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Identification of compounds from extract methanol of ketepeng leaves (*Cassia alata*)

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ABSTRACT

Cassia alata known as ketepeng is one of traditional herb that has been used as ointment. In Indonesia, its components information has not been much explored yet. The purpose of this research is to characterize one or more compounds from its leaves methanolic extract. C. alata leaves powder was macerated by methanol in room temperature for 24 hours. Chlorophyll and tannin from its leaves methanolic extract were removed by using methanol-water (1:1) and EtOAc, respectively. The EtOAc extract was then fractionated by Liquid Vacuum Chromatography (LVC) by applying gradient elution system of n-hexane-EtOAc and 8 fractions (A-H) were obtained. From B fraction was obtained one of phenolic compounds, methyl p-hydroxybenzoate, has been isolated for the first time from the leaves of this species. The structure of the compound has been established by ^{1}H and ^{13}C NMR spectroscopic methods. Meanwhile from the H fraction was obtained 21 fractions. One of them, the H_9 fraction then was analyzed further by using LC-MS and ^{1}H NMR. The chromatogram and ^{1}H NMR spectrum of this fraction still indicated mixture of compound, but a dominant peak at retention time (t_R) 6.7 min was observed. Further analysis to this peak, particularly to its mass spectrum, indicated that the peak is predicted as a flavonoid adduct, that is 4'-hydroxy-6-C-xylofuranosyl flavanone.

Keywords: *Cassia alata*, ^{1}H NMR, ^{13}C NMR, LC-MS, methyl *p*-hydroxy benzoate, flavonoid adduct 4'- hydroxy-6-*C*-xylofuranosyl flavanone

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INTRODUCTION

Cassia is a main genus of Fabaceae family consisting of around 600 species. Some of them are spread out widely, particularly in tropical countries and they are abundant in India. *C. alata*, *C. tora*, *C. siamea*, and *C. fistula* are some species researched frequently [1]. *C. alata* is a herb which is well known in Indonesia as ketepeng. *C. alata* generally used as traditional medicine mainly in tropical area such as Malaysia, Brazil, and Indonesia. This plant is believed to be able to cure constipation, hernia, syphilis, diabetes [2], malaria and influenza [3], bronchitis, asthma [4], as anti-obesity [5], strong laxative, and insects repellant [6]. Its leaves methanolic extract was proved to has anti-fungi and antibacterial activity [2]. Chloroform fraction of leaves methanolic extract was proved to have antimalarial activity [7]. Besides, leaves methanolic extract also has anti-angiogenic and cytotoxic activity in breast cancer cell lines [8]. In Indonesia, one of cleaning tissue product made from this plant claimed to be able to prevent and treat leucorrhoea (fluor albus) [9].

Varieties of active compounds were isolated from *C. alata.* Essential oil of *C. alata* leaves from Nigeria contains 1,8-cineole (39.8%), β-caryophyllene (19.1%), caryophyllene oxide (12.7%), limonene (5.2%), germacrene D (5.5%), and α-selinene (5.4%) [10]. *C. alata* leaves from Brazil contain quinone compounds such as emodin, aloe-emodin, chrysophanol, isochrysophanol, and rhein [11]. The methanolic extract of *C. alata* leaves growing in Bangladesh contains tannin which is ellagic acid derivative, that is 2,3,7-tri-*O*-methylellagic acid [12]. *C. alata*

leaves ethanolic extract from Nigeria contains flavonol glycosides, 5,7-dihydroxy-2-(4-hidroxyphenyl)-3-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hidroxymethyl) oxan-2-il] oxy-chromene-4-one [11]. The seed ethanolic extract contains alkaloid compound, cannabinoid dronabinol ((*S*)-3-buthylamino-9-methyl-10,10*a*-dihydro-7*H*-benzo[*c*] chromene-1-ol) [13]. A flavonoid compound, kaempferol also found in *C. alata* leaves methanolic extract from Jamaica [14].

A typical research to *C. alata* leaves has not been much conducted yet in Indonesia, mainly related to chemical components information of its leaves. Growing site difference will influence the type and amount of secondary metabolites existing in this plant. Therefore, this research was conducted to characterize chemical components in *C. alata* leaves growing in Indonesia. Isolation and characterization were conducted to leaves methanolic extract. This result is desired to be able to enlarge information database of secondary metabolites existing in *C. alata* which can be used to explore this plant potency further next time.

MATERIALS AND METHODS

Materials and Equipment

Materials used were ketepeng leaves (obtained from Cikasungka village, Solear, Tangerang, Indonesia), methanol, ethyl acetate (EtOAc), chloroform-ammonia, concentrated sulphuric acid, Mayer, Dragendorf, and Wagner reagent, diethyl ether, concentrated acetic acid, Mg, HCl, $FeCl_3$ 1% reagent, *n*-hexane, acetone, chloroform ($CHCl_3$), dichloromethane (DMC), silica gel Merck 60G, silica gel Merck 60 PF_{254}, silica gel 60 (0.2–0.5 mm), and TLC on silica gel GF_{254}. The equipment used were liquid vacuum chromatography (LVC) kit, column for gravitational column chromatography (CC), liquid chromatography-mass spectrometer (LC-MS) with electrospray ionization (ESI-MS) mode Mariner Biospectrometry, proton nuclear magnetic resonance (^{1}H NMR) spectrometer Agilent 500 MHz (^{1}H), the ^{1}H NMR spectra were recorded on a JEOL ECA 500 (500 MHz) instrument in $CDCl_3$ with TMS as an internal standard (chemical shifts in δ, ppm), and ^{13}C NMR spectra were recorded on a JEOL ECA 500 (125 MHz) instrument.

Extraction and Isolation of C. alata leaves

C. alata leaves powder (1 kg) was macerated by methanol (4 L) in room temperature for 24 hours. Macerate was separated, then the residue was macerated again with the same type and amount of solvent. This step was repeated for 3 times. All of macerate were collected and concentrated. A dark green crude methanolic extract obtained and its recovery was counted with water content as correction factor. Then, phytochemical test was conducted to that extract. The chemical components process separation of *C. alata* leaves methanolic extract was initiated by separating chlorophyll and tannin. This separation was conducted by diluting crude methanolic extract (150 g) with 1 L of methanol: water (1:1), shaking it, and letting it for a night. The precipitated chlorophyll was separated and weighed, then free-chlorophyll methanolic extract was extracted by EtOAc and was let for a while until 2 layers formed. The bottom layer was predicted as water extract containing tannin, while the other one was predicted as EtOAc layer containing chemical components without chlorophyll and tannin. The EtOAc extract obtained was concentrated by rotary-evaporator, and then fractionated by LVC with increasing polarity of *n*-hexane-EtOAc eluent system and 8 fractions were obtained (A-H fractions). B fraction (68 mg) was fractionated further by radial chromatography (RC) with the former eluent system in LVC and 8 fractions were obtained (fraction-B_{1-8}). B_5 fraction (9.40 mg) then was fractionated further by preparative-thin layer chromatography (TLC) with *n*-hexane-$CHCl_3$-EtOAc (7:1:2) eluent system and 4 fractions were obtained (fraction-B_{51-54}). The B_{53} fraction then was analyzed further by using ^{1}H and ^{13}C NMR. H fraction (4 g) was then fractionated by gravitational column chromatography by *n*-hexane-EtOAc gradient elution system and 21 fractions were obtained. The H_9 fraction was further purified by liquid partition with *n*-hexane-methanol (1:1). Methanol-soluble component was then analyzed under TLC to ensure that was a single component and analyzed further by ^{1}H NMR and LC-MS spectrometer.

RESULTS AND DISCUSSION

The crude methanolic extract obtained is 380.5 g (40.41% based on dried weight) and 150 g of this extract was used further. Precipitated chlorophyll is 69 g (46%), sticky like a gum, and is a dark green precipitate. Chlorophyll disappearance from extract was proved by green spot disappearance under UV lamp 254 nm and red spot under UV lamp 366 nm. This color is typical for chlorophyll as reported by Khasanah *et al*. (2013). Separated tannin is 57 g (38%) and EtOAc extract obtained is 26 g (17%) which is a dark brown extract and sticky like a gum. The EtOAc extract (20 g) was impregnated and chemical components inside were separated based on polarity by LVC. The elution was conducted by using some solvents, respectively, *n*-hexane (once), *n*-hexane-EtOAc with ratio 8:2, 7:3, 6:4, and 4:6 (each 3 times), 3:7 and 1:9 (each 2 times), EtOAc (twice), and methanol (6 times). The 8 fractions (A-H) were obtained from this separation. B and H fractions were then further analyzed.

Phytochemical Constituents in Crude Methanol Extract

Phytochemical test result of its extract showed the existence of phenolics, flavonoids, steroids/triterpenoids, saponins, and alkaloids. Flavonoids and steroids/triterpenoids have ever been reported by Soetjipto *et al*. [15], Veerachari and Bopaiah [16]. Phenolics and saponins were not found by Veerachari dan Bopaiah [16], while alkaloid was not found by Soetjipto *et al.* [15]. In Bangladesh, 2,3,7-tri-*O*-methylellagic acid which belongs to phenolic group was successfully isolated from *C. alata* methanolic leaves extract by Alam *et al.* [12]. Kaempferol-3-*O*-gentio-bioside, a flavonoid compound, which detected in *C. alata* methanolic leaves extract in Indonesia was already reported by Moriyama *et al.* [17]. Singh *et al*. [18] isolated flavonoid compounds, kaempferol and rhein, from hydromethanolic leaves extract in India. An alkaloid, cannabinoid dronabinol, was successfully isolated from ethanolic seed extract in Nigeria [13]. This difference of secondary metabolites constituents is caused by age difference and place where the plant grows.

Methyl p-hydroxybenzoate

Result of B fraction (68 mg) which was fractionated further by radial chromatography (RC) with the former eluent system in LVC obtained 8 fractions (fraction-B_{1-8}). B_5 fraction (9.40 mg) then was fractionated further by preparative-thin layer chromatography (TLC) with *n*-hexane-$CHCl_3$-EtOAc (7:1:2) eluent system were obtained 4 fractions (fraction-B_{51-54}). The B_{53} fraction then was analyzed further by using ^1H and ^{13}C NMR and . Fraction-B_{53} was predicted as methyl *p*-hydroxy benzoate or *p*-methoxybenzoate acid.

Analysis on ^1H NMR spectrum (Figure 1) shows proton chemical shifts (δ) at 3.90; 6.85, and 7.95 ppm. A singlet signal (3H) at 3.90 ppm is identified as proton chemical shift of methoxy group. Two doublet signals (2H) at 6.85 and 7.95 ppm with same coupling constant value (J = 8.43) are identified as proton chemical shift of *para* disubstituted-benzene aromatic ring. One signal in upfield area (6.85 ppm), might be caused by electron donation from substituent in *ortho* position. That substituent with electron donating property may be a methoxy (–OCH_3) or hydroxy (–OH) group. Another signal at 7.95 ppm (in downfield area) indicates the existence of electron attracting substituent in *ortho* position. Substituents predicted having this property are –CO_2H, –CO_2R, and –COH. Generally, proton chemical shift of aromatic without substituents is around 7.4 ppm.

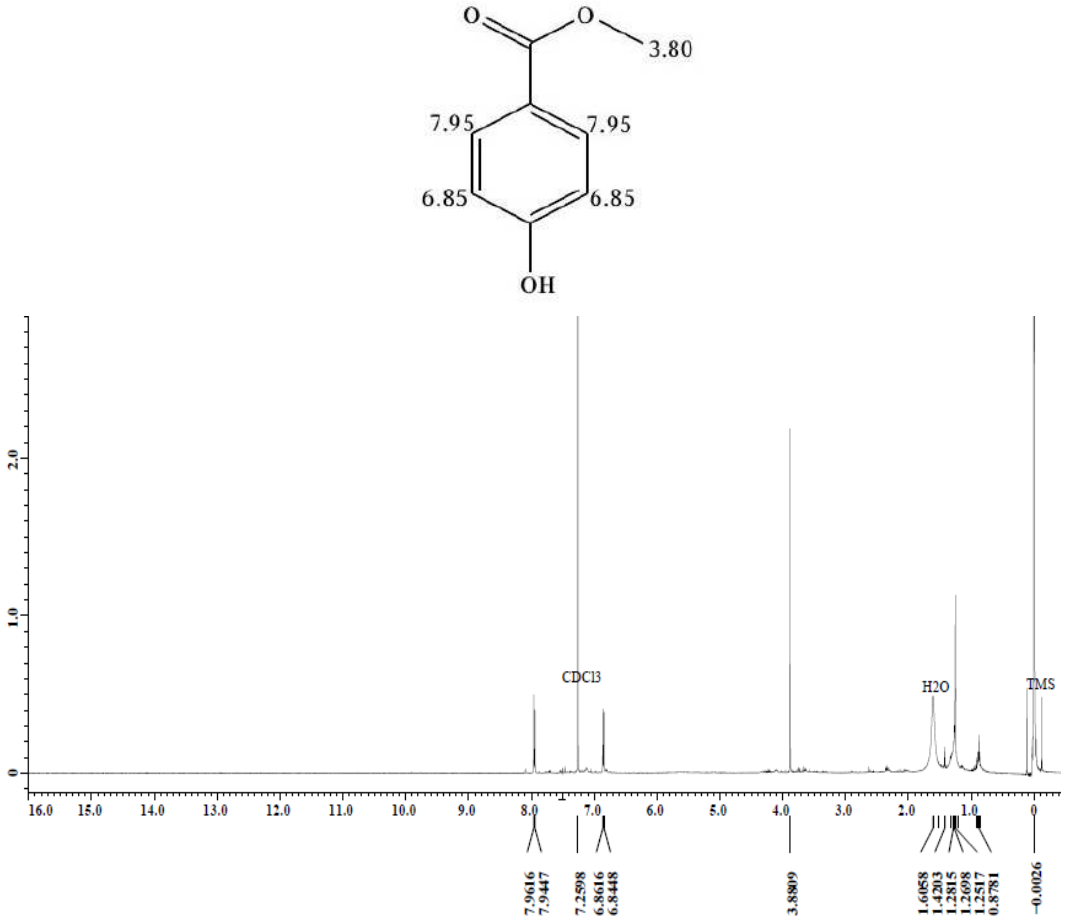


Figure 1. The ^1H NMR spectrum ($CDCl_3$) of fraction-B_{53}

The ^{13}C NMR spectrum (Figure 2) shows signals at 52.09; 115.35; 122.98; 132.09; 159.92; and 167.00 ppm. Signals at 52.09 ppm and 167.00 ppm are identified as carbon signal from methoxy group and carbonyl group of carboxyclic acid or ester. Two sp^2 carbon signals at 132.09 and 115.35 ppm shows twice as higher intensity indicating that each of them represents 2 equivalent C atom (homotopic), while sp^2 carbon signals at 122.98 and 159.92 ppm with lower intensity are predicted as quaternary C. Signal which is more downfield (159.92 ppm) is predicted because of stronger electron attracting substituent attached to quaternary C. The resulted ^1H and ^{13}C NMR data spectrum from this study and the calculated ^1H and ^{13}C NMR chemical shift [19] are compared to strengthen the compound prediction of B_{53} fraction.

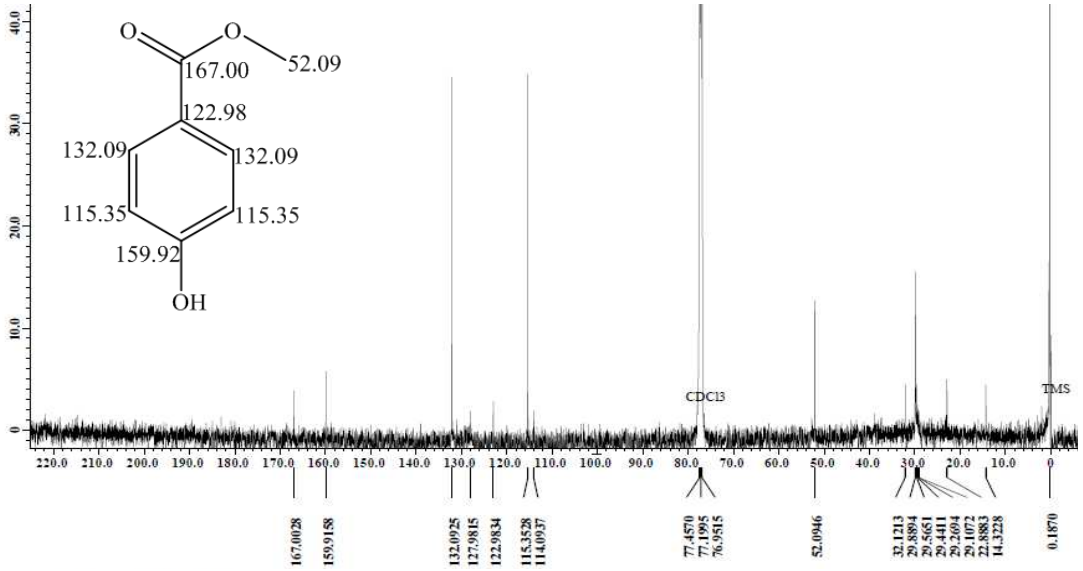


Figure 2. The ^{13}C NMR spectrum of fraction-B_{53}

Table 1 shows comparison between chemical shifts of B_{53} to calculation of methyl *p*-hydroxybenzoate and *p*-methoxybenzoate acid based on theory [19] and *Spectral Data Base* (SDBS) literature [20,21], respectively. SDBS is an organic compounds databases spectrum built by Japan to whole world. The result showed the ^1H and ^{13}C NMR chemical shifts of B_{53} have suitable values with calculated chemical shift values of methyl *p*-hydroxybenzoate, either according to theory or SDBS literature. Base on this, therefore, B_{53} was deducted as methyl *p*-hydroxybenzoate.

Table 1 The^1H and ^{13}C NMR chemical shifts of methyl *p*-hydroxybenzoate and *p*-methoxybenzoate acid as compared to theoretical calculation and SDBS

Methyl *p*-hydroxybenzoate –B_{53} ($CDCl_3$)			Methyl *p*-hydroxybenzoate (Theoretical*)		*p*-Methoxy-benzoate acid (Theoretical*)		Methyl-*p*-hydroxybenzoate (SDBS)		*p*-methoxy-benzoate acid (SDBS)	
Atom C/H	δ_H 500 MHz (ppm) (multiplicity, *J* in Hz, H amount)	δ_C 500 MHz (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_C(ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)
1		122.98	-	123.20	-	123.70	-	122.11	-	131.46
2/6	7.95 (*d*, 8.43, 2H)	132.09	7.90	131.30	8.06	130.80	7.84	131.98	7.93	131.46
3/5	6.85 (*d*, 8.43, 2H)	115.35	6.91	115.70	7.02	114.50	6.88	115.39	7.04	113.84
4	-	159.92	-	159.90	-	164.20	-	160.62	-	162.97
OCH_3	3.9 (*s*, 3H)	52.09	-	-	-	-	3.80	52.12	3.84	55.44
C=O		167.00	-	-	-	-	-	167.72	-	167.14
OH			-	-		-	-	-	12.70	-

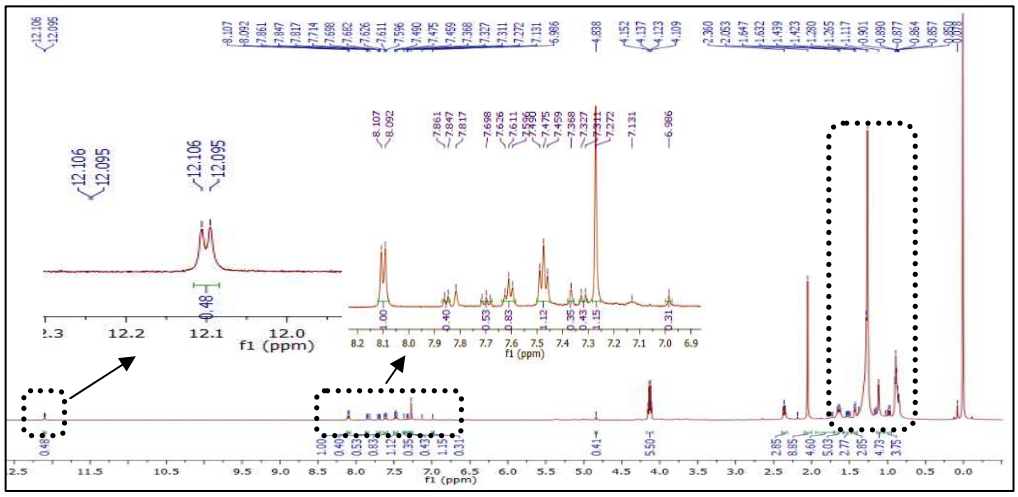
**Silverstein et al. (2005)*

Methyl *p*-hydroxybenzoate has not ever reported yet whether from *C. alata* leaves or from *Cassia* genus and Fabaceae family [22], but this existence has ever been reported from the other family. This compound was isolated from *Nerium oleander* leaves which come from Shambat [23], *Stocksia brahuica* in Pakistan [24], from *Oxalis teburosa* root [25], and methanolic root peel extract of *Zanthoxylum ailanthoides* from Taiwan [26].

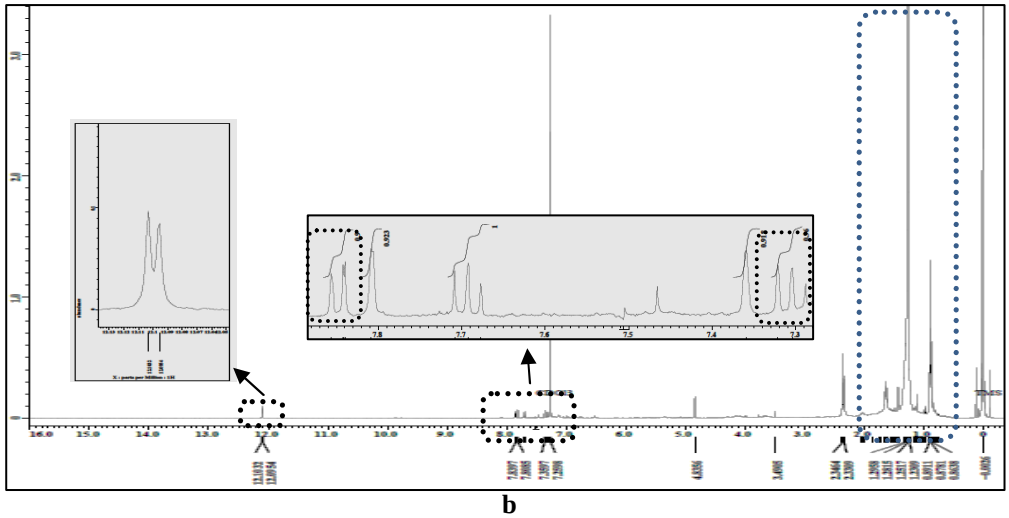
Identification of H Fractions

Fractionated by gravitational column chromatography for H fraction (4 g) was obtained 21 fractions. The H_9 fraction was further purified by liquid partition with *n*-hexane-methanol (1:1). Methanol-soluble component was then analyzed under TLC and analyzed further by ^{1}H NMR and LC-MS spectrometer.

The H_9 fraction ^{1}H NMR spectrum shows that H_9 still not pure. (Figure 3a). It is indicated with the existence of complex enough signal peaks in aliphatic range (δ_H 0.7–1.7 ppm) and signal peak at δ_H 12.1 ppm (indicates –OH carboxylic acid functional group) splitting into 2 peaks (Figure 3b). Further analysis with LC-MS strengthen the former hypotheses since the chromatogram (Figure 4) shows 4 peaks with retention time (t_R) respectively 3.0, 3.7, 4.7, and 6.7 minute with the area 325.30, 302.264, 264.99, and 2822.93 respectively.



a



b

Figure 3 The H_9 fraction ^{1}H NMR spectrum before partition (a) and after partition by *n*-hexane-methanol (b)

H_9 peak with t_R 6.7 minute (then signed as H_{94}) has the highest abundance in H fraction. Therefore, secondary metabolite structure characterization was focused to compound in that fraction. Fragmentation pattern obtained is relatively simple. (Figure 5). Due to that fact, compound inside was predicted to contain relative stable functional group and was not fragmented overall. This may be caused by low energy used in ionization process. Tsimogiannis *et al*. (2007) [27] said that if the collision energy in ionization process is low, spectrum obtained from $[M+H]^{\cdot+}$ cannot be fragmented overall. This kind of fragmentation pattern was predicted to be produced by compound with aromatic cyclic structure stabilized by conjugated system. This compound does not contain aliphatic hydrocarbon chain since fragment having $[M+H-14]^{\cdot+}$ or multiples is not observed. Based on those characterizations, this compound was predicted to have flavonoid basic skeleton. The ^{1}H NMR spectrum also shows signals in aromatic range (δ_H 6.5–8.0 ppm), that are at δ_H 7.2 ppm (1H, *d*) and 7.8 ppm (1H, *dd*) with coupling constant respectively 9.1 and 7.8 Hz ($^3J_{ortho}$ = 7–10 Hz) (Figure 3b). This pattern in accordance with benzene structure unit substituted in *para*

position. One signal which is more unshielded (δ_H 7.8 ppm) than the other (δ_H 7.2 ppm) indicated that 2 substituents in *para* position have different electron-withdrawing power.

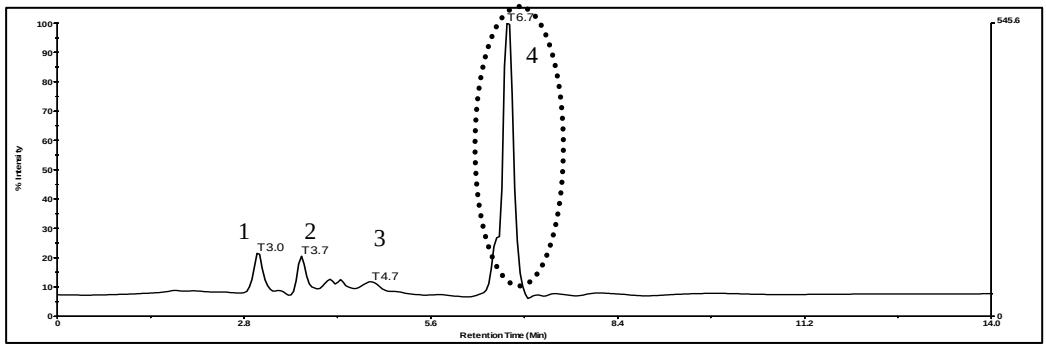


Figure 4 H_9 fraction-methanol soluble LC chromatogram with methanol-water 95:5 as eluent and flow rate 1 mL/minute

Flavonoid has C_6-C_3-C_6 basic skeleton. Based on C ring structure, flavonoid is distinguished to 5 big groups. Those are flavonol, flavone, dihydroflavonol or flavanonol, flavanone, and flavanol. Flavonoid compounds isolation and identification frequently are hard to do since structure and polarity similarity [27]. Flavonoid is generally found as flavonoid *O*-glycoside form in nature that one or more of hydroxyl groups from aglycone are bound to sugar through hemiacetal bond which labile to acid. Yet, glycosylation may also happens through C-C bond resisting to acid, forms *C*-glycoside flavonoid. The *C*-glycoside flavonoid generally found are mono-*C*-glycoside, di-*C*-glycoside, and *C*-glycosyl-*O*-glycoside flavonoid. Sugar group usually found in nature is glucose. Galactose, rhamnose, xylose, and arabinose are not common. Similarly, manose, fructose, glucuronic acid, and galacturonic acid are also rare. That sugar group is substituted at position 3, 5, and 7 in flavonoid *O*-glycoside, while in flavonoid *C*-glycoside is only found at position 6 and 8 [28].

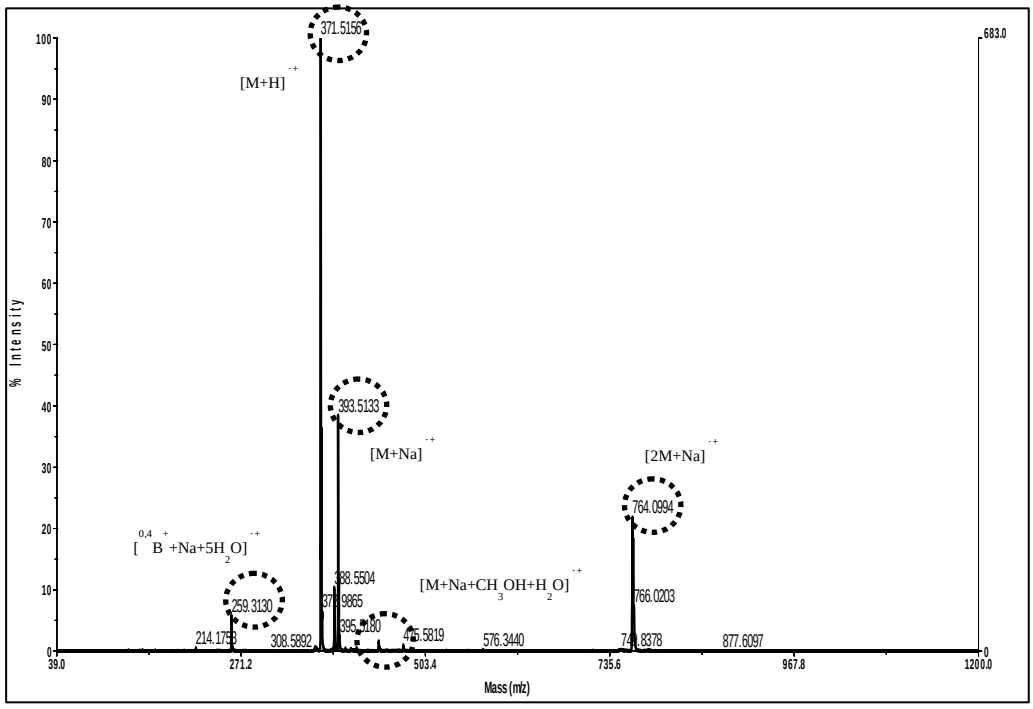


Figure 5 The H_9 fraction mass spectrum with t_R 6.7 minute

Based on C-C bond resistibility of sugar group with flavonoid aglycone compared to O-C bond of *O*-glycoside flavonoid, this H_{94} compound was predicted belong to *C*-glycoside flavonoid. The bonded sugar probably was a pentose. A pentose was predicted bonded to flavonoid basic skeleton based on some combinations made which were suitable with base peak appearing at *m*/*z* = 372 (Figure 6), with assumption that the base peak showed molecular ion

$[M+H]^{\cdot+}$ from H_{94} fraction. This pentose sugar existence was predicted gave weak signals in ^1H NMR spectrum at δ_H 3.4–4.3 ppm (Figure 7).

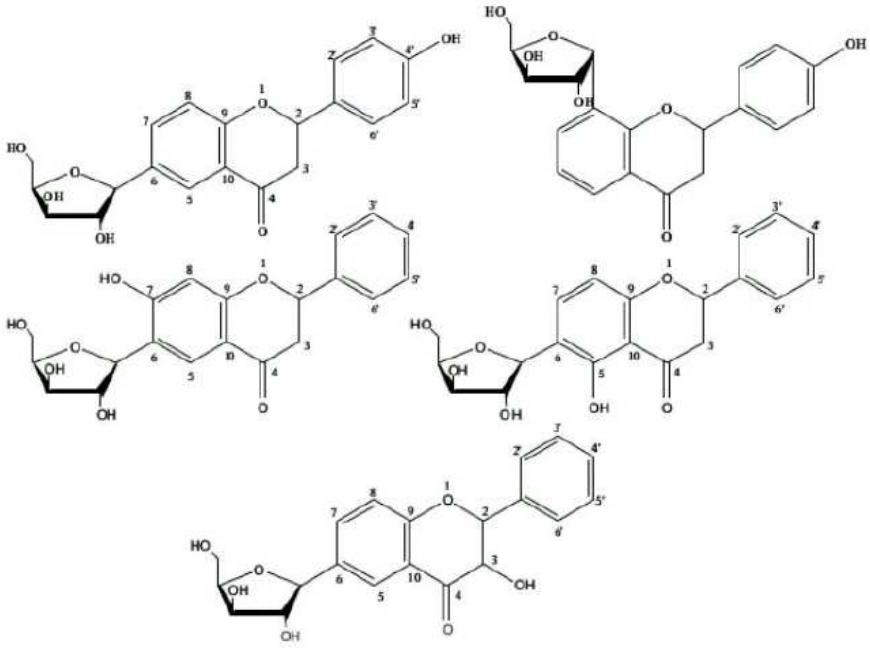


Figure 6 Some predictions of compounds having molecular weight 371 g·mol^{-1}

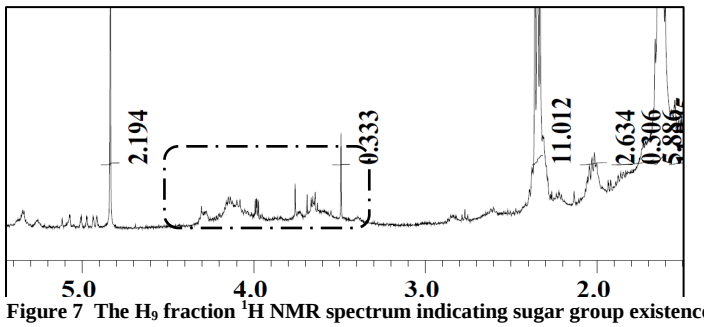


Figure 7 The H_9 fraction ^1H NMR spectrum indicating sugar group existence

Based on structure prediction made before, the H_{94} compound having *m/z*= 371 most probably belong to flavanone derivative compound hydroxylated and glycosylated by a pentose sugar. A hydroxyl group was predicted at position 4' of B ring based on ^1H NMR spectrum analysis showing *para*-disubstituted existence. While a pentose sugar group was predicted to be bound at position 6 or 8 since naturally sugar groups usually exist at those positions in *C*-glycoside flavonoid (Figure 8). Pentose sugar groups which have ever been identified to be combined with flavonoid are xilose and arabinose [28].

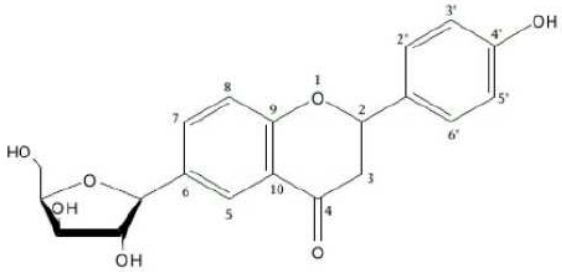


Figure 8 Flavanone derivative compound glycosylated by pentose sugar at position 6

Fragmentation pattern of this compound was proposed as in Figure 9, yet none of m/z values of these fragments was observed in mass spectrum (Figure 5). It may due to the low energy used in ionization process. When a weak ionization method like ESI and APCI are used to analyze flavonoid compounds, generally fragmentation is not

observed. This problem can be overcome by using CID integrated with MS-MS [29]. This compound was predicted to have molecular weight, 372 g/mol with $[M+H]^{\cdot+}$ at m/z= 371. The $[M+H+1]^{\cdot+}$ fragment also appears at m/z= 372.

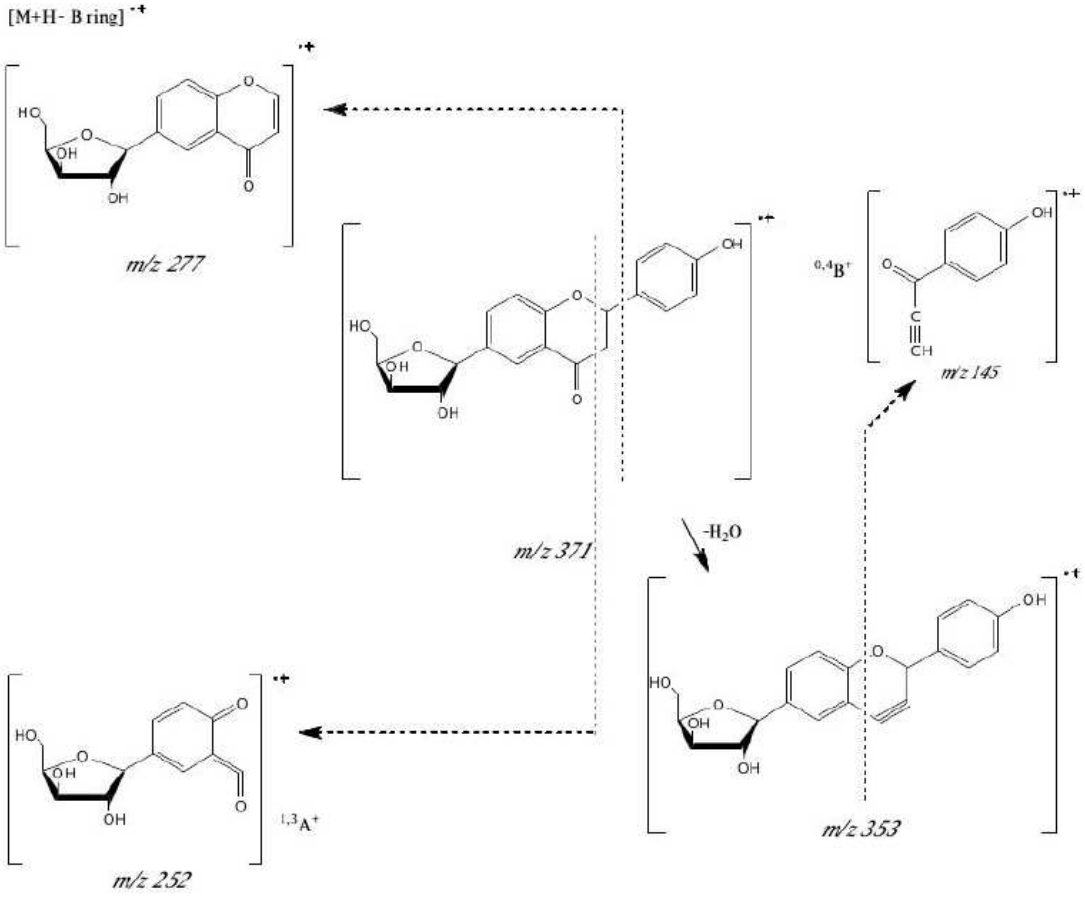


Figure 9 The H_{94} presumed compound fragmentation pattern

The molecular ion existence at this m/z value is supported by probability of *pseudomolecular ion* formation. This ion is formed as adduction result between molecular ion or its fragments and cation existing in the solvents or with the solvent mediated by that cation. In ESI-MS, complex formation from analite with alkali metal cations commonly is observed since Na^+ or K^+ cation exists in the solvent or glassware used [30]. Those cations generally are extracted from glassware during storage and are easier formed in flavonoid substituted at position 3, like flavonol 3-*O*-glycoside and isoflavone [28]. The presumed compound, H_{94} has ketone functional group with free electron pair as a ligand which will complex metal cation, Na^+ or K^+. Besides, Na^+ ion can form 5 or 6 coordination with ligand so that one Na^+ ion can coordinate with free electron pair from that ketone functional group and with the other molecules like solvent [31]. Based on this fact, it was predicted that molecular ion appearing at m/z= 371 formed an adduct as $[^{0,4}B^+ + Na + 5H_2O]^{\cdot+}$ and $[M+Na]^{\cdot+}$ so peaks having low abundance appear respectively at m/z= 259 and m/z= 393 with abundance less than 10% and around 39%. Besides, this molecular ion can also form $[M+Na+H_2O+CH_3OH]^{\cdot+}$ and $[2M+Na]^{\cdot+}$ adduct so peaks having low abundance also appear respectively at m/z= 444 and m/z= 765 with abundance less than 10% and around 18% (Figure 10).

The m/z values obtained and probability of those adducts formation strongly supported the former hypotheses that H_9 compound having retention time (t_R) equal with 6.7 minute, or simply the H_{94} is predicted belong to C-glycoside flavonoid, that is 4'-hydroxy-6-*C*-xylofuranosyl flavanone. This compound has not been found in *C. alata* yet.

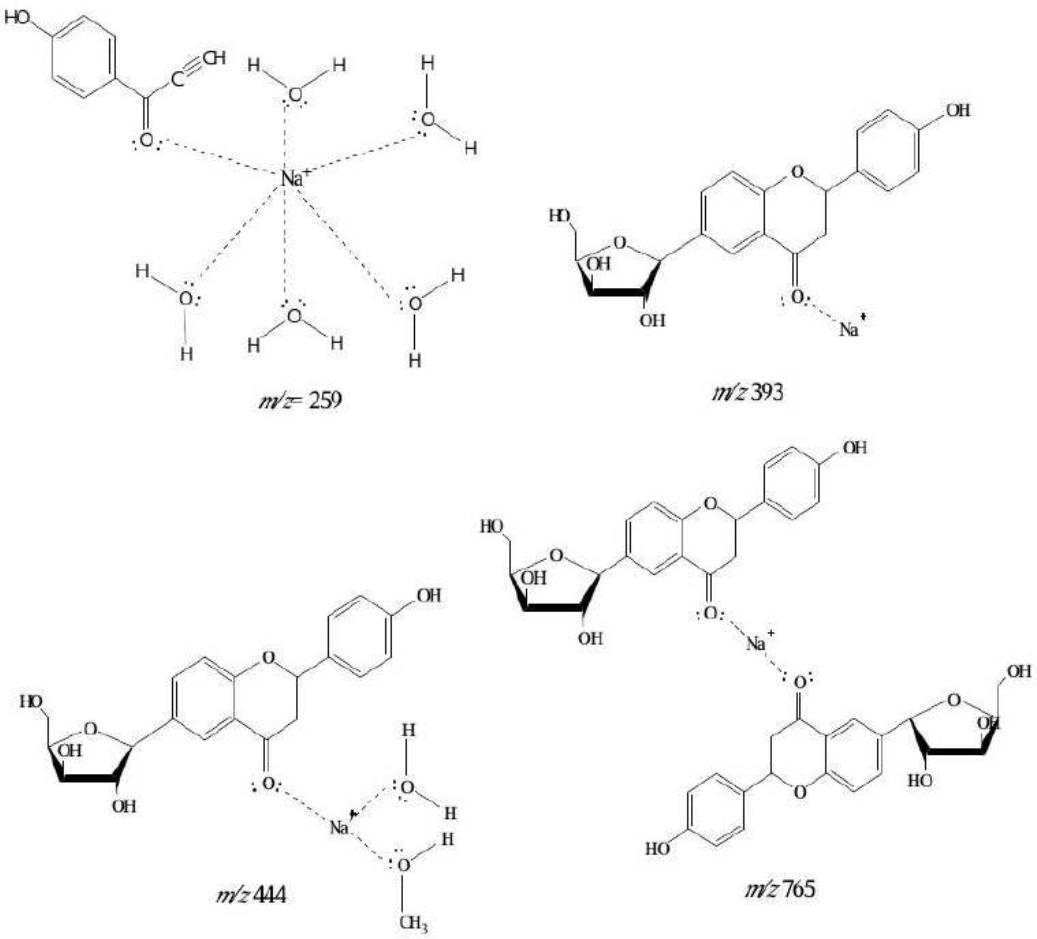


Figure 10 **Adducts** formed in ESI-MS positive ion mode between molecular ion, fragment, Na^+ ion, and solvent molecule

CONCLUSION

The phytochemical result of *C. alata* leaves methanolic extract from Cikasungka village, Solear, Tangerang, Indonesia was identified to contain phenolics, flavonoids, steroids/triterpenoids, saponins, and alkaloids. Two components characterized successfully were belong to phenolic compounds, methyl *p*-hydroxybenzoate, and flavonoid adduct which presumed as pelargonidin-3-(feruloyl)diglucoside-5-(malonyl)glucoside, respectively.

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