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Isolation of two methoxy flavonoid compounds from *kumis kucing* (*Orthoshipon stamineus*, Benth.) a popular plant in Indonesian herbal medicine *Jamu*

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ABSTRACT

Kumis kucing (*Orthosiphon stamineus,* Benth.) is a medicinal plant that has been used traditionally in Indonesia. Its traditional uses have been also proved by scientific researches. This plant has been developed from traditional use to rational phytotherapy with some indications. Since *kumis kucing* has many pharmacological activities, it is assumed that the compounds which are responsible for the activities not only sinensetin, other compounds could probably be a marker compound of this plant. Extraction was done using soxhlet apparatus and ethyl acetate as a solvent, then separated with $CuCl_2$ and NaOH solution to clean up the chlorophyll contents. Subfractination was done with serial chromatographic methods. Isolated compounds were characterized by UV-Vis spectrophotometry and nuclear magnetic (NMR)-^{1}H. UV spectra showed that compound **X** had λmax at 267 and 319 nm while compound **Y** had λmax at 269 and 328 nm. After adding shift reagents, there were not bathochromic or hipsochromic shift in the spectra. NMR-^{1}H spectra showed that compound **X** had chemical shift at 3,82; 3,88; 3,90; 4,01,6,55; 7,10 and 7,99 ppm**.** Compound **X** was assumed to be 5,7,8,4'-tetramethoxy–flavone which has one free OH group in ring A or ring B and isolate **Y** was concluded to be sinensetin.

Keywords: Kumis Kucing, *Orthosiphon stamineus*, Indonesian Medicinal Plant, Methoxy Flavones, Sinensetin.

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INTRODUCTION

Orthosiphon stamineus, Benth belonging to family Lamiaceae known as *kumis kucing* is one of important medicinal plant in Indonesia as well as in Shout East Asian region that has been used to treat several diseases and be one of the components for many marketed herbal medicines (*jamu*) produced by industries [1,2]. *O. stamineus* contains some active compounds which have been tested their pharmacological activites. Around 116 compounds have been reported belong to group of compounds such as monoterpenes, diterpenes, triterpene, saponin, flavonoids, essential oil and organic acid. Flavonoid group is an important secondary metabolites found in this plant. The highest flavonoid contents from this plant is methoxylated flavonoid such as sinensetin which is one of flavonoid reported to have pharmacological activities [3]. As a biggest natural fenolic compound, at least 2% out of all carbon synthesized by plants are converted to flavonoid and related compounds [4]. It could be found in all part of plants such as root,steem, leaf, wood, flower, fruit and aerial part, so that almost of plants contains flavonoid [5]. Sinensetin ($C_{20}H_{20}O_7$) is a 3',4',5,6,7-pentametoksiflavon, a colorless powder with molecular weight and melting point are 372.37 g/mol and 174-176 °C respectively and semipolar compound. Sinensetin content in *O. stamineus* is relatively low. It is around 2,1µmol/g (purple flower) and 2,9 µmol/g (white flower) [6]. Due to the good stability, sinensetin could be used as a marker in the herbal preparation. According to the determination of herbal medicines quality containing this plant, sinensetin has been used as a marker [7] . Leaves of *O. stamineus* have been used to treat kidney stones, urolithiassis and diuresis [8]. It has been also reported to be antioxidant, antidiabetes, antitumor, anti hpertension, diuretic, anti inflammation and hepatoprotector [10,11]. Sinensetin has been reported to have the diuretic, antioxidant anti antibacterial. Diuretic activity of sinensetin was shown in the rat at the dose of 10 mg/kg BB after intravenous administration. Hydroxy alcohol extract of *O. stamineus* leaves at the dose of 2g/kg BW showed the diuretic activity in the rat through enhancement the urine volume and the concentration of sodium and kalium. this effect is comparable to positive control hydroklortizid at the dose of 10 mg/kg BW. While this extract at the dose of 0,5, 1 and 2 g/kg BW could reduce the concentration of uric acid of blood serum. Polysaccharide isolated from *O. stamineus* was reported to reduce the urine volume and calcium oxalate excretion and inhibit the formation of calcium oxalate of the rat which was induced nephrolitiasis using ethyilene glycol 1% and ammonium chloride1 % in 7 days [12,13,14] . Methyilpariochromene A isolated from *O. stamineus* has antihypertension effect through reducing blood pressure, vasodilatation, cardiac output and enhancing urine volume as well as enhance the excretion of sodium, calium and chloride [15]. Chloroform extract of *O. stamineus* at the dose of 1 g/kg BW reduce the blood glucose level of rat which has been induced diabetes using glucose subcutaneous at the dose of 150 mg/kg BW. Its fractions also showed the antidiabetic activity through the same mechanism with metformin which has antihyperglycemic effect in normal rate [16]. Water extract of leaves at the dose of 0,2-1,0 g/kg BW reduce the plasma glucose level of normal and diabetic rat, while at the dose of 1 g/kg BW has the similar effect with antidiabetic drug glibenclamid at the dose of 5 mg/kgBW [17]. Acetone extract has higher antioxidant activity than water, hydroalcohol and chloroform using 1,1-diphenyl-2-picrylhydrazide (DPPH). In this research total phenolic compounds have antioxidant effect comparable to quersetin and buthyl hydroxyl anisol (BHA) [10]. Antioxidant activity has also been shown by *O. stamineus* by inhibiting of NO formation in lipopolysacharide-activated macrophage cell. The compounds from this plant tested such as siponol A-C, E, 2-*O*-deacetylortoshipol J, orthosipol A, B, D, H, K, M, N, O, X, Y, staminol A, neoortosipol B, staminol C and D, orthosiponon C and D and 14-deokso-14-*O*-asetilortosipol Y, norortosiponolid A, ortosiponon A, sekoortosipol B and C have higher antioxidant activity compare to positive control NG-monomethyl-L-arginin. 2-*O*-deacethylorthosiponon A is the most potential antioxidant with IC_{50} 35.0 μM. Inhibition effect in NO formation is assumed to be related to the strukture of diterpen [18] . Water extract of *O stamineus* has antimicrobial activity against *Staphylococcus aureus* with inhibition zone and minimum concentration (MIC) of 10,5 mm and 1,56 mg/ml respectively [19]. This study was aimed to isolate the methoxyflavone compounds from *O. stamineus* and modify the isolation procedure in order to optimize the process.

MATERIALS AND METHODS

Plant Material

Orthosiphon stamineus, Benth. herb were collected in Manoko Lembang West Java, Indonesia and determined in Herbarium Bandungense, Biology Study Program, School of Life Sciences and Technology, ITB. The plants were dried in oven and powdered

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Extraction

Extraction was performed using two strategies to optimize the yield. The first strategy was done by Soxhlet apparatus using three solvents with different polarity i.e n- hexane, ethyl acetate and methanol. The second strategy was done using Soxhlet apparatus using ethyl acetate as a single solvent. All extract were evaporated their solvents using rotary evaporator providing concentrated extract. These extracts were used for further isolation.

Isolation and structure elucidation of methoxylated flavonoids from the extracts

Ethyl acetate extract which contain flavonoid was further fractionated using liquid-liquid extraction using $CuCl_2$ and NaOH to eliminate chlorophyll in order to simplify the isolation process. Ethyl acetate fraction was evaporated using rotary evaporator. Flavonoid content of fraction was monitored by thin layer chromatography using silica gel GF_{254} and chloroform-ethyl acetate (6:4) as stationary and mobile phase respectively then visualized with UV light 254 and 365 nm in wavelength and H_2SO_4 10% in methanol. Ethyl acetate fractions were further isolated by vacuum liquid chromatography using silica gel 60 H as stationary phase and n-hexane : ethyl acetate with gradient concentration for mobile phase. The collected fractions were monitored by TLC with the system as mentioned above. The fractions containing flavonoid were combined and evaporated their solvent, then further continued isolation using chromatotron. To purify the isolated compounds, preparative TLC was performed. The purity of isolated compounds was monitored using TLC 2 dimensions and co-chromatography using three different solvent with different polarity. Isolated compounds were characterized using TLC with specific spray reagent, spectrofotometry ultraviolet and nuclear magnetic resonance spectrometry.

RESULTS AND DISCUSSION

The yield of extraction using several solvents was vary as stated in Table 1. The highest yield which high content of flavonoid was provided by ethyl acetate solvent with direct extraction with Soxhlet apparatus (Table 2). This extract was used furthermore for isolation of methylated flavonoid.

Table 1: Extract yield and sinensetin content of *Orthosipon aristatus* with several solvents

Extraction methods	Solvent used	Extract yield (% b/b)
Soxhlet using several solvent	n-hexane	1.51
	ethyl acetate	3.11
	metanol	6.51
Soxhlet using single solvent	ethyl acetate	6.53

Table 2: Sinensetin content of different strategy and solvents of extraction using TLC densitometer

Extraction methods	Sinensetin content (Area in %)
A. Soxhlet with n-hexane	11,49
B. Soxhlet with ethyl acetate	11,12
C. Soxhlet with methanol	6,29
D. Soxhlet with ethyl acetate (single solvent)	12,36
E. Soxhlet with methanol (single solvent)	10,24
F. Maceration with methanol	10,83
S. Sinensetin standard	86,85

Based on the TLC chromatogram of extracts as shown in Fig 1, there are some compounds available in the extract including chlorophyll with the red color band and a methoxylated compound with the yellowiest white fluorescence which was assumed as sinensetin when it was compared to standard sinensetin compound. In addition, some bands showed the same fluorescence with sinensetin. It was predicted that those bands are also methoxylated flavonoid. Furthermore the isolation was focused to the methoxylated flavonoid.

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Quantitative determination of sinensetin using TLC densitometer showed that ethyl acetate extract form direct Soxhlet extraction contains highest level of sinensetin (Table. 2).

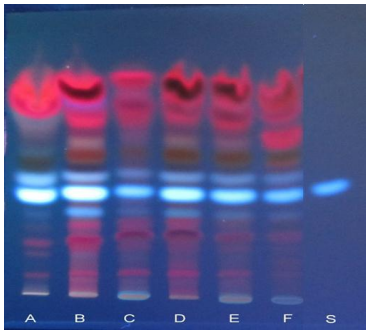

Figure 1: TLC chromatogram of extract from Soxhlet extraction using different solvent with different polarity: A) n-hexane, B) ethyl acetat, C) methanol, Soxhlet extraction with single solvent D) ethyl acetate, E) methanol, F). Extraction with maceration using methanol and S) sinensetin standard. Note: stationary phase silica gel GF_{254} and mobile phase chloroform-athyl acetate (6:4)

Results of phytochemical screening showed that flavonoid, phenol, quinone, and steroid/triterpenoid group of compounds were detected in both crude drug (dried and powdered plant) and ethyl acetate, saponin group has been only detected in ethyl acetate extract, while alkaloid has not been found in both samples (data not shown)

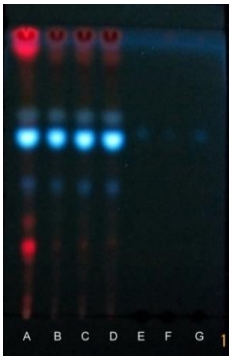

Figure 2: TLC chromatogram of ethyl acetate extract after treatment with $CuCl_2$ 1 M and NaOH 0,1 N (A) ethyl acetate extract, (B) with $CuCl_2$ 10 % and NaOH, (C) with $CuCl_2$ 20 % and NaOH, (D) with $CuCl_2$ 30 % and NaOH, (E, F and G), $CuCl_2$ fraction of extract B, C and D. Note: stationary phase silika gel 60 GF_{254}, mobile phase chloroform-ethyl acetate (6:4),

Liquid-liquid extraction of ethyl acetate extract using $CuCl_2$ and NaOH could reduce the chlorophyll content which was shown in TLC chromatogram with red color (Fig. 2). Reducing of chlorophyll was useful for the next isolation process. The chlorophyll are usually exist in every fraction of chromatography results. It would disturb the isolation process, so there is a need to eliminate them in the previous isolation process. This is a first attempt to pretreatment of isolation process to eliminate the chlorophyll and related contents in plant. This is probably applicable for simplification of isolation process of secondary metabolites from leaves which contain many chlorophyll.

Around 18 g of ethyl acetate after reducing chlorophyll have been fractionated by vacuum liquid chromatography using gradient elution with n-hexane and ethyl acetate yielded 21 fractions. All fractions were monitored by TLC using chloroform-ethyl acetate (6:4) as mobile phase. According to TLC chromatogram, 9 fractions contain sinensetin and other flavonoid (Fig. 3). Three of fractions with less impurity were combined and further isolated using radial TLC (chromatotron) yielded 32 subfractions. Subfractions 13-20 were combined and further isolate using coloum chromatography then followed by TLC preparative. There were three dominant bands in TLC preparative chromatogram. Two bands were successfully scrapped and re-

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dissolved with ethyl acetate. Ethyl acetate solvent was evaporated resulting pure crystal named isolated compound X and Y

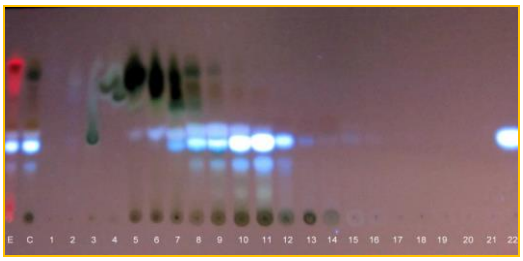

Figure 3: TLC chromatogram of fractions after isolation with vacuum liquid chromatography. Note: stationary phase silica gel GF_{254}, mobile phase chloroform-ethyl acetate (6:4) and visualization with UV light λ 366 nm

To check the purity of isolated X and Y, TLC with three different mobile phases and TLC two dimensions were done. All TLC chromatogram showed the only one band is each mobile phase used. It was assumed that the isolated compounds were pure enough for identification

Isolated compound Y is white yellow fine powder. Based on UV spectra, this compound showed two peaks which are specific for flavonoid group i.e λ 269 and 328 nm, while isolated compound X is also white yellow fine powder showing two peaks in λ 267 and 319 nm (Fig. 4). According to TLC, UV spectra, NMR-^{1}H, NMR-^{12}C data and compared to standard compound, it is concluded that the isolated compound Y is sinensetin.

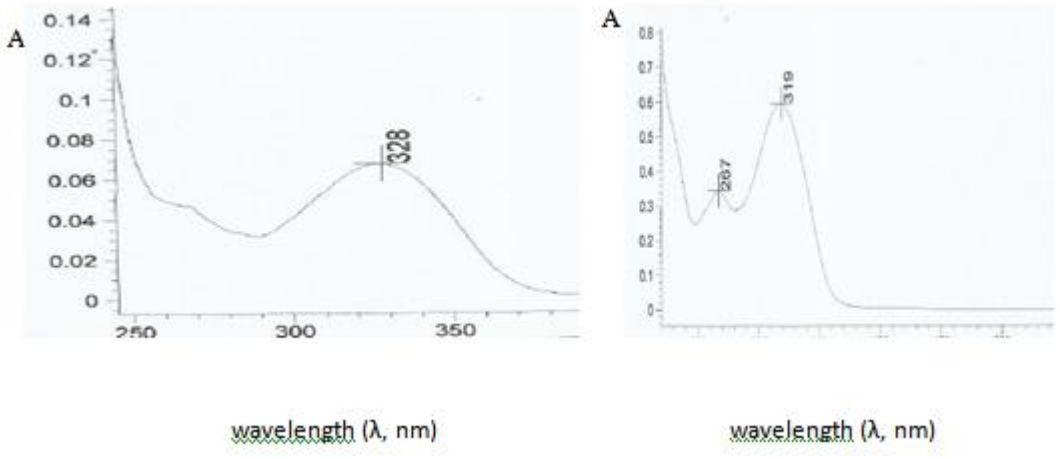

Figure 4: UV spectra of isolated compound Y (left) and X (right) in methanol, note: A =absorban

Analysis of isolated compound X using TLC with spray reagent of $FeCl_3$ showed that this compound was a flavonoid having OH group in aromatic ring. Further identification using shift reagents sodium hydroxide, sodium acetate, aluminium chloride, hydrochloride acid and boric acid showed no batocrhomic and hipsochromic shifts in the both peak of UV spectra (Table 3). It showed that there is no additional OH group in ring A and B.

Table 3: Interpretation of UV spectra of isolated compound X after reaction with shift reagents

Sift reagent	Maximum wavelength (λ, nm)		Shift of λ (Δ nm)	Interpretation
	Peak I	Peak II		
MeOH	319	267	-	-
NaOH	319	267	-	No OH group in position 3 and 4'

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NaOAc/ H_3BO_3	319	267	-	No o-di-OH in ring A and B
$AlCl_3$/HCl	319	267	-	No OH group in position 5 and o-di-OH in ring B

Further identification of isolated compound X was done using NMR spectrometry (Fig. 5). RMI-^{1}H spectra showed 3 proton at δ 3,5-4 according their integration. In aromatic area of δ 6-8 showed three signals which are a singlet, doublet and a triplet signals in one proton in δ 6-8. Unsubstituted flavon should has ten proton signals. In RMI-^{1}H spectra, there are five proton signals that have been identified. Other five proton signals could be substituted by 4 methoxy groups which are detected in δ 3,8-4 and another OH group in ring A or B which are not in position 3, 5 and 4′. These results were agreed with the interpretation of UV spetra which are no shifts showing OH groups in these positions. Taken together, identification data using TLC, UV and RMI-^{1}H spectra showed that isolated compound X was assumed 5,7,8,4'-tetramethoxy-flavone with a free OH group in ring A or B.

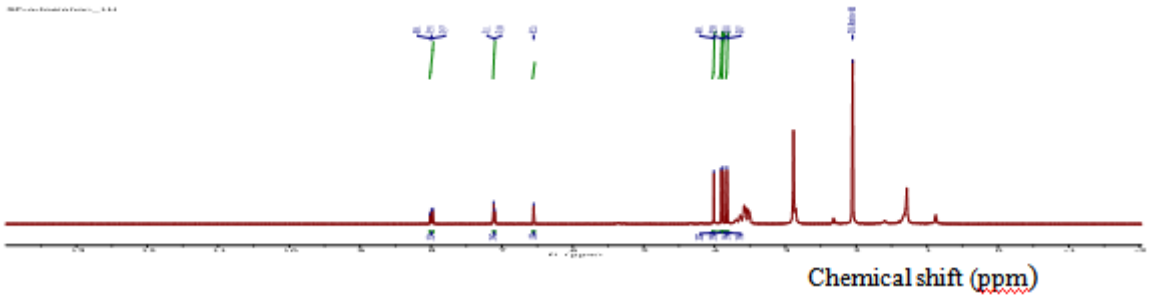

Fig 5: RMI-^{1}H Spectra of isolated compound X

CONCLUSION

Two methoxy flavonoid compound have been successfully isolated and identified from leaf of *Orthosiphon stamineus*, Benth which are known compound sinensetin and a 5,7,8,4'-tetramethoxy-flavon with a free OH group in ring A or B. Extraction by Soxhlet using ethyl acetate as a solvent continuing with liquid-liquid extraction by CuCl2 and NaOH reducing the effect of chlorophyll content in the isolation process.

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REFERENCES

[1] Ohashi K, Bohgaki T, Matsubara T, Shibuya H, Indonesian medicinal plants XXIII. Chemical structures of two new migrated pimarane-type diterpenes, neoorthosiphols A and B, and suppressive effects on rat thoracic aorta of chemical constituents isolated from the leaves of *Orthosiphon aristatus* (Lamiaceae). Chem Pharm Bull 2000; 48: 433-435

[2] Elfahmi, Woerdenbag HJ, Kayser O, Jamu: Indonesian traditional medicines toward rational phytopharmacological use. J Herbal Med 2014; 4: 51-73

[3] Franz-Christian, C. Herbal Drugs and Phytopharmaceuticals. Medipharm Scientific Publishers, Stuttgart. 1994

[4] Harborne JB, Mabry TJ, The Flavonoid : Advances in Research,Chapman and Hall, London, 1982, 1-16

[5] Markham KR, Cara Mengidentifikasi Flavonoid. Penerbit ITB Bandung. 1998, 15-27, 39-42.

[6] Sumaryono W, Analisis Profil Metabolit Sekunder Beberapa Kultivar *Orthosiphon aristatus.* Badan Pengkajian dan Penerapan Teknologi, Jakarta, 1994.

[7] Anggraeni, Kandungan utama kumis kucing. In Hasil Penelitian Plasma Nutfah dan Budidaya Tanaman Obat, Prosiding Forum Komunikasi Ilmiah. Bogor, 1992. Bogor: Pusat Penelitian dan Pengembangan Tanaman Industri, 1992, 165-170.

[8] De Waard PWF, Goan LT, Agricultural, Technological, and Economic Aspect of some Selected Medicinal Plants. Amsterdam: Selected Medicinal Plants. Department of Agricultural Research, Amsterdam, 1978
[9] Ohashi K, Bohgaki T, Shibuya H, Antihypertensive substance in the leaves of kumis kucing (*Orthosiphon aristatus*) in Java Island. J Pharm Soc Jpn 2000; 120: 474-482.
[10] Akowuah GA, Ismail Z, Norhayati I, Sadikun A, Radical scavenging activity of methanol leaf extracts of *Orthosiphon stamineus*. Pharm Biol 2005; 42: 629-635.
[11] Sumaryono W, Proksch P, Wray V, Witte L, Hartmann T, Qualitative and quantitative analysis of the phenolic constituents from *Orthosiphon aristatus*. Planta Med, 1991, 57, 176-180.
[12] Schutz GA, Zwaving JH, Pharmacological investigation of some lipophilic flavonoids from *Orthosiphon aristatus*. Fitoter 1993; 64: 99-102.
[13] Arafat OM, Tham SY, Sadikun A, Ismail Z, Haughton PJ, Asmawi MZ, Studies on diuretic and hypouricemic effects of *Orthosiphon stamineus* methanol extracts in rats. J Ethnopharm, 2008; 18: 354-360.
[14] Zhong YS, Yu CH, Ying HZ, Wang ZY, Cai HF, Prophylactic effects of *Orthosiphon stamineus,* Benth. extracts on experimental induction of calcium oxalate nephrolithiasis in rats. J Ethnopharm, 2012; 144: 761-767.
[15] Matsubara T, Bohgaki T, Watarai M, Suzuki H, Ohashi K, Shibuya H, Antihypertensive actions of methylripariochromene A from *Orthosiphon aristatus,* an Indonesian traditional medicinal plant. Biol Pharm Bull 199; 22: 1083-1088.
[16] Mohamed EA, Mohamed AJ, Asmawi MZ, Sadikun A, Ebrika OS, Yam MF, Antihyperglycemic Effect of *Orthosiphon Stamineus* Benth Leaves Extract and Its Bioassay-Guided Fractions. Molecules. 2011; 16: 3787-3801.
[17] Sriplang K, Adisakwattana A, Rungsipipat A, Yibchokanun S, Effects of *Orthosiphon stamineus* aqueous extract on plasma glucose concentration and lipid profile in normal and streptozotocin-induced diabetic rats. J Ethnopharm 2007; 109: 510-514.
[18] Awale S, Tezuka Y, Banskota AH, Shimoji S, Taira K, Kadota S, Norstaminane and isopimarane-type diterpenes of *Orthosiphon stamineus* from Okinawa. Tetrahed. 2002; 58: 5503-5512.
[19] Alshawsh MA, Abdulla MA, Ismil S, Amin ZA, Qader SW, Hadi HA, Harmal NS, Free radical scavenging, antimicrobial and immunomodulatory activities of *Orthosiphon stamineus***.** Molecules. 2012; 17: 5385-5395.