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RESEARCH ARTICLE

Anticancer Property of *Orthosiphon stamineus* Benth. Extracts in Different Solvent Systems against T47D Human Breast Cancer Cell Lines

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SUMMARY

Solvent system is an important factor in extraction process in order to obtain compounds that have pharmacological activity. The aim of this research is to develop a comprehensive extraction methods by modification of solvents used that might produce compounds possessing pharmacological activity for anticancer. In this study, Orthosiphon stamineus Benth. extract was used as sample in different solvent systems to observe their metabolite profiles. Extraction carried out using sonication techniques with ethanol solvents in three types of concentrations (96%, 70% and 50%). Then, the extracts introduced into anticancer activity profiles in order to find its active compounds. The anticancer activity had explored against breast cancer cells (T47D) using the MTT assay and doxorubicin as a positive control. The best IC_{50} value obtained from the 50% ethanol extract of Orthosiphon stamineus Benth. Based on the scanning chromatogram at 366 nm using Thin Layer Chromatography, each sample contains sinensetin and rosmarinic acid. The largest percentage of rosmarinic acid area was found on 70% ethanol extract of Orthosiphon stamineus Benth, while the highest percentage of sinensetin was found on 50% ethanol extract of Orthosiphon stamineus Benth. Thus, it can be concluded that sinensetin which has the most influence on anticancer activity.

Key Words: *Orthosiphon stamineus, breast cancer, rosmarinic acid, sinensetin, T47D, anticancer*

Orthosiphon stamineus Benth'in farklı Çözücü Ekstrelerinin T47D İnsan Meme Kanseri Hücre Hattına Karşı Antikanser Özelliği

ÖZ

Solvent sistemi, farmakolojik aktiviteye sahip bileşiklerin elde edilmesinde ekstraksiyon sürecinde önemli bir faktördür. Bu araştırmanın amacı, antikanser farmakolojik aktiviteye sahip bileşikler için çözücüleri modifiye ederek kapsamlı bir ekstraksiyon yöntemi geliştirmektir. Bu çalışmada Orthosiphon stamineus Benth. ekstraktı metabolit profillerini gözlemlemek için farklı solvent sistemlerinde numune olarak kullanılmıştır. Ekstraksiyon, üç farklı konsantrasyonda (% 96, % 70 ve % 50) etanol çözücülerle sonikasyon teknikleri kullanılarak gerçekleştirildi. Daha sonra ekstraktlar, aktif bileşiklerini bulmak için antikanser aktivite profillerine sokuldu. Antikanser aktivitesi, meme kanseri hücreleri (T47D) kullanılarak MTT analizi ile araştırılmıştır, pozitif kontrol olarak doksorubisin kullanılmıştır. Orthosiphon stamineus Benth'in elde edilen en iyi IC_{50} değeri % 50 etanol ekstraktında bulunmuştur. İnce Tabaka Kromatografisi kullanılarak 366 nm'de elde edilen kromatogramına göre, her numune sinensetin ve rosmarinik asit içermektedir. Rosmarinik asit alanının en büyük yüzdesi Orthosiphon stamineus Benth'in % 70 etanol ekstresinde bulunurken, en yüksek sinensetin yüzdesi Orthosiphon stamineus Benth'in % 50 etanol ekstresinde bulunmuştur. Böylece antikanser aktivitesi üzerinde en fazla etkiye sahip olanın sinensetin olduğu sonucuna varılabilir.

Anahtar Kelimeler: *Orthosiphon stamineus, meme kanseri, rosmarinik asit, sinensetin, T47D, antikanser*

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INTRODUCTION

Recently, the efforts to treat cancer patients have focused on inhibiting the growth or killing cancer cells. The investigations to find an ideal drug that targets cancer cells with minimal side effects are ongoing. Some Indonesia medicinal plants may have a potential bioactive compound to be developed into an ideal drug for cancer. One of them is *Orthosiphon stamineus* Benth.

Orthosiphon stamineus Benth. (Lamiaceae) is a Indonesia plant native, traditionally used for diuretics, rheumatism, diabetes and hypertension (Fei *et al*., 2010). Previous study reported the activity of this plant extract inhibits human oral cancer cells (Younis *et al*., 2013). The methanol extract of this plant enhanced tamoxifen on breast cancer cell (MCF 7) proliferation (Sahib *et al*., 2009). In addition, 200 mg/kg ethanol extract of this plant showed no tumor cell growth compared to control group using xenograph method of tumor models that transplanted with breast cancer cells (MCF7) and colon cancer cells (HCT116) (Ahmad *et al*., 2010).

The bioactive compound will become lead compound to find an effective drug for cancer. One of the bioactive compounds found in this plant is sinensetin (5,6,6,7,3',4'-pentamethoxy flavone). It also contains orthosiphol D, orthosiphol E (Takeda *et.al*, 1993), orthosiphol A, orthosiphol B, 3'- hydroxy-5,6,7,4'-tetramethoxyflavone, neoorthosiphol A, neoorthosiphol B, α-amyrin, β-amyrin, maslinic acid, urosolic acid, orthosiphonone A, orthosiphonone B, myo-inositol, β-caryophyllene, caffeic acid, sinensetin, tetra-methyl scutellarein, eupatorin, cirsimaritin, acetovanillochromene, orthochromene A, methylripario chromene, agermacrene-D, β-selinen, α-cadinol, choline, betaine, O-cyamenea-terpineol, lyrol, valencene, nephthalin, camphor, α-elemene (Singh *et al* 2015), 5,6,7,8-tetra hydroxy-6-methoxy-flavones (Hossain *et.al*, 2008), potassium, flavonol glycosides, caffeic acid (rosmarinic acid) (Sumaryono, *et al*., 1991), essential oils, diterpenes, lipophilic flavones such as eupatorin, (6-hydroxy-5,7,4-trimethoxy flavone), and TMF (3'-hydroxy-5, 6, 7, 4 'tetrametoxy flavone) (Awale *et.al*, 2001), triterpenes such as betulinic, ursolic, oleanolic acids, β sitosterol (Tezuka *et al*., 2000) and flavonoids such as 5-hydroxy-6,7,30,40-tetrametoxiflavone, salvigenin, 6-hydroxy-5,7,40-trimethoxyflavone, 5,6,7,30-tetramethoxy-40-hydroxy-8-Cprenylflavone (Hossain and Rahman, 2015). The chloroform extract of this plant contained 1.48% of sinensetin, 2.26% of eupatorin, and 0.58% of 30-hydroxy- 5,6,7,40-tetrametoksiflavon (Mohamed *et al*., 2013, Yam *et al*., 2012).

Previous studies showed that the 50% methanol extract of this plant using freezed and sprayed dried methods contained protein, polysaccharides and saponins (Siddiqui *et al*., 2009). Research on metabolite profiles in this plants had been carried out using chromatographic and spectroscopic techniques combined with chemometrics (Akowuah *et al*., 2004; Sumaryono, *et al*., 1991; Saidan *et al*., 2015a). Ethanol extract using maceration method contained high phenolics and flavonoids, (rosmarinic acid and eupatorin) as antioxidants, while 50% ethanol and methanol extracts using soxhlet contained high protein and glycosaponin. Water extracts using reflux and maceration showed high polysaccharides (Saidan *et al*., 2015b).

Solvent system is an important factor in extraction process in order to obtain compounds that have pharmacological activity. In this study, the effect of different solvents on the metabolites profile in each extract had been determined. Extractions with 96, 70 and 50% ethanol were carried out according to the previous study with different method (Arifianti *et al*., 2014). Arifianti had extracted this plant using maceration method while this study used ultrasonic method to accelerate the extraction process and % yield with optimum results at same concentrations. It correlated to the anticancer activity (breast cancer) and their secondary metabolites are responsible for their activity.

MATERIAL AND METHODS

General Experimental Procedures and Materials

The plant was extracted on CAMAG ultrasonic and then evaporated by BUCHI rotary evaporator. The metabolite profiles of *Orthosiphon stamineus* Benth leaves were measured on a CAMAG Scanner 3 Densitometer and Linomat 5. The solvent extracts were combination between ethanol p.a (Merck) and aquadest. Sinensetin and rosmarinic acid from Sigma used as standard. The material used for breast cancer activity of T47D cells were RPMI 1640 medium (Gibco, Invitrogen), Fetal Bovine Serum (FBS, Sigma), Penicillin-Streptomycin (Sigma), Amphotericine B (Sigma), Dimethyl sulfoxide (DMSO, Sigma), Phosphate Buffer Saline (PBS, Sigma), 3-(4,5-dimethylthiazol-2-yl)2-5- diphenyl tetrazolium bromide (MTT, ThermoFisher) and sodium dodecylsulfate (Sigma). The cancer cells inhibition was determined with Robonik Elisa Reader.

Plant Materials

Orthosiphon stamineus Benth leaves were obtained on late April 2018 from Balai Materia Medika, Malang and voucher specimens were deposited in Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Universitas Airlangga.

Extraction of plant materials

Extraction was carried out by ultrasonic method according to the previous published method by modification (Juliana *et al.*, 2019). The dried leaves of *Orthosiphon stamineus* Benth (50 g) were extracted with 250 mL of each 96%, 70% or 50% ethanol (3 x 10 minutes) using CAMAG ultrasonic. Then the extracts were separated by filtration. The residue was re-extracted by using same procedure (3 times repeated). The filtrates were evaporated by BUCHI rotary evaporator to dryness under *vacuo* to get 96%, 70% or 50% ethanol extracts. The extracts were used to examine bioassay activity and phytochemical analysis. It performed by thin layer chromatography (TLC) to observe the sinensetin and rosmarinic acid profile.

Phytochemical analysis of plant extracts using TLC

The 10 mL of each 96%, 70%, 50% ethanol extracts of *Orthosiphon stamineus* Benth, sinensetin and rosmarinic acid were applied on a pre-coated TLC plate, silica gel 60F 254 (10 cm x 20 cm) as 7 mm bands using a Camag automatic TLC sampler (Linomat 5) spray-on band applicator equipped with 100 mL syringe, and the space between two spots was 2 mm of the plate. The extracts were each applied duplicates on the plate. The TLC plates were developed with chloroform-ethyl acetate in a ratio of 6:4 as mobile phase. Then, they were identified using a UV lamp at 365 nm. The phytochemical were analyzed based on chromatogram pattern using a CAMAG TLC Scanner 3 Densitometer and winCATS software, using a deuterium light source, the slit dimension was 6.00 x 0.45 mm. Peak areas were recorded and the spot of sinensetin and rosmarinic acid in the sample were confirmed by comparing the RF and spectra of the spot with that of sinensetin and rosmarinic acid standard (Arifianti *et al.*, 2014, Hossain and Ismail, 2016).

Cell line

The T47D Human breast cancer cell lines were obtained from the CCRC (Cancer Chemoprevention Research Center), Gajah Mada University, Indonesia and a modification method described by Fresney Method (Freshney, 2005). The T47D Human breast cancer cells were maintained in RPMI 1640 that contained 10% of FBS, 2% of Penicillin-Streptomycin and 1% of Amphotericine B. It was stored at 37°C with humidified atmosphere of 5% CO_2 (Eppendorf). The cells were routinely observed to keep them from contamination.

Measurement of inhibition of cancer cell by MTT method

The MTT method used was a method that has been modified by Freshney (Freshney, 2005). The 5 x 10^4 cells/wells of T47D cells with or without samples (96%, 70%, 50% ethanol extracts of *Orthosiphon stamineus* Benth) were cultured in RPMI 1640 medium that contained 10% of FBS and 1 % (v/v) of penicillin-streptomycin into 96 well plate then incubated for 24 hours at 37°C and 5% CO_2 (70-80% confluent). The samples were dissolved in DMSO and further diluted with medium to make series of concentrations (15 – 1,000 mg/mL). The final concentration of DMSO in the test solution should not more than 1%. Control cell was treated with 1% DMSO. Cells were then treated with a serial dilution of tested samples. The doxorubicin concentrations of 2.5-100 mg/ml were used as positive control. After 24 h incubation, 0.5 mg/ml of MTT was added to each well and incubated for 4 hours. Then, the stopper solution (sodium dodecylsulfate 10% in 0.1 N HCl) was added to dissolve the formazan crystal and incubated overnight at room temperature and dark. Finally, the cells viability was measured using ELISA reader at l 570 nm. The absorbance of each well then converted into percentage of viable cells using calculation below and the IC_{50} values were determined by Probit analysis using SPSS software. Experiments were done in triplicates.

$$\%cellviability = \frac{samp\ absorbance - medium\ control\ absorbance}{cell\ control\ absorbance - medium\ contro\ labsorbance} \times 100\%$$

RESULT AND DISCUSSION

Extraction

Several ways can be do to obtain phytochemicals from plants, one of which is extraction. Extraction efficiency is influenced by the chemical properties of the compound, the extraction method used, the particle size of the sample, the solvent used, and the presence of disturbing substances (Stalikas, 2007). Conventional extraction techniques are often associated with long heating times and a risk of bioactive compounds degradation. This has led to sophisticated techniques such as ultrasonic extraction which are efficient in terms of extraction time and solvent consumption. In view of this method, ultrasonic cavitation produces shock waves that are able to disrupt the external structure of plant samples and release plant bioactives effectively (Budynas & Nisbett, 2008; Floros & Liang, 1994).

Ultrasonic extraction using ultrasonic frequencies at >20 kHz can accelerate the contact time between samples and solvents at room temperature. It causes the mass transfer of bioactive compounds from plant cells to solvent to be faster. Sonication relies on sound energy that causes the cavitation. It forms small bubbles due to ultrasonic frequency transmission to help the diffusion of solvents into plant cell walls (Ashley *et al.*, 2001).

General extraction parameters such as concentration and ratio of solvents using ultrasonic method were first optimized. The solvent used was ethanol because it is non-toxic, good polarity for the sound energy and ultrasonic frequencies, so it is able to dissolve interesting bioactive compounds (Xiao *et al.*,2008). The extraction yield depends on the solvent with various polarity, pH, temperature, extraction time, and sample composition. At the effect of the same extraction time and temperature, the solvent and sample composition are the most important parameters. In this study, *Orthosiphon stamineus* Benth. extracts were obtained using ethanol and water at various concentrations (50%, 70%, and 96%). Their extraction yields were ranged between 15.64%, 12.39% and 3.44%, respectively (Table 1). The results of extraction with various solvents decreased in the following order: 50% ethanol> 70% ethanol> 96% ethanol. It showed that the extraction yield increases with increasing polarity of the solvent used in the extraction. Increasing the concentration of water in the solvent can increase the extraction yield. Compounds other than sinensetin and rosmarinic acid in the extract, it may have been extracted and contributed to higher yields. This might be caused by higher solubility of protein and carbohydrate in water-ethanol than pure ethanol (Zieliński and Kozłowska, 2000). The use of a combination of water in organic solvents can facilitate the extraction of water-soluble chemicals and/or organic solvents. This might be the reason why the ethanol extract yield is 50% higher than other extracts. The results of this study are in accordance with the results of extraction in *Limnophila aromatica* (Do *et al.*, 2014) and several medicinal plants (Sultana *et al.*, 2009).

Table 1. % yield of *Orthosiphon stamineus* Benth extracts

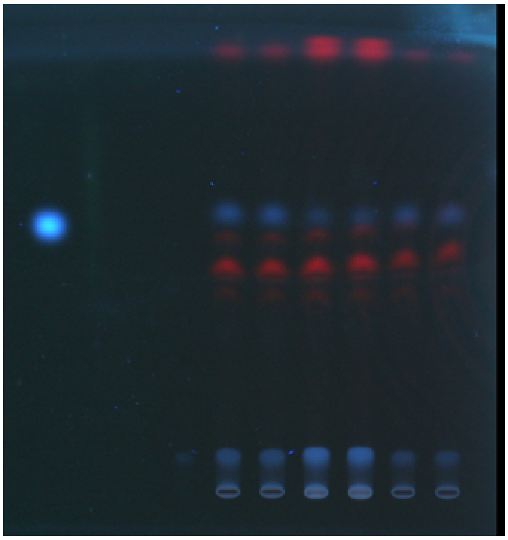
Sample	% yield
96% ethanol extract	3.444
70% ethanol extract	12.390
50% ethanol extract	15.636

The addition of water in the extraction solvent showed that the extraction yield is improved, because the presence of water increased heating efficiency due to its high dielectric constant (Sato & Buchner, 2004), and increased the permeability of plant matrices to encourage mass transfer and diffusion of bioactive compounds (Boeing *et al.*, 2014). The effects of aqueous ethanol have different effects, the optimum solvent concentration was found to be 50% (v/v) ethanol.

The phytochemical analysis

A number of sinensetin and rosmarinic acid found in the leaves of *Orthosiphon stamineus* Benth. TLC-densitometry is the current method for the quantization of some flavonoids and caffeic acid derivatives in pharmaceutical formulations. It is quickly gaining widespread acceptance in pharmaceutical analysis. This is due to simplicity, accuracy, cost effectiveness and possibility of simultaneous determination of a number of samples on a single TLC plate. HPTLC allows the identification and quantification of more than 20 samples in the same chromatographic process and requires more than 2 hours. Whereas TLC takes only 15-30 minutes because it does not require conditioning steps, such as in HPLC, and is cheaper.

A fingerprint chromatography was performed to describe components found in sinensetin & rosmarinic acid-rich extract using TLC densitometry according to the modified method of Hossain and Ismail (2016) as well as Hossain and Ismail (2009). Samples (96, 70 and 50% ethanol extracts of *Orthosiphon stamineus* Benth) and standard (sinensetin and rosmarinc acid) were explored on a silica gel GF 254 and developed with chloroform-ethyl acetate in a ratio of 6:4. under UV observation at 366 nm by TLC visualizer (Fig. 1). The advantage of using the TLC-visualizer method is easy, fast, accurate, inexpensive and most suitable for natural material analysis. A sample chromatogram showed the presence of spots of the same color and at the same Rf value as the standard (Fig.1). A single peak at Rf 0.49 was observed in the chromatogram of sinensetin standard and Rf 0.06 as rosmarinic acid standard. At 70% ethanol extract had more intensity spot because it contained higest rosmarinic acid compare than other extracts.



Sinensetin Ros Ac. 50% 50% 70% 70% 96% 96%

Figure 1. The chromatograms of samples and standard without any spray reagent, in UV light at 366 nm

TLC analysis results showed bright blue fluorescent spots and the ultraviolet spectrophotometry showed the spectra images that were identical between samples with standards spectra. Two spectra are said to be identical if they have a MF (Match Factor) price > 95. In this study, the price of a match factor from the sample against sinensetin and rosmarinic acid standard obtained 0.99559 and 0.99985, respectively, so that they can be said to be identical (Fig. 2). The presence of sinensetin and rosmarinic acid in samples were proven by comparison of standards spectra with components that are separate from the samples UV-VIS spectra. Figure 2a showed the standard UV-VIS spectra of sinensetin (black) with samples, while in figure 2b was rosmarinic acid (pink) with samples. It can be observed the presence of sinensetin and rosmarinic acid peak in a sample at the same Rf value. It showed the similarity of spotting between sinensetin and rosmarinic acid standard in each sample. This data is supported by a standard spectrum profile of rosmarinic acid that has similarities with the spectrum of 96, 70 and 50% ethanol extracts of *Orthosiphon stamineus* Benth. Furthermore, a similar spectrum image was also obtained between sinensetin standard and them, but there was a slight shift in the sinensetin spectrum of 96% ethanol extract of *Orthosiphon stamineus* Benth (Fig.2).

a. b.

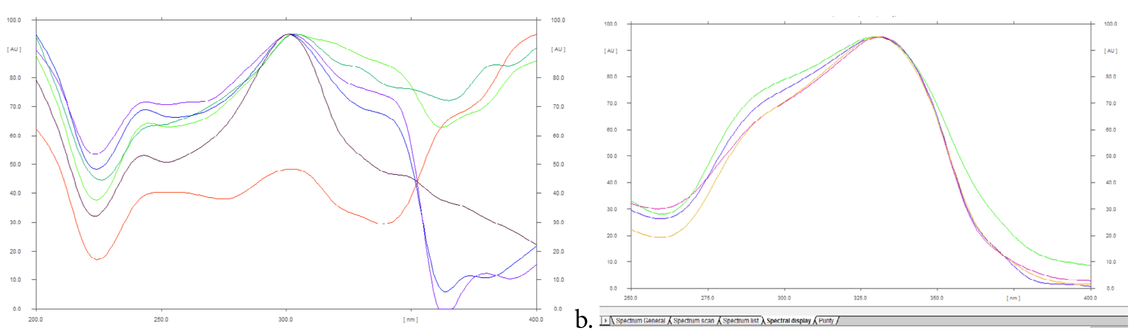


Figure 2. The UV-VIS Spectra overlay results of sinensetin (a, black) and rosmarinic acid (b, pink), together with samples of 50 % ethanol extract (blue), 70% ethanol extract (green) and 96% ethanol extract (yellow).

Quantitative determination was done by TLC-densitometry using the calibration curve method. The calibration curve was performed by the winCATS software program. In table 2, the percentage data of each chromatogram area refered to the sinensetin and rosmarinic acid standard. The largest percentage of rosmarinic acid area was found on 70% ethanol extract (74.61±0.03), while the highest percentage of sinensetin was found on 50% ethanol extract (32.97±0.06) of *Orthosiphon stamineus* Benth. So 70% ethanol is the best solvent system for extracting rosmarinic acid while 50% ethanol for sinensetin.

Table 2. Peak identification by TLC-densitometry

Samples	Start position	Start Height	Max position	Max Height	Max %	End Position	End Height	Area	% Area	Mean % area
50% EtOH Ex_1	0.06 Rf	0.4 AU	0.11 Rf	102.3 AU	72.22%	0.16 Rf	0.0 AU	1330.3 AU	65.85%	67.04±0.02
	0.48 Rf	4.3 AU	0.55 Rf	39.4 AU	27.78%	0.60 Rf	2.9 AU	689.9 AU	34.15%	32.97±0.06
50% EtOH Ex_2	0.06 Rf	0.6 AU	0.11 Rf	103.4 AU	73.36%	0.14 Rf	1.2 AU	1338.7 AU	68.22%	
	0.49 Rf	3.3 AU	0.55 Rf	37.6 AU	26.64%	0.59 Rf	3.2 AU	623.7 AU	31.78%	
70% EtOH Ex_1	0.06 Rf	1.0 AU	0.12 Rf	192.9 AU	80.66%	0.18 Rf	0.0 AU	2774.9 AU	76.45%	74.61±0.03
	0.49Rf	7.0AU	0.56 Rf	46.3 AU	19.34%	0.60 Rf	2.8 AU	855.0 AU	23.55%	22.15±0.47
70% EtOH Ex_2	0.06 Rf	0.2 AU	0.12 Rf	191.2 AU	76.66%	0.15 Rf	1.2 AU	2821.9 AU	72.77%	
	0.50 Rf	5.9 AU	0.56 Rf	46.0 AU	18.45%	0.61 Rf	0.3 AU	804.1 AU	20.74%	
96% EtOH Ex_1	0.07Rf	0.0AU	0.11 Rf	73.6 AU	72.24%	0.15 Rf	0.1 AU	892.7AU	70.10%	71.37±0.02
	0.54Rf	6.3 AU	0.57 Rf	28.3 AU	27.76%	0.61 Rf	2.7 AU	380.8 AU	29.90%	28.63±0.02
96% EtOH Ex_2	0.07Rf	0.3AU	0.12 Rf	75.4 AU	74.20%	0.18 Rf	0.0 AU	942.1 AU	72.64%	
	0.56Rf	4.6AU	0.60Rf	26.2 AU	25.80%	0.64 Rf	0.4 AU	354.9 AU	27.36%	

Anticancer activity

The anticancer properties of the ethanol extracts of 96, 70 and 50% of *Orthosiphon stamineus* Benth. were determined by MTT test. This test was chosen because it is reliable, simple, applies to a variety of cells, and can be done in microtiter plates. The test was based on the reaction of colorimetry of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide with the enzyme dehydrogenase in living cells to form a colored formazan corresponding to a viable cell numbers (McCauley et al., 2013).

Table 3. IC_{50} value of the samples on breast cancer activity

Sample	IC_{50} (ppm)
96% ethanol extract	259.016 ± 18.3
70% ethanol extract	390.521 ± 14.5
50% ethanol extract	159.049 ± 12.9
Doxorubicin	63.916 ± 5.5

In this study, the anticancer activities of the extracts were examined against human breast carcinoma cells (T47D). Based on the bioactivity results, the best IC_{50} value was obtained from 50% ethanol extract of *Orthosiphon stamineus* Benth. (table 3) and TLC-densitometry results also showed that this extract contains the higest sinensetin. Sinensetin was able to inhibit proliferation of gastric cancer cell, arterial blood gas (ABG) cancer cells by apoptosis mechanism through P53 and P21 regulation cell using Western Blot Technique (Dong *et al.*, 2011). While rosmarinic acid had known to prevent cell damage caused by free radicals, thereby reducing the risk of cancer and osteosclerosis (Fernando *et al.*, 2016) and is a major compound of polyphenol that can be used as a nutraceutical product that helps improve body immunity in cancer patients (Moore *et al.*, 2016). Therefore, the 50% ethanol extract from this plant showed the highest cytotoxic activity against T47D breast cancer cells compared to other extracts and sinensetin has an important role for anticancer properties in the extracts.

CONCLUSION

The 50% ethanol extract from *Orthosiphon stamineus* Benth showed the highest cytotoxic activity against T47D breast cancer cells compared to other extracts. This extract contains highest of sinensetin (32.97±0.06) compared to other extracts. This compound may be responsible for anticancer properties in the extracts.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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