Antioxidant and Xanthine Oxidase Inhibitory Activities of Kecapi (*Sandoricum koetjape* (Burm.f) Merr.) Leaf Extract

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Antioxidant and Xanthine Oxidase Inhibitory Activities of Kecapi (*Sandoricum koetjape* (Burm.f) Merr.) Leaf Extract

Fajar Nur Hamzah[1], Subandi[1], Wawan Sujarwo[2], Abdi Wira Septama[3] and Tjandrawati Mozef[3]*

[1]Dept of Chemistry, Faculty of Mathematics and Natural Sciences, State University of Malang, Malang
[2]Bali Botanic Gardens, Indonesian Institute of Sciences, Tabanan
[3]Research Centre for Chemistry, Indonesian Institute of Sciences, Serpong

*Corresponding author: tjandrawati@lipi.go.id

Abstract. *Sandoricum koetjape* has been used as folk medicine for the treatment of several diseases. The study was undertaken to determine antioxidant property of *S. koetjape* extract. The xanthine oxidase inhibitory effect was also evaluated. The dried powder of *S. koetjape* leaves was extracted with methanol. The extract was then successively partitioned with different solvents: n-hexane, ethyl acetate, buthanol and water. Phytochemical, total phenolic contents (TPC) and total flavonoid compounds (TFC) were analysed. DPPH scavenging assay was conducted to evaluate antioxidant activity. Xanthine oxidase inhibitor were determined using spectrophotometer. The results showed that amongst of tested extract, methanol extract contained high amount of phenolic and flavonoid compounds. For antioxidant and XO inhibitor, ethyl acetate fraction revealed strong activity with IC_{50} values of 3.70 and 50.18 µg/mL, respectively. XO inhibition by ethyl acetate extract was found to be a competitive inhibition that mimics inhibition mechanism of allopuriol. LC-MS/MS chromatogram exhibited that the extract contains dehydrotumulosic acid, methyl ophiopogonanone A, quercetin-4′-glucoside, luteolin-7-β-D-glucopyranosid and neokurarinol. The result of this study indicated that the potency of *S. koetjape* extract as antioxidant and XO inhibitor.

1. Introduction

Antioxidant activity can prevent several chronic diseases such as cell damage by reactive oxygen species (ROS) from the environment and those that are induced by the activity of several enzymes in the body [1, 2]. Antioxidants also has a role in the inhibition of oxidoreductase enzymes groups such as xanthine oxidase (XO) to reduce uric acid levels in the body [3]. Xanthine oxidase catalyzes oxidation of hypoxanthine to xanthine becoming uric acid in purine base metabolism pathway [4]. Increased levels of uric acid in the blood lead to gout incidence in which uric acid forms crystals in the joints, bones, tissues and organs of the human body [5]. Recently, the prevalence of gout is around 1-4% of the total population. In western countries, cases of gout occur around 3-6% in men and 1-2% in women per total population. The number of gout sufferers' worldwide increases gradually due to diet, lack of exercise, obesity and metabolic disorders [6]. One of medications for gout, can be done by reducing uric acid synthesis in the body, using xanthine oxidase inhibitors (XOI). Allopurinol is an XOI that is used clinically but still has many side effects such as hypersensitivity, Stevens Johnson syndrome and is toxic to the kidneys [4]. Therefore, exploring the other drugs, especially herbal drug, is still important.

Kecapi or *Sandoricum koetjape*, a tropical plant belonging to family *Meliceae,* is abundantly found in Southeast Asian region. [7]. The seeds of Kecapi contain sandoricin and 6-hydroxidandoricin that can be used as pesticide [8]. The fruit of this plant exhibited anti-tumor activity [9]. Conic acid, koetjapic acid and indicic acid in the bark of this plant possessed anti-cancer activity [10]. It has been reported that the fruit extract has high antioxidant activity [2]. However, less information is available about xanthine oxidase inhibitor which induces gout. Therefore, the present study is conducted to determine antioxidant, XOI activities as well as total phenolic and flavonoid contents from this plant. Possible bioactive compounds are also identified using LC-MS/MS spectroscopy.

2. Experiment Methods

2.1. Materials and Instrumentation

Sandoricum koetjape leaves were collected from Bali Botanic Gardens. Methanol, chloroform and formic acid were technical grade, while acetonitrile was LC grade. Potassium iodide, iodine, mercury(II) chloride, glacial acetic acid, zinc powder, hydrochloric acid, sulfuric acid, Folin-Ciocalteu, sodium nitrite, aluminum chloride, sodium bicarbonate, sodium hydroxide, sodium dihydrogen phosphate, dimethyl sulfoxide) were obtained from Merck. DPPH (2,2-diphenyl-1-picrylhydrazyl), xanthine and xanthine oxidase were from Sigma Aldrich. Allopurinol was purchased from Indofarma. The apparatus used in this study were rotary evaporator (Buchi), UV-Vis Spectrophotometer (Agillent), ELISA Reader (Multiscan Go, Thermo Scientific), LC-MS/MS (Agillent).

2.2. Procedure

2.2.1. Extraction. Samples were dried in the oven at 50^{o}C, reduced to powder using grinder and weighed. The powder was then macerated using methanol with a solvent replacement every 24 hours. The filtrates were concentrated using a rotary evaporator at 50^{o}C at rotation of 70-75 rpm. The concentrated extract was weighed and labelled as crude extract. The yield of crude extract were determined following equation 1, where W_1 is weight of crude extract after concentrated and W_2 is weight of dried leaf.

$$\%Yield = \frac{W_1}{W_2} \times 100\% \quad (1)$$

2.2.2. Phytochemical Test. The phytochemical screening was conducted following the Indonesian Ministry of Health protocols [12] to identify flavonoid, terpenoid and steroid, saponin and alkaloid. a) The qualitative test for flavonoids was done by taking a few milligrams of extract, added with 4 mL of ethanol, 2 mL of test solution was added by 0.5 g of Zn powder and 2 mL of 2 N HCl, then was incubated for a minute, then was added by concentrated HCl per drop to form an intensive red color which means positive flavonoids. b) Qualitative test for tannins in the extract was done by taking a few milligrams of extract, then was added with hot water and boiled for 5 minutes, then added by a few drops of $FeCl_3$ 1%. Positive reaction was shown in green-violet. c) Qualitative test for terpenoids and steroids in extracts was carried out by taking a few milligrams of crude extract then was dissolved in 2 mL chloroform in a test tube then added by 10 drops of anhydrous acetic acid and 3 drops of concentrated sulfuric acid. Positive reactions are shown in Green for steroid and in purplish red as triterpenoids. d) Qualitative test for saponins in the extract was done by taking a few milligrams of extract and added by hot water and shaken vigorously until intensive foam formed. The addition of a few drops of HCl 2 N into foam does not disappear, then indicates positive saponins. e) Qualitative test for the alkaloid (Buchard test) was done by taking about 500 mg of extract then was added with 1 mL of HCl 2 N and 9 mL of water and heated in a water bath for 2 minutes. The filtrate was taken as many as a few milliliters into the drip plate and added with the Burchard reagent (2 g iodine and 4 g KI dissolved in 100 mL distilled water) a few drops. A positive reaction is shown by the formation of brown-black precipitate. Qualitative Meyer's alkaloid test was performed by taking about 500 mg

extract and was added 1 mL of HCl 2 N and 9 mL of water and then heated in a water bath for 2 minutes. The filtrate was taken as many as a few milliliters into the drip plate and was added a few drop of Meyer reagent (1.358 g $HgCl_2$ in 60 mL of distilled water and 5 g KI in 10 mL of distilled water). The positive reaction is indicated by the formation of white or yellow precipitate.

2.2.3. Partition of Crude Extract. One gram of crude extract of kecapi was successively partitioned using n-hexane (Hex), ethyl acetate (EtOAc), buthanol (BuOH) and water. Fractions were concentrated under reduced pressure at a temperature setting of 50 ^oC.

2.2.4. Determination of Total Phenolic Compounds (TPC). Total phenolic content was performed according to previous study with slight modification [13]. In brief, Extract stock solution was made with a concentration of 1000 µg/mL. The sample was piped 250 µL into a test tube then added 250 µL of Folin Ciocalteu reagent. A number of 3.5 ml of distilled water was added to the sample and was incubated for 8 minutes at room temperature. Then, the sample was added 0.75 mL of 20% Na_2CO_3 (20 grams Na_2CO_3/100 mL) and added to 5 mL with distilled water. The samples were incubated for 2 hours then absorbance was measured with a UV-Vis spectrophotometer at a wavelength of 765 nm. The concentrations of phenol in the samples were determined by the gallic acid calibration curve with a concentration of 0, 5, 10, 20, 30 µg/mL.

2.2.5. Determination of Total Flavonoid Contents (TFC). From the stock solution of 1000 µg/mL sample was taken 500 µL then added 2 mL of distilled water and 150 µL of 5% $NaNO_2$ (5 g $NaNO_2$ /100 mL). The samples were incubated for 5 minutes then added with 10% $AlCl_3$ (10 g $AlCl_3$ dissolved in glacial acetic acid up to 100 mL) of 150 µL. The samples were incubated for 6 minutes. A total of 2 mL of 1 M NaOH (2.5 g/100 mL) was added to the sample and then made final volume of 5 mL by water. Total flavonoid contents were measured using the UV-Vis spectrophotometer at a wavelength of 510 nm. The flavonoid concentration in the sample was determined by the calibration curve of the standard quercetin with concentrations of 0, 10, 30, 50, 100 µg/mL [1].

2.2.6. DPPH Scavenging. Crude extracts and the fractions of the kecapi were made a solution stock of 1000 ppm in methanol. Stock DPPH solution was made 4 mg/25 mL in methanol. All test substances are made in a standard series from 0.5 to 50 µg/mL. The screening results were compared with blank and quercetin standards with 1000 µg/mL stock with a series of concentrations of 1, 2, 3, 4 and 5 µg/mL. Percent inhibition was calculated using bellow formula (2):

$$\%DPPH\ inhibition = \frac{A\ blank - A\ sample}{A\ blank} \times 100\% \quad (2)$$

IC_{50} values were determined by drawing a linear regression curve y = ax + b where y is the percent of inhibition and x for the concentration of samples [14].

2.2.7. Xanthine Oxidase Inhibitory Activity Test. Stock solutions of crude extract and fractions were made in 100% DMSO of 10,000 µg/mL. Stock sample solutions were tested by making crude extract concentration series (10, 20, 50, 100 µg/mL), n-hexane and water fractions series (20, 50, 80, 100 µg/mL), ethyl acetate and butanol fractions series (20, 50, 100, 200 µg/mL) with a final reaction volume of 300 µL in a 96-well microplate and a final DMSO concentration of maximum 1% in diluting phosphate buffer at pH 7.4. Then 10 µL 0.05 units/mL of XO enzyme was added to the mixture and pre-incubated at 25 ^oC for 15 minutes. After pre-incubation, 0.375 mM 100 µL of xanthine was added and incubated at 25 ^oC for 10 minutes. The reaction was then stopped by adding 100 µL of 1 N HCl. Samples were measured by Multiscan Go at a wavelength of 295 nm. Percent inhibition was determined by equation (3)

$$\%\ XO\ Inhibition = \frac{(A-B)-(C-D)}{(A-B)} \times 100\ \% \quad (3)$$

Where A is the absorbance of without inhibitors, B is absorbance of the control of substrate, C is the absorbance of inhibitor tests, D is absorbance of inhibitors and substrates. Allopurinol solutions was made in a concentrations series of 0.5, 0.8, 2.0, 4.0 ppm and a final volume of 300 µL [15].

2.2.8. Determination of Enzyme Inhibition Kinetic. Variations in xanthine concentration were made with the same inhibition test procedure, measurements were made based on time variations during the incubation process. Absorbance values were read at specified intervals and expressed as t values to get the v values. The obtained absorbance values were used to make the Lineweaver-Burk line curve, with the y axis is 1/v and the x axis is 1/[Substrate] to determine the value of Km, Vmax and the type of inhibition.

2.2.9. Identification of Candidate Compunds by LC-MS/MS. About 5 mg of the test sample was weighed and dissolved into HPLC grade methanol at a concentration of 10000 µg/mL then injected into LC-MS/MS. The effluent used was 0.1% formic acid: water (eluent A) and acetontril: 0.1 formic acid (eluent B). The column used was C-18.

3. Results and Discussion

3.1. Result of Preparation and Phytochemical Screening

The stability of phenolics and flavonoids of leaves material can be affected by the drying process [16], thus the drying must be performed at temperatur of only 50^{o}C. Beside of that, the refining has also been done to expand the surface area of the leaves material so that the surface contact of the material with the solvent will be greater and can facilitate the penetration of the solvent into the sample, then can attract more compounds [17]. The extraction temperature was carried out at room temperature in order to prevent destruction of secondary metabolites [18]. In this study methanol was selected due to its ability to afford more polyphenol components in plants, besides its boiling point is relatively lower than ethanol or water [19]. It has been known that several phytochemical compounds including flavonoid and polyphenol possessed antioxidant activity by the formation of ROS or reducing free radicals directly [20]. Based on phytochemical screening (Table 1), it indicated that the extract contains several secondary metabolites such as flavonoids, tannins, steroids, saponins, and alkaloids.

Table 1. The Result of phytochemical screening

Phytochemical Test					
Flavonoids	Tanins	Steroids	Saponins	Alkaloids	
				Buchard	Meyer
+++	++	+++	++	+	+

Notes: + (low contents), ++ (moderate contents), +++ (high contents)

3.2. TPC, TFC, Antioxidant Activities and XO Inhibitory Activities

The methanol extract was then successively partitioned using different solvents, starting from the most non-polar solvent to the most polar solvent. Partition was conducted to observe the distribution of compounds which have potential to be used as antioxidant and anti-gout. The process is also allowing to eliminate other compounds that are suspected to be antagonistically against compounds that have the potential as anti-gout. The result of antioxidant activity, TPC, TFC and XO inhibition of the crude extract and the fractions result, are shown in Table 2.

Table 2. Yield, TPC, TFC, DPPH scavenging and XO inhibitory activities of Kecapi leaf extract, and Quercetin or Allopurinol as Standard

Samples/ Fractions	Yield (%)	TPC (%)	TFC (%)	IC_{50} DPPH (µg/mL)	IC_{50} XO (µg/mL)
Crude extract	66.83	33.67	22.20	8.14	54.85
n-hexane fraction	62.60	11.97	43.09	31.08	74.03
Ethyl acetate fraction	5.67	35.72	75.13	3.70	50.18
n-Butanol fraction	24.34	9.15	18.79	12.62	138.05
Water fraction	3.16	6.22	22.75	28.65	142.11
Quercetin	-	-	-	3.20	-
Allopurinol	-	-	-	-	1.07

It can be seen that ethyl acetate fraction exhibit strong activity as antioxidant and XOI compared to others with IC_{50} values of 3.70 and 50.18 µg/mL. Moreover, this fraction also has high percentage in total phenolic and flavonoid contents (35.72 and 75.13%, respectively). Interestingly, this active fraction revealed more potent antioxidant effect compared to quercetin as positive control. Thus, ethyl acetate fraction was selected for further assay, e.g. XO inhibitory mechanism study.

3.3. Xanthine Oxidase Inhibitory Mechanism

The xanthine oxidase inhibition mechanism of ethyl acetate fraction was carried out using the Lineweaver-Burk equation curve. Based on the result, it showed dose-dependent manner. This indicates that the extract had a potency as XO inhibitor. It has been reported that some polyphenol compounds enable to inhibit XO [4]. According to the Lineweaver-Burk curve (Figure 1), the ethyl acetate fraction gave a competitive inhibition. This type of inhibition mimics inhibition mechanism of allopurinol.

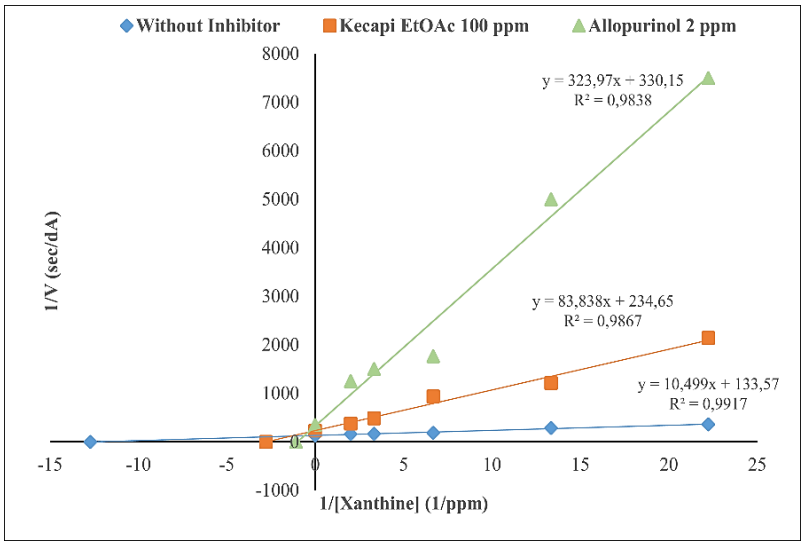

Figure 1. Lineweaver-Burk plot of XO inhibition

Table 3. Km and Vm of XO inhibitory activity of ethyl acetate fraction

Variables	Vm (µg/mL/sec)	Km (µg/mL)
Without Inhibitors	0.007487	0.078603
Allopurinol 2 µg/mL	0.003029	0.981281
Kecapi EtOAc 100 µg/mL	0.004262	1.526827

3.4. Identification of Candidate Active Compounds in The Crude Extract using LC-MS/MS

In order to know the profiling of secondary metabolites in the extract, identification was carried out using LC-MS/MS analysis. The principle of detection is separation of the components in the liquid chromatography column, while the detection was carried out on the abundant compounds in the crude extracts. The selected peaks are the highest peak, with the correction of the solvent blank, and then the m/z of selected peak was adjusted to the Agillent software database of LC-MS/MS. Analysis of crude extracts was carried out by considering the results of phytochemical tests as the first step in the exploration of a natural ingredient as the gout medecine.The results of detection of the predicted active compounds in crude extracts are shown in Figure 2.

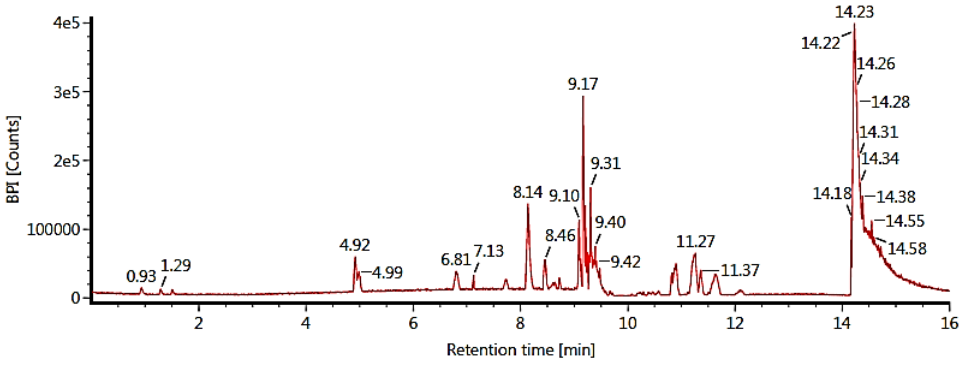

Figure 2. Chromatograms of methanol extract of *S. koetjape*

The results are presented in the Table 4 and Fig 3. Dehydrotumulosic acid is a triterpenoid group. This compound has been reported having activity as antihyperglycemia [21]. Phenolic compounds are not the only major components for XO inhibition and DPPH radical inhibiton activities, a previous study proves that terpenoids have XO inhibitory activity and antioxidant effects on DPPH free radicals [15]. The methyl ophiopogonanone is a homoisoflavone that has quite high antioxidant activity [22], quercetin and luteolin are flavonoid groups which have activity as competitive inhibitors of XO [23], while neocurarinol has been reported to modulate gamma-aminobutyric acid type A receptors [24].

Table 4. Compounds Identification based on LC-MS/MS data

Compounds	Retention Time (min)	*m/z*	Mass (Da)
Dehydrotumolosic acid	1.51	485.3565	484.35526
Methyl ophiopogonanone A	1.16	365.1024	342.11034
Quercetin-4′-glucoside	5.09	465.1001	464.09548
Luteolin-7-β-D-glucopyranoside	5.41	449.1047	448.10056
Neokurarinol	5.98	493.2244	470.23045

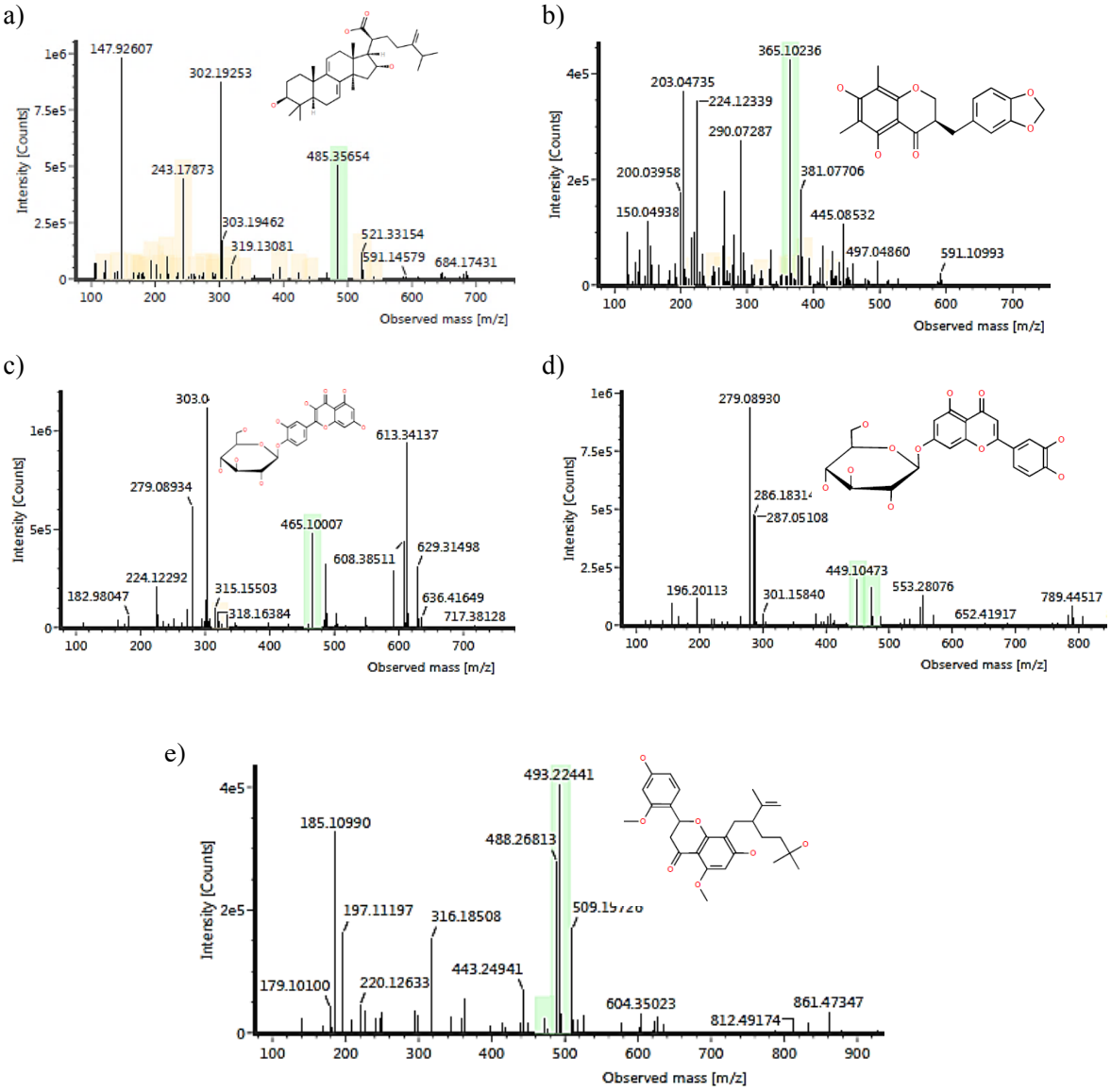

Figure 3. Mass Spectra and structures of candidate active compounds in the kecapi leaf crude extract. Dehydrotumulosic acid (a), Methyl ophiopogonanone A (b), Quercetin-4′-glucoside (c), Luteolin-7-β-D-glucopyranoside (d) and Neocurarinol (e)

4. Conclusion

It can be concluded that *S. koetjape* leaf extract has a potency as antioxidant and xanthine oxidase inhibitors. The extract contains high amount of phenolic and flavonoid compounds. The type of xanthine oxidase inhibition by ethyl acetate fraction is competitive inhibition, and based on spectroscopic analysis, predicted active compounds in the extract particularly are dehydrotumulosic acid, methyl ophiopogonanone A, quercetin-4′-glucoside, luteolin-7-β-D-glucopyranoside and neokurarinol. However, further study is still needed to elucidate others active components.

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