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The effect of drying treatment to metabolite profile and cytotoxic potential of *Rhizophora apiculata* leaves

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Abstract. *Sibero MT, Siswanto AP, Pribadi R, Sabdono A, Radjasa OK, Trianto A, Frederick EH, Wijaya AP, Haryanti D, Triningsih DW, Hayuningrat SJ, Igarashi Y. 2020. The effect of drying treatment to metabolite profile and cytotoxic potential of Rhizophora apiculata leaves. Biodiversitas 21: 2180-2187.* Coastal communities in Indonesia have utilized *Rhizophora* spp. leaves as a traditional medicine for many years. The previous studies have succeeded in extracting bioactive compounds from this mangrove after drying treatment, but there is a possibility of the compound decomposition or breakdown. This study aimed to determine the effect of drying treatment on the metabolite profile of *R. apiculata* leaves which were taken from mangrove forests in Rembang, Central Java. The effect of pre-drying treatment was examined by comparing the metabolites profiles of fresh, oven-dried, and sun-dried leaves crude extracts. Extraction was carried out using maceration method with agitation (110 r.p.m.) for 24 hours in methanol. The metabolite profile was analyzed using high-performance liquid chromatography (HPLC) with diode array detector (DAD) and thin layer chromatography (TLC), while secondary metabolites were studied by phytochemical test. The phytochemical results showed that there were no differences in metabolites in dried and fresh *R. apiculata* leaves. Crude extract of fresh and oven-dried gave 10 spots on the TLC, while sun-dried crude extract had 9 spots. The one missing spot (R_f value of 0.79) in the sun-dried crude extract might have unstable compounds that are easily degraded or damaged by the sunlight. Moreover, HPLC chromatogram indicated the pre-drying treatment gave alteration to the *R. apiculata* metabolites that only detected at 400 nm. Cytotoxic assay against P388 murine leukemia cell indicated that oven-dried treatment gave the best anticancer activity with IC50 value of 0.0323 mg/mL.

Keywords: Bioactive, chromatography, cytotoxic, drying, *Rhizophora*

INTRODUCTION

FAO (2005) and Darajati et al. (2016) stated that Indonesia as a maritime country has abundant mangrove genera such as *Avicennia*, *Bruguiera*, *Ceriops*, *Excoecaria*, *Lumnitzera*, *Rhizophora*, *Sonneratia*, and *Xylocarpus*. These coastal plants provide sufficient ecological services for its associated organisms. In terms of ecosystem services for humans, mangrove has been used as traditional medicine especially in Asian countries for many years (Tarman et al. 2013; Saranraj and Sujitha 2015; Saranya et al. 2015). Bibi et al. (2019) stated that China, India, Malaysia, Philippines, and Thailand utilized various mangroves to cure diarrhea, vomiting, diabetes, fever, intestinal worm infection, stings from venomous fishes, etc. Furthermore, the medicinal properties in mangrove are influenced by the production of secondary metabolites (Aljaghthmi et al. 2018). The secondary metabolites of mangroves help themselves to adapt to the extreme habitat (Basyuni et al. 2012, 2019).

Local communities in several coastal regions in Indonesia utilize mangrove metabolites for natural dye, paralytic substance for fishing, as well as traditional medicine (Purwanti 2016; Kusmana 2018; Pringgenies et al. 2018). Among all mangrove species, *R. apiculata* is commonly found in most of Indonesia's mangrove forests. Thus, this species is used as traditional medicine. However, the study of *R. apiculata* as biomedicine is less done rather than *R. mucronata*. Tarman et al. (2013) reported the alkaloid derivatives in the crude extract of *R. mucronata* leaves successfully inhibited diarrhea causative agents. Several studies also reported the effectiveness of *R. mucronata* against plenty of pathogens (Joel and Bhimba 2010; Saranraj and Sujitha 2015; Saranya et al. 2015; Sumardi et al. 2018). Nevertheless, study from other countries showed outstanding antimicrobial and anticancer properties of *R. apiculata* (Seepana *et al*. 2016; Ramalingam and Rajaram, 2018). Therefore, *R. mucronata* and *R. apiculata* are recognized as a traditional medicinal herb.

As an herbal plant, it is really important to understand the appropriate handling of *R. apiculata* to maintain the bioactive contents to be not degraded. Joel and Bhimba (2010) did a shade drying before extraction of the bioactive

in the leaves, on the other hand, Sumardi et al. (2018) dried the leaves at 60-75 ℃ before extraction. In addition, this mangrove also reported as a potential source of anticancer agents (Diastuti and Warsinah 2010; Palaniyandi et al. 2018). Nonetheless, there is no further study about the effect of the drying treatment on the metabolite profile and its biological activity, especially anticancer. Hence, this study was conducted to understand the impact of different drying treatment on the metabolite profile.

MATERIALS AND METHODS

General information

Methanol for extraction was purchased from PT. Brataco Chemicals, Indonesia. Evaporation using rotary evaporator (EYELA N-1001S-W, USA) then dried using vacuum centrifuge (Speed Vac ® Plus) SC210A combined with refrigerated vapor trap (RVT 400, Thermo Scientific). Acetonitrile, chloroform, formic acid, and methanol for chromatography analysis were purchased from Wako, Japan. Thin-layer chromatography (TLC) glass plate using silica 60 F_{254} base from Merck, Germany. High-performance liquid chromatography (HPLC) instrument from Agilent 1100 series (Agilent Technologies, USA) with diode array detector (DAD) and column COSMOSIL $3C_{18}$-AR-II (4.6ID × 100 mm) from Nacalai Tesque, Japan.

Sampling

R. apiculata leaves were collected from a mangrove forest in Rembang, Central Java, Indonesia with coordinate 6°41'57.4"S 111°23'18.6"E (Figure 1) and kept in a zip-lock plastic bag then brought to the laboratory for further step. Sample was taken in September 2019.

Sample preparation

Sorting was done to discard the infected, old, and broken leaves. All green and healthy leaves (without any infection symptom) were resized using a mechanical grinder then divided into three groups for drying treatments: fresh (75.5 g), sun-dried (75.5 g), and oven-dried at 40 ℃ (75.5 g). Further, samples were extracted using methanol by maceration combined with agitation (110 r.p.m.) at room temperature (27 ℃) for 24 h. Samples were separated using cotton to obtain the solvent phase and then concentrated using rotary evaporator at 30-35 ℃. The crude extracts were dried using cold vacuum centrifuge for 3 × 24 h then stored at-20 ℃ to prevent degradation of components.

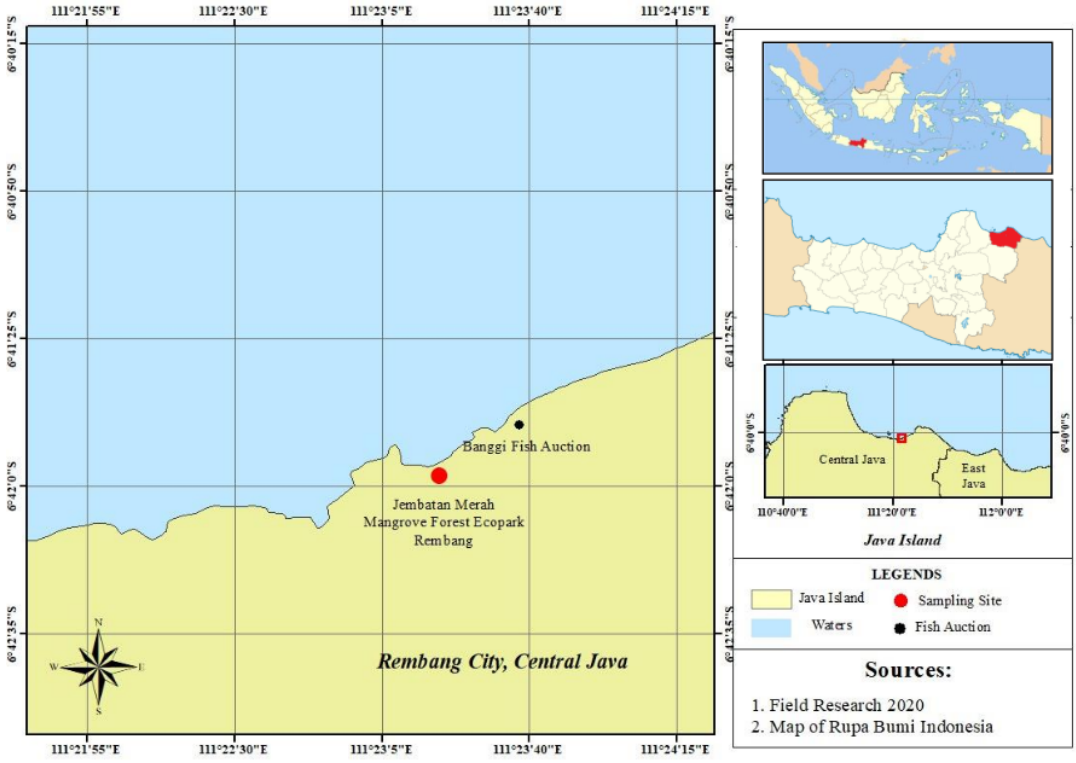


Figure 1. Sampling location in Rembang, Central Java, Indonesia

Metabolite profiling

Phytochemical test

Phytochemical test was performed for alkaloid, saponin, and steroid according to Sibero et al. (2019) while flavonoid and tannin were referred to Khan et al. (2011).

Thin-layer chromatography (TLC) method

A TLC glass plate F_{254} (Merck) was prepared for this analysis. The crude extracts with concentration 4 mg/mL were dotted onto the TLC plate then run in a TLC chamber. Chloroform (Wako, Japan), methanol (Wako, Japan), and water with ratio 7:3:0.5 (v/v) were prepared as the eluent system. A sulfuric acid (5%) (Wako, Japan) in 1-butanol (Wako, Japan) was sprayed onto the TLC plate then heated at 60-80 °C for spot visualization.

High-performance liquid chromatography with diode array detector (HPLC-DAD)

A total of 100 μL sample with concentration 1 mg/mL was injected into HPLC-DAD for metabolite profiling. Eluent system for this chromatography was acetonitrile (Wako, Japan) and 0.1% formic acid (Wako, Japan) solution. Metabolite profiling using HPLC was carried out with the following condition: 0-40 % of acetonitrile for 0-25 min, 40-85 % for 25-28 min, 85 % for 28-30 min, and 85-90 % for 30-35 min. The flow rate was 1.2 mL/min, pressure 160 bar with column COSMOSIL 3C$_{18}$-AR-II (4.6ID × 100 mm) from Nacalai Tesque.

Cytotoxicity assay

Cytotoxicity potential of *R. apiculata* leaves was evaluated to inhibit P388 murine leukemia cells according to Sharma et al. (2019). The cells were recultured in RPMI-1640 medium containing and HEPES (product no. 189-02145) supplemented with 10% fetal bovine serum, 0.1 mg/ml gentamicin sulfate, L-glutamine, and phenol red. The crude extracts were diluted in DMSO with concentration 2 to 2 ×10-3 mg/mL. Doxorubicin was set as positive control with concentration 1 × 10-1 to 1 × 10-4 μg/mL, while DMSO as negative control. P388 murine leukemia cells with density 104 cells/mL were prepared and added into 96-well round-bottom microtiter plate with volume of 200 μL/well while samples and control were added with volume of 1 μL/well with 3 repetitions. Plate was homogenized and then incubated for 72 h at 37 °C in an atmosphere of 5% CO_2 in air with 100% humidity. After that, 50 μL of XTT was added and incubated for 4 h to visualize the cell viability. The cell viability was counted using microplate reader at 450 nm. The data was plotted into a linear regression equation with formula Y = a+bX utilized to count the IC_{50} to understand the cytotoxicity potential (Sharma et al. 2019).

RESULTS AND DISCUSSION

Mangrove produces bioactive compounds to adapt to their extreme habitat (Joel and Bhimba 2010; Sumardi et al. 2018). The bioactive compound is defined as a chemical substance that gives biological effects on organisms in in vitro and/or in vivo test (Guaadaoui et al. 2014; Dewanjee et al. 2015). Unfortunately, several unstable bioactive compounds are broken down or decomposed after processing, such as drying (Angiolillo et al. 2015; Altemimi et al. 2017). In this study, the influence of sun-drying and oven-drying treatment on the metabolite profile of *R. apiculata* leaves was observed. The result of phytochemical comparison is shown in Table 1.

The result of phytochemical test indicates that all crude extracts gave positive results for alkaloid, flavonoid, saponin, and steroid/triterpenoid. Nonetheless, previous studies reported various results of this qualitative test. Poompozhil and Kumarasamy (2014) dried *R. apiculata* leaves by shade-dried method at room temperature then extracted using methanol showed positive results for alkaloids, flavonoids, phenols, saponins, steroids, and terpenoids. A similar result was reported by Muthulingam and Chaithanya (2018). The shade-dried *R. apiculata* leaves gave positive results on phenolic, alkaloid, flavonoids, tannin, saponin but it did not show positive results of steroids. Nevertheless, a recent study stated that the shade-dried methanol extract of the leaves contained alkaloid, flavonoid, phenol hydroquinone, tannin, and saponin, whereas the extract gave negative results for triterpenoid and steroid (Mulyani et al. 2019). The various results of phytochemical content of *R. apiculata* might be caused by the different production of secondary metabolites by this plant. As noted, plant produces secondary metabolites to adapt to environmental stress such as salinity, pH, light uptake, moisture, drought, tide and nutrient uptake from the soil (Dasgupta et al. 2014; Iwuala and Alam 2017; Uddin 2019). The presence of steroid/triterpenoid content in our crude extracts was suggested as the response of salinity fluctuation in its habitat, even less our samples were taken from the tree which grew in the tidal area. The salinity fluctuation in the sampling location was reported by Ariyanto et al. (2018). They stated that the lowest salinity that recorded was 16.67±2.87 psu (practical salinity unit), while the highest salinity was 34.46±0.78 psu. This contention is supported by Basyuni et al. (2009, 2012) who stated mangrove produces triterpenoid derivatives to tolerate the salinity fluctuation in the environment. Moreover, steroid derivatives such as brassinosteroid, known as plant hormone, also play an important role in salt tolerance, development, and growth (Ryu and Cho 2015; Su et al. 2019). Further analysis to understand the effect of drying treatment on the metabolite by thin-layer chromatography (TLC) is shown in Figure 2.

Metabolite profiling through TLC led to a fact that the pre-drying treatment gave chemical alteration in the *R. apiculata* leaves (Figure 2). Crude extracts of fresh and oven-dried leaves were noted to have 10 spots on the TLC plates after visualization, on the other hand, the sun-dried leaves merely gave 9 spots because it lacked one spot at R_f value of 0.61 (Table 2). This might have happened because the missing spot contained unstable or photolabile compounds. Consequently, the compounds were degraded and subsided, or the amount was decreased by the exposure to sunlight during the drying. Ademiluyi et al. (2018)

showed that sun-drying method for plant leaves causing a significant reduction of certain metabolite contents such as alkaloid, flavonoid, saponin, oxalate and vitamin C. Interestingly, most of studies stated that oven-drying treatment resulted in higher amount of beneficial bioactive substances such as flavonoid, tannin, and phenolic rather than the sun-drying (Roshanak et al. 2016; Mbondo et al. 2018). Furthermore, the chemical alteration after the drying treatment also could be detected using HPLC which is presented in Figure 3.

The application of HPLC-DAD for observing the effect of drying treatment on metabolite profile of *R. apiculata* leaves was performed according to the report by Ademiluyi et al. (2018) which showed HPLC-DAD has decent sensitivity to compare metabolite profiles in particular samples with or without any marker standard in several UV-wavelengths. Chemical alteration was not detected by UV monitoring at 210 and 254 nm, whereas UV monitoring at 400 nm successfully showed chemical alteration at retention times (R_t) 29 to 30 mins caused during drying process. The retention time (R_t) of the altered peaks is shown in Table 3.

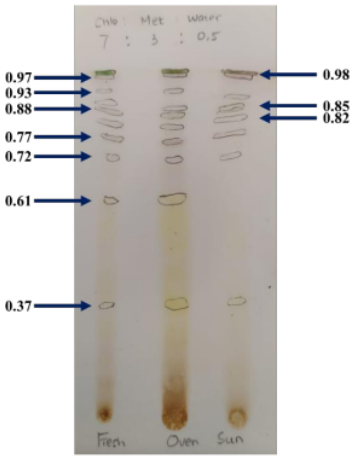
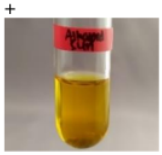
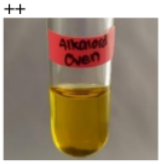
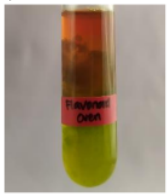
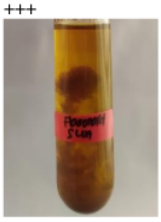
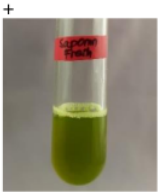
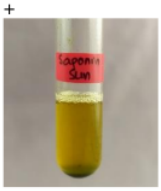
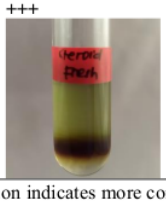
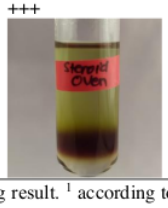
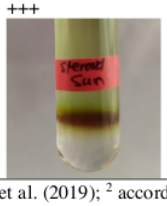


Figure 2. TLC chromatogram of *R. apiculata* leaves crude extracts in different treatments after spraying 5% sulfuric acid in 1-butanol

Table 1. Phytochemical content in fresh, oven-dried and sun-dried *R. apiculata* leaves

Phytochemical test	Positive result	Fresh	Oven-dried	Sun-dried
Alkaloid	Presence of yellow to deep orange precipitate[1]	+++ 	+ 	++ 
Flavonoid	Presence of yellow to orange color after addition of 1% KOH[2]	+ 	+ 	+++ 
Saponin	Formation of a stable foam after addition of 2 N HCl[1]	+ 	+++ 	+ 
Steroid/ Triterpenoid	Formation of green color in the upper layer and deep red to color in the lower layer[1]	+++ 	+++ 	+++ 

Note: +: positive;-: negative. The more "+" notation indicates more convincing result. [1] according to Sibero et al. (2019); [2] according to Khan et al. (2011)

Table 2. Retention factor (R_f) of TLC spots from *R. apiculata* leaves crude extracts

Treatment	Retention factors (R_f)									
	0.98	**0.97**	**0.93**	**0.88**	**0.85**	**0.82**	**0.77**	**0.72**	**0.61**	**0.37**
Fresh	●	●	●	●	●	●	●	●	●	●
Oven-dried	●	●	●	●	●	●	●	●	●	●
Sun-dried	●	●	●	●	●	●	●	●	×	●
Color	Intense green	Pale purple	Pale yellow	Pale yellow	Pale purple	Grey	Grey	Pale yellow	Yellow	Yellow

Note: "●" indicates the presence of the same R_f; while notation "×" indicates the absence of the same R_f in the crude extract)

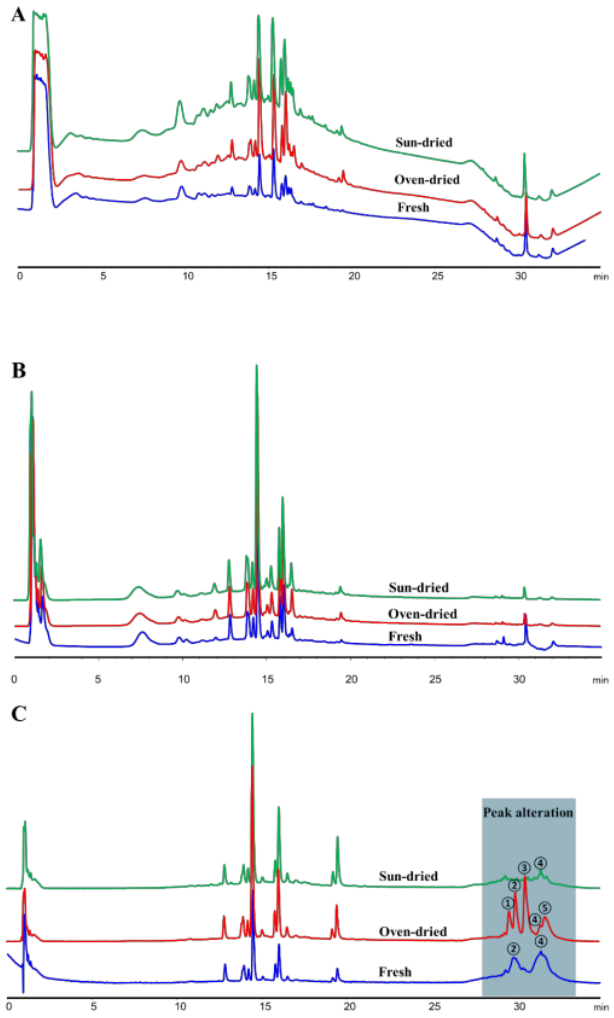


Figure 3. HPLC chromatogram of fresh (blue), oven-dried (red) and sun-dried (green) *R. apiculata* crude extracts. HPLC chromatograms were recorded at 210 nm (A), 254 nm (B) and 400 nm (C)

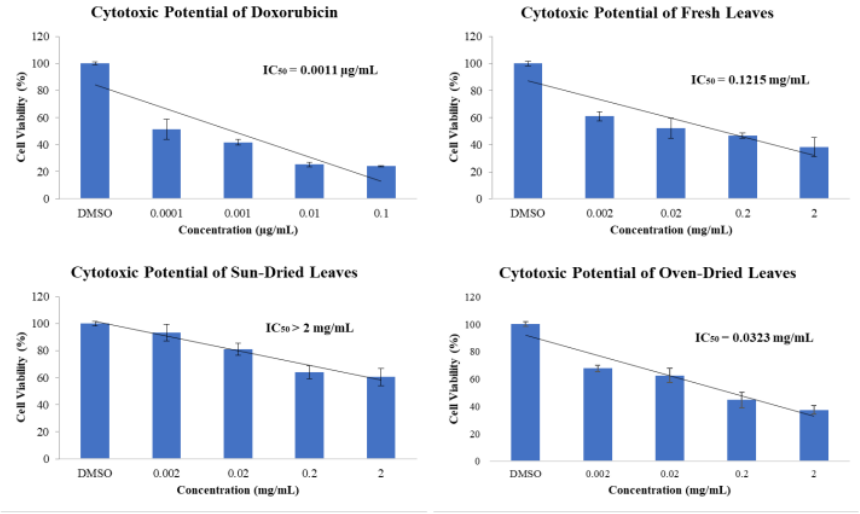


Figure 4. IC_{50} value of Doxorubicin and crude extracts of *R. apiculata* leaves against P388 murine leukemia cells

Table 3. Retention time (R_t) of altered peaks in the *R. apiculata* leaves extracts which detected at 400 nm

Samples	Retention time (R_t) (min)				
	1	2	3	4	5
Fresh	-	29.8	-	31.4	-
Oven-dried	29.6	30.0	30.6	31.5	31.7
Sun-dried	-	-	-	31.4	-

Note: Peaks were detected by UV lamp at 400 nm

Compounds detected by UV absorption at 400 nm are usually contained chromophore that gives color to the compounds. This compound type is usually easily degraded by unfavorable environmental condition such as heat and light. Peak number 4 which detected by UV lamp at 400 nm in fresh (R_t value of 31.4 min), oven-dried (R_t value of 31.5 min), and sun-dried (R_t value of 31.4 min) crude extracts is suggested as thermo and photo-stable compounds. These compounds are not degraded by exposure to sunlight and heat during the drying. Then, peak number 2 appeared at fresh and oven-dried chromatogram suggested to be the thermo-stable but photolabile. Interestingly, two peaks (numbers 3 and 5) appeared merely in oven-dried crude extract at R_t value of 30.6 min and 31.7 min. Roshanak et al. (2016) stated that drying treatment will inactivate certain enzyme that probably causes degradation of metabolites in the plant. The dry samples did not have sufficient water to activate the enzyme, thereby limiting, the degradation of metabolite (Mediani et al. 2014). This explanation rationalizes the previous studies which reported higher phenolic content in dried samples than the fresh sample. Hence, the peak numbers 3 and 5 were suggested as the thermo-stable compounds. However, these compounds were either photolabile or were prone to degradation by particular endogenous enzymes in fresh extracts.

The effect of drying treatment to cytotoxic property of *R. apiculata* leaves and Doxorubicin are presented in Figure 4. The results of cytotoxicity assay show that the highest toxicity (IC_{50} value of 0.0323 mg/mL) was observed in extracts of oven-dried leaves, followed by that of fresh leaves (IC_{50} value of 0.1215 mg/mL). In contrast, the sun-dried leaves had lowest cytotoxicity (IC_{50} value of > 2 mg/mL) whereas Doxorubicin as the positive control had IC50 value of 0.0011 µg/mL. It is highlighted that the oven-drying treatment gave the best IC_{50} value, while the sun-drying treatment weakened the cytotoxicity against P388 murine leukemia cells. Interestingly, the weakening of bioactivity in sun-drying might be correlated to the loss of several metabolites which emphasized by Figure 3 and Table 3. Hence, it is expected that the lost metabolites might play important role in the anticancer activity of *R. apiculata* leaves.

As a summary, this study showed that drying treatment caused alteration of metabolite profile in the *R. apiculata* leaves. TLC and HPLC analyses explained that oven drying caused the disappearance of heat-intolerant compounds, while the heat and light-sensitive compounds were disappeared in the sun-drying crude extract. However,

the result of the phytochemical test indicated that there was no difference in bioactive compounds in fresh and dried leaves, qualitatively. Furthermore, this study showed that oven-drying treatment can preserve the anticancer potential of *R. apiculata* leaves.

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