang *et al*. (2016) explained that persimmon leaves extracted using ethyl acetate was mainly consisted of flavonoids and triterpenoids. The identification of the compounds in the semi-polar fraction was separated using the TLC technique, in which it used ten combinations of mobile phase (Table 03).

Table 01: Water and phytochemical content of the crude ethanol extract of GJE leaves

Components	Unit	Values ± SD
Water content simplicia powder	% wb	7.60 ± 0.07
Extract yield	% wb	9.39 ± 0.08
Water content crude extract	% wb	37.47 ± 0.16
Total flavonoid content	mg QE/100 g wb	1,622.66 ± 8.68
Total phenolic content	mg GAE/100 g wb	5,225.78 ± 6.47
Antioxidant capacity	mg GAEAC /L wb	15,160.64 ± 206. 26
IC_{50}	ppm	74.54 ± 0.40
AAI		0.54 ± 0.00

QE = quercetin equivalents, GAE= gallic acid equivalents, GAEAC=gallic acid equivalents antioxidant capacity, AAI = antioxidant activity index (weak <0.5, moderate 0.5-1, strong 1-2 and very strong> 2)

Table 02: Phytochemical non-polar, semi-polar and polar fractions of the ethanol extract GJE leaves

Component	Unit	Values ± SD		
		Non polar fraction	Semi polar fraction	Polar fraction
Extract yield	% wb	26.10 ± 1.39	36.46 ± 1.05	29.24 ± 0.83
Total flavonoid content	mg QE/100 g wb	4,878.30 ± 30	25,209.96 ±156.37	19,637.46 ± 636.12
Total phenolic content	mg GAE/100 g wb	21,547.48 ± 967.78	35,863.47 ± 456.79	20,000.42 ± 177.13
Antioxidant capacity	mg GAEAC /L wb	5,363.13 ± 635.35	8,688.93 ± 267.26	281.77 ± 10.45
IC_{50}	ppm	75.56 ± 0.83	62.50 ± 1.45	435.40 ± 5.40
AAI		0.53	0.64	0.09

Table 03: The type of mobile phase for separation in semi-polar fraction

No	Mobile phase	Number Spot	Rf value	Separated
1.	N-hexane : ethyl acetate (6,5 : 3,5)	8	0.07;0.12;0.19;0.25;0.51;0.65;0. 70;0.95	good
2.	Water : butanol: acetic acid: (5:4:1)	4	0.49;0.58;0.61;0.92	not good
3.	Methanol: n-hexane (4:6)	1	0.96	not good
4.	Methanol: chloroform (2:8)	1	0.95	not good
5.	Butanol: water : ethyl acetate (4:2:1)	2	0.92;0.98	not good
6.	Methanol: ethyl acetate (8 :2)	2	0.89;0.96	not good
7.	Ethyl acetate: water: formic acid: acetic acid (10: 2,6:1:1)	4	0.85;0.90;0.95;0.98	not good
8.	Chloroform: ethyl acetate (6,5:3,5)	2	0.13;0.98	not good
9.	Ethyl acetate: butanol: acetic acid (5:3:2)	3	0.59;0.85;0.91	not good
10.	n-hexane: chloroform: ethyl acetate (6:1,5:1)	12	0.09;0.16;0.26;0.35;0.41;0.52;0.5 6;0.65;0.73;0.87;0.92;0.94	very good

The best separation was obtained using a mixture of n-hexane: chloroform: ethyl acetate (6:1.5:1) with 12 well separated spots, which were selected to separate in the column chromatography.

Column chromatography semi polar fraction with the best mobile phase

The fractionation by column chromatography obtained 295 fractions while using silica gel (SiO_3) as a solid phase. The collected fractions were identified by TLC to combine the fractions with the same spot and retention factor (Rf) value. By combining the 295 fractions, 11 fractions were attained (Table 4).

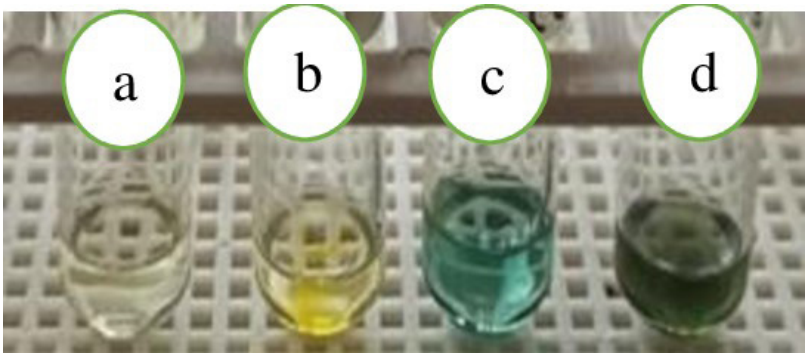
The antioxidant activity analysis of the 11 collected isolate fractions showed that fK isolate had the highest percentage of 90.38% (Table 04). All the isolate fractions had an active antioxidant, which was confirmed by the changing of the DPPH 0.1 mM solution color from purple to yellow. The IC_{50} value was 50%. Furthermore, the IC_{50} value of the fK isolate was compared with gallic acid as the standard antioxidants, 15.74±0,3 and 1.01±0.01 ppm, respectively, in which the classified antioxidant activity of fK isolate was very strong because the antioxidant activity index was greater than 2 (2.59 and 38.80, respectively). The ethyl acetate extract of "puspa" leaves was the most active as an antioxidant, compared to the other solvents (water, n-hexane, methanol, and buthanol). The obtained IC_{50} values were 11.44; 171.94; 257.92; 17.57, and 12.55 ppm. In addition, the IC_{50} of the active isolate after fractionation by column cromatography was 48.04 ppm (Widiyarti *et al.,* 2018), which was different from the antioxidant activity of the GJE isolates. This showed that it was stronger than the fractionation yield, although the value was still higher than IC_{50} gallic acid and quercetin (9.35 ppm). This was probably due to the isolated active component showing synergistic properties to reduce DPPH radicals.

Identification of active isolate fK by color test

The identification of compounds in fK isolate with sodium hydroxide, hydrochloric acid, Mg powder (Wilstater reagent), sulfuric acid, and heating (Bate Smith-Metcalfe) showed that a certain color was formed (Figure 01). Specifically, with the addition of 5% NaOH, the color of the isolate began to turn yellow, while with the addition of HCl 37% and Mg powder, the colour was blue. In addition, it also reacted with H_2SO_4 plus heating, in which a greenish yellow color was obtained. These results demonstrated flavonoid compounds (Vimalkumar *et al.,* 2014). Strong acid was also mixed into the isolate solution, which was then hydrolized (glycoside-flavonoid to aglycone-flavonoid). Subsequently, yellow color was observed in the leaves, which indicated flavones or isoflavones (Saptarini *et al.,* 2016).

Table 04: The fractionation result from the incorporation of semi-polar fraction chromatography column and antioxidant activity

No	Collected fractions	Spot value	Rf value	Antioxidant activity (%)
1	fA (1-29)	1	0,97	12,64 ± 0,27
2	fB (30-49)	1	0,92	8,17 ± 0,17
3	fC (50-65)	1	0,82	12,41 ± 0,10
4	fD (66-76)	-	-	6,67 ± 0,20
5	fE (77-89)	1	0,62	1,97 ± 0,10
6	fF (90-114)	1	0,50	8,64 ± 0.36
7	fG (115-159)	1	0,48	2,20 ± 0,27
p8	fH (160-184)	1	0,42	5,22 ± 0.46
9	fI (185-193)	7	0,17;0,25;0,35;0,47;0,52;0,69;0,80	8,52 ± 0,30
10	fJ (194-269)	9	0,16;0,25;0,30;0,36;0,49;0,55;0,65;0,80;0,82	24,12 ± 0,27
11	fK (270-295)	9	0,16;0,20;0,26;0,35;0,49;0,55;0,59;0,67;0,83	90,38 ± 0,10

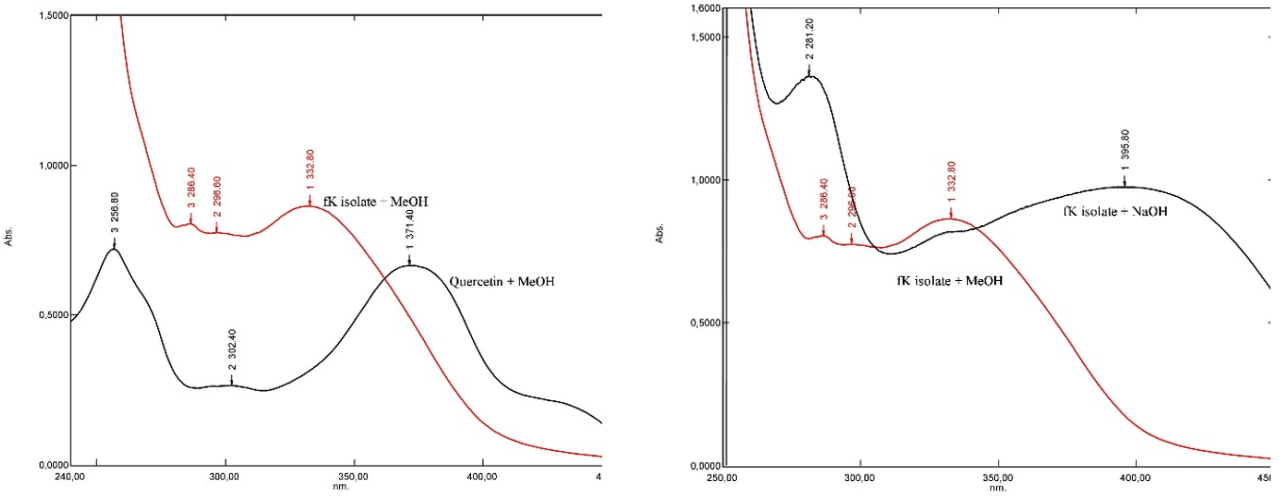


a = sample of the most active isolate in methanol, b = active isolate + 10% NaOH, c = active isolate + Wilstater reagent, d = active isolate + Bate Smith-Metcalfe reagent

Figure 01: The results of identification of active isolates by color test

Shifting of wavelength of the fK isolate by UV-Vis spectra with shear reagent

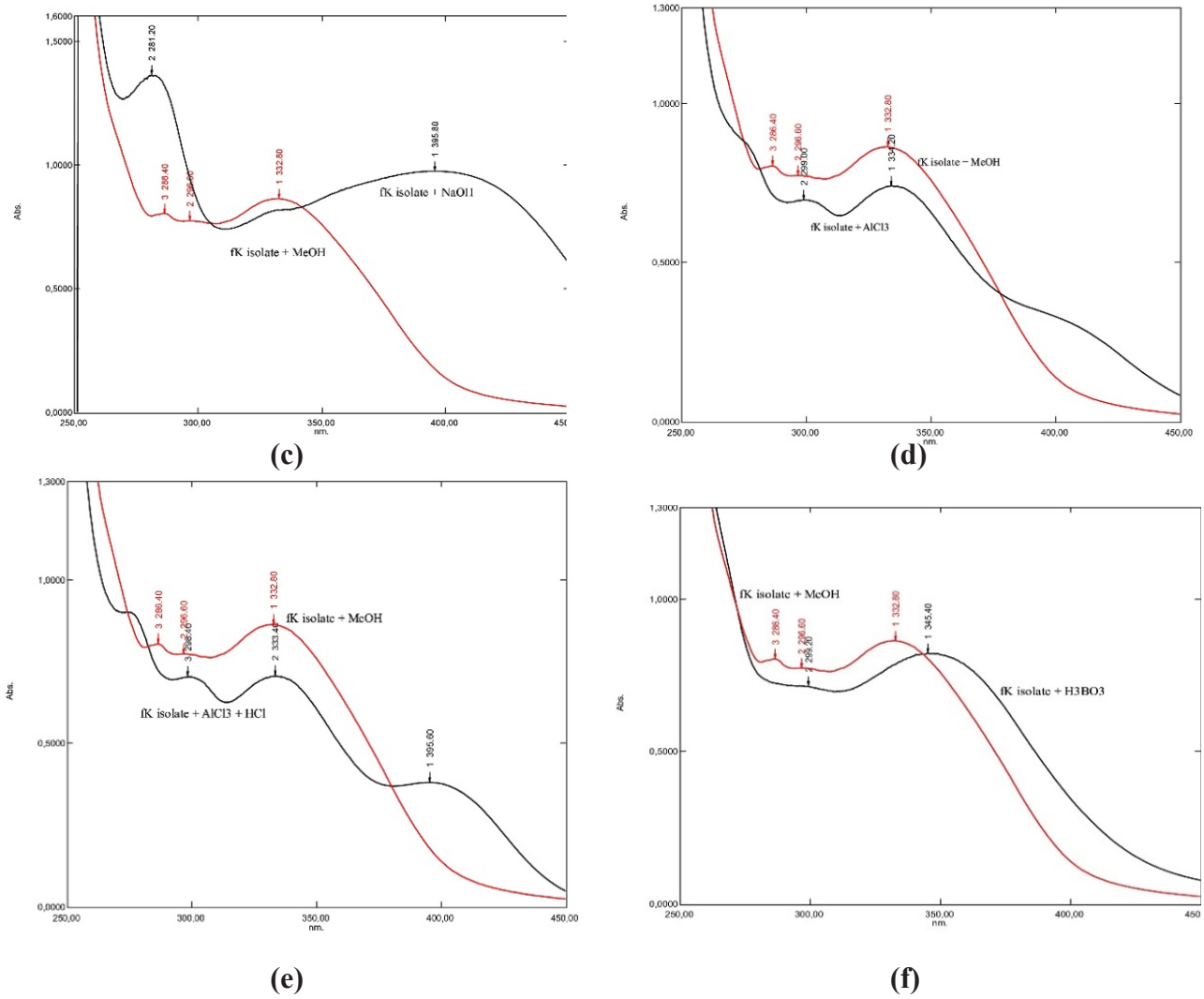
The addition of shear reagents into the active isolate was to identify the interpretation of the flavonoid group using chemical reagents such as sodium hydroxide (NaOH), aluminum chloride ($AlCl_3$), hydrochloric acid (HCl), sodium acetate (NaOAc), and boric acid (H_3BO_3). The shift in wavelength of the pure flavonoid isolates with the addition of the shear reagents can be seen in Figure 02. The UV-Vis readings of fK isolate in methanol obtained two maximum wavelenghts of 332.2 (band I)/ 286.2 (band II), which are known as flavonoids in the flavonol group. Haggag (2021), identified a compound in the leaves of *Catharranthus roseus* (L) G. Don which had wavelengths at 255 and 360 nm detected as flavonols. The maximum absorption that occurred at the wavelength indicated by band II was due to the electronic transition from n - π *, which indicated that there was a C=O bond, as a single chromophore group, while the maximum absorption that occurred at the wavelength shown by band 'I' owed to the excitation of electrons from π - π *, which indicated a conjugated C=C chromophore group.The compounds with a UV-Vis spectrum in band II (λ max 250-280 nm) and band I (λ max 330-360 nm) were flavonol group (substituted 3-OH).



(a) **(b)**

(a), fK isolate + NaOH (b), fK isolate + NaOAc

Figure 02: The spectrum of isolate active (fK) with the share reagent, fK isolate and quercetin in methanol



solate + NaOH (b), fK isolate + NaOAc (c), fK isolate +$AlCl_3$ (d), fK isolate + $AlCl_3$ + HCl (e), fK isolate+ H_3BO_3 (f)

Figure 02: The spectrum of isolate active (fK) with the share reagent, fK isolate and quercetin in methanol

Sodium hydroxide is a strong base that is used to detect the presence of unsubstituted hydroxyl groups. The occurrence of degradation or reduced absorption over time is an indication of a sensitive group to bases. The results of the addition of NaOH to fK isolate illustrated a clear bathochromic shift in band 'I' (+66,73 nm), and this indicated the presence of a hydroxyl group at the position of atom C number 4' on ring B. The addition of NaOAc reagent reacted by ionizing the most acid resistant hydroxyl groups of flavonoids, the 7-OH group, which then caused a bathochromic shift in band II (5-20 nm). Also, the addition of NaOAc reagent in the fK isolate showed a bathochromic shift in band 'I' (+2.80 nm) and 'II' (+19 nm), which indicated the presence of groups 6, 7 or 3', 4' diOH. Moving on, the addition of aluminum chloride formed a complex with an ortho-dihydroxy or hydroxy ketone group. Meanwhile, the addition of HCl caused the complex to break down again due to the unstable Al formed in the ortho-dihydroxy group. Next, the addition of aluminum chloride resulted in a bathochromic shift of 2.67 nm in band 'I' and 12.93 nm in band 'II', while the addition of hydrochloric acid showed a bathochromic shift of +1.47 nm in band 'I' and +12.87 nm in band 'II'. This indicated a hydroxy group on ring A, namely at C-5, and an ortho dihydroxy group on ring B, while the addition of H_3BO_3 related the two hydroxyl groups in the ortho-dihydroxy group to be a borate complex, in which there was a bathochromic shift in band 'I' and band 'II' (+14.07 nm and +7.47 nm) respectively. Based

on the identification results of fK isolate with a UV-Vis spectrophotometer from the addition of shear reagents, it could be assumed that the content of flavonoid compounds belonged to the flavonol group.

Observations of fK isolate using FTIR and LC-MS/MS spectrophotometer

Infrared spectrum can be referred as the molecular fingerprint of a compound. A compound has different bonds and vibrational frequencies, giving rise to a specific spectrum for each compound. Screening is performed at wave numbers 4000 to 667 cm^{-1} (2.5-15 nm). The fingerprint spectrum of the compounds lies in the area below 1200 cm^{-1}, which is caused by the vibrations of the whole molecule, while the vibrations of functional groups or chemical bonds occur in the spectrum of above 1200cm^{-1}. The results of the analysis with an IR spectrometer is illustrated in Figure 03.

The spectra FTIR showed that the fK isolate had a functional group (Figure 03), including the wave number 3666,84 cm^{-1}, which showed absorption as stretching vibration of –OH, thus supporting the presence of flavonoid compounds that had free and reinforced OH groups by bending the vibration of alcoholic C-O (1054,14 cm^{-1}). The vibration at 2968,58-2860,56 cm^{-1} indicated the presence of alifatic C-H stretching. The characteristic aromatic ring C=C, was shown by the absorption at the wave number 1528,65 cm^{-1} and, 1708,04 cm^{-1} was carbonyl ring stretch C=O . This is a chromophore group that is common in flavonoids in the conjugated bond system (Parwata *et al.,* 2018). Based on the results, the fK isolate was assumed as flavonoid compounds 3,5,7,4 'tetrahydroxy flavonol, and 3,5,7,3,4' penta hydroxy flavonols.

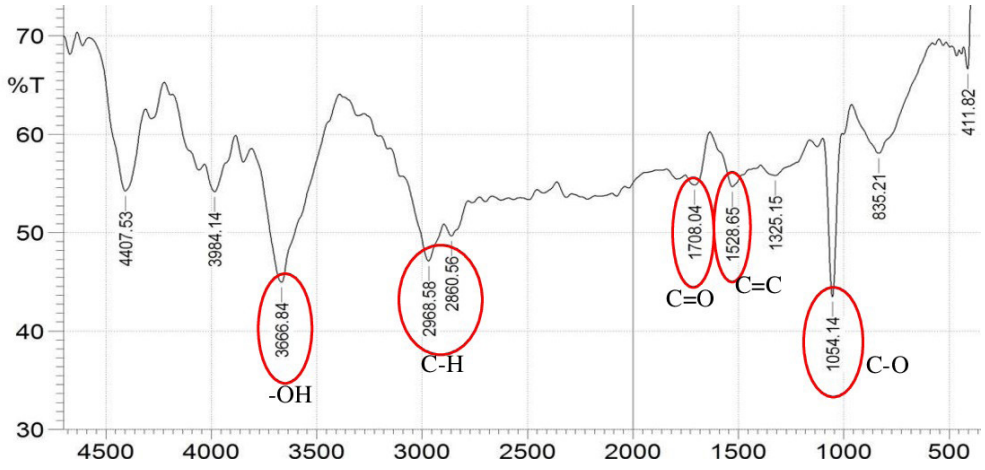


Figure 03: FTIR Spectra of fK isolate

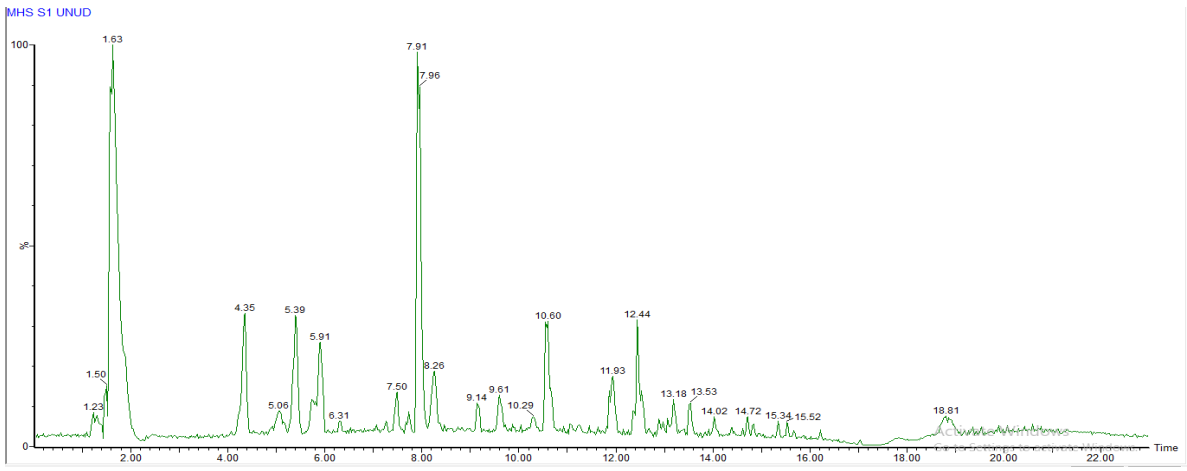


Figure 04: Chromatogram of fK isolates based on retention time (minutes)

Besides, LC-MS/MS was also used to identify the fK isolate compound with a chromatogram and fragmentation pattern. The sugar groups in the glycosylated flavonoids were also broken-down, which served as additional evidence in relation to the sugar groups that were bound to the flavonoid base framework. The chromatogram pattern showed that there were peaks at the retention time of 5.91 and 5.06 (minutes) (Figure 04), which were thought to be flavonoids.

The retention time mass spectrum of the fK isolate was at 5.91 minutes (Figure 05), which showed the peak of the precursor ion with $(M + H)^+$ 595.1650 ($C_{27}H_{30}O_{15}$) and product ion with m/z 287.0547 ($C_{15}H_{10}O_6$) (base peak). The peaks were with m/z 449.1067 $[(M + H)^-146]$, m/z 287 $[(M + H)^- 146\text{-}162]$ and their ion fragmentation. Based on this identification, the active compound in fK isolate demonstrated antioxidant potency is flavonoid compound in the flavonol group, namely kaempferol 3-O-rutinoside. The same compound was previously detected in banana leaves (Yingyuen, 2020). Kaempferol 3-O-rutinoside was also previously isolated and purified from aerial parts of *Anchusa italica*, which was found to contain both antibacterial and antifungal active ingredients (Osw and Hussain, 2020).

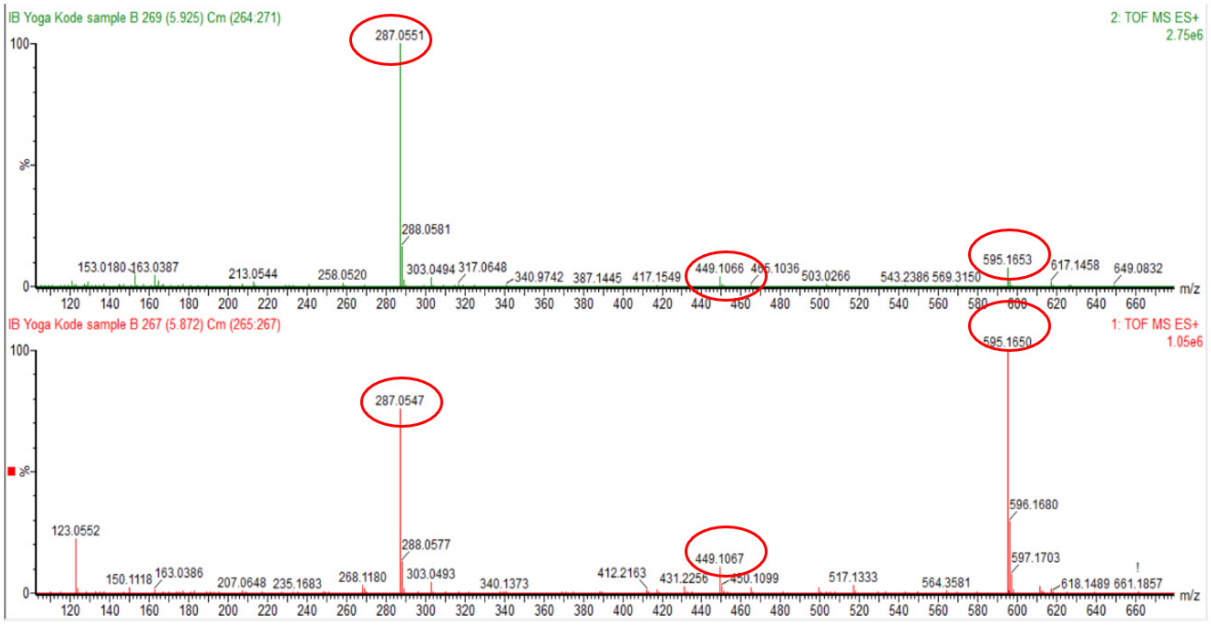


Figure 05: Mass spectrum of the compound at retention time (tR) of 5.91 minutes.

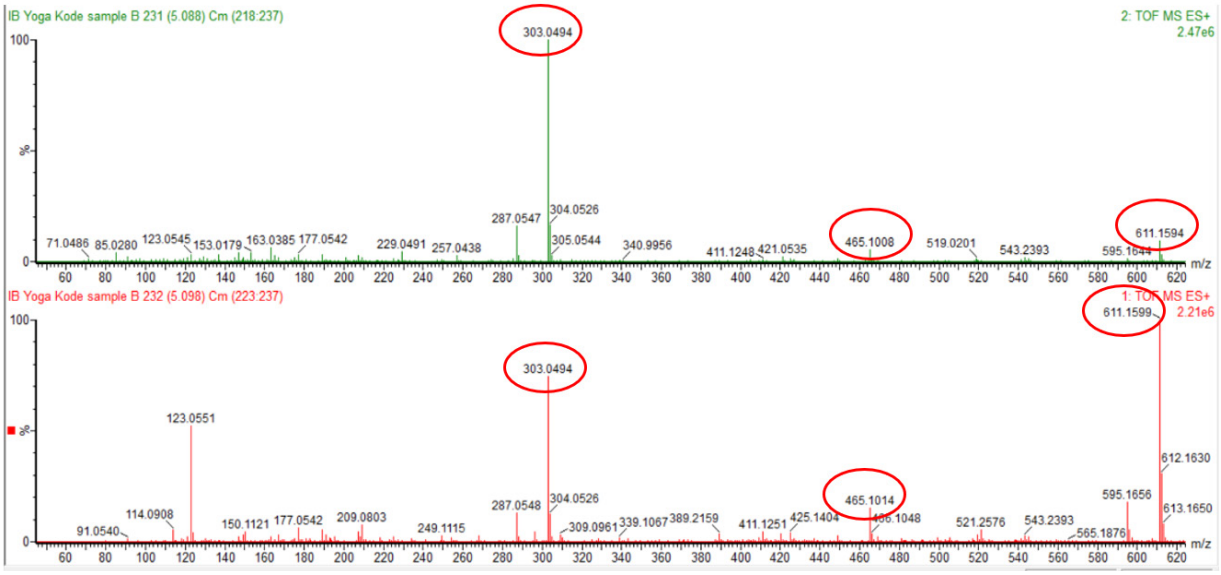


Figure 06: The mass spectrum of the compound at retention time (tR) of 5.06 minutes

Identification of compounds at 5,06 minutes (Figure 06) saw peak spectrum appearing with m/z 465 $[(M + H)-146]^+$ due to the release of the $C_6H_{10}O_4$ group, m/z 447 $[(M + H) -146-18]^+$ with the release of one water molecule, and m/z 303 [(M + H) 146-18-144] + with the release of the $C_6H_{10}O_5$ group. Based on the identification, the active compounds were categorised in the flavonol group, specifically rutin. Rutin, which is also known as quercetin-3-rutinoside, sophorin, and rutoside, is a flavonoid that is found naturally in many plants. Rutin is a water soluble compound and it is more soluble in alcohols. In addition, it has many health benefits (Habtemariam and George, 2015), and has been used as an antimicrobial, antifungal, antioxidant, anti-allergic, and anti-cancer drug, including for hypertension and diabetes therapy (Frezza *et al.,* 2018). It is widespread in the leaves, seeds, and flowers of buckwheat (Hussain *et al*. 2017), citrus and other edible plants (Suzuki *et al.,* 2005).

CONCLUSIONS

The present study ascertained that the semi-polar fraction of the crude extract of GJE leaves contained the highest active components of total flavonoids, total phenolic, and antioxidant activity. The best mobile phase in the separation of the semi-polar fraction was n-hexane, chloroform, and ethyl acetate (6:1.5:1). Separation on column chromatography resulted in 11 groups of fractions (fA-fK). Among them, fK isolate had the highest antioxidant activity of 90.4%, and IC_{50} 15.74 mg/L. Additionally, identification using color test and shear reagent showed that the fK isolate was a group of flavonoid compounds (from the flavonol group), which was further strengthened by the results of the FTIR test, which showed the presence of functional groups with the estimation of the compounds 3,5,7,4 'tetrahydroxy flavonols and 3,5,7,3,4 'penta hydroxy flavonol. Finally, identification by LC-MS/MS illustrated that the fK isolate was an active flavonoid compound, namely, kaempferol-3-O-rutinoside and rutin.

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