

## Phytochemical Constituent's Comparison using Various Drying Effects on *Rubus Fraxinifolius* Pour. Leaves

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### Abstract

*Rubus fraxinifolius* is a genus that belongs to the Rosaceae family, which grows in the mountainous forests in Indonesia. The study aimed to determine the effects of air-drying (AD) and oven drying (OD) of the young *R. fraxinifolius* leaves and compare them to the fresh leaves (FL) to observe the phytochemical content and bioactivities. Phytochemical contents of the samples were determined based on qualitative assay, total phenolics, and total flavonoid, whereas bioactivities were evaluated on antioxidant DPPH free radical scavenging effect,  $\alpha$ -glucosidase inhibitory activity, and antibacterial assays. LCMS-MS was used for the quantitative investigation of naturally occurring phytochemicals. The methanol extract of FL had the highest free radical scavenging activity against DPPH with  $IC_{50}$  of  $45.51 \pm 5.03 \mu\text{g/mL}$ , total phenolic content ( $25.35 \pm 1.06 \text{ mg GAE/100 mg extract}$ ), and total flavonoid content ( $28.71 \pm 2.07 \text{ equivalent quercetin mg/100 mg extract}$ ) compared to those of AD and OD. However, AD had the highest  $\alpha$ -glucosidase inhibitory activity ( $IC_{50} = 8.86 \pm 1.41 \mu\text{g/mL}$ ) among others. The antibacterial activity of the young leaves showed that there was no activity difference in both fresh and dried conditions. Based on LCMS-MS analysis, it was revealed that Luteolin-7-O-glucoronide, an active antioxidant flavonoid



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
### Keywords:

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glycoside, had been detected in FL and AD leaf extracts, but it was not found in OD leaf extract. Our findings showed that drying could influence the phytochemical contents, which significantly affect the production of characteristic active compounds in *R. fraxinifolius* leaves.

## Introduction

Herbal medicines have gained worldwide attention for their use in treating chronic diseases due to their higher effectiveness and less side effects than those of synthetic drugs. Rubus is a genus that belongs to the *Rosaceae* family, which grows naturally in the mountainous forests of Indonesia. It has a high potential to be developed as crop cultivated plant. In fact, Rubus is one of the largest genera within *Rosaceae* that consists of 740 species. This plant is also native to six continents and can grow in several locations. It is reported that 46 species of Rubus were found in Malesia Region and 25 species in Indonesia.<sup>1,2</sup> *R. fraxinifolius*, belonging to the Sub genus Malachobatus, is one of Raspberry, which is distributed in mountainous forests of Indonesia. Kalkman (1993)<sup>1</sup> reported that this species was found in Borneo, Java, Celebes, Moluccas, Lesser Sunda Islands, at an altitude of 0-2500 meter above sea level. Furthermore, Cibodas Botanical Garden, an institute of ex situ conservation, has collected eleven Rubus species some of which are from mountains in Indonesia<sup>3</sup> included *R. fraxinifolius*.<sup>4</sup>

The traditional medicinal use of Rubus in South east Asia was very similar to the one in Europe. Some Rubus were reported to produce various substances that possess pharmacological effects, including antidiabetic, antibacterial, anti-inflammatory, antioxidant, antidiarrheal, anti-tumor, anti-fertilization, neuroprotective and wound healing properties.<sup>4,5</sup>

The drying process is an efficient way to maintain the phytochemical content in plant especially for medicinal herbs.<sup>6</sup> Furthermore, drying can help reduce the cost of the final product since product weight determines transportation and storage costs.<sup>6</sup> The biological activity of Rubus leaves has been previously reported<sup>4, 6</sup>, furthermore, Abu Bakar (2016)<sup>7</sup> reported the phytochemical content and activities of *R. fraxinifolius* fruits, while

Shamsudin (2019)<sup>8</sup> reported Antioxidant activities and phytochemicals content in three different parts (ie, fruit, stem and leaves) of *R. fraxinifolius* which dried with freeze dryers. However, still no study has ever been conducted on understanding the effect of drying *R. fraxinifolius* leaves on their phytochemical content and bio-activity. Therefore, the aim of this study is to evaluate the chemical compositions, especially is phenolic and flavonoid compounds of the selected young *R. fraxinifolius* leaves that underwent different drying processes by using qualitative analysis and biological properties such as antioxidant, antidiabetic, and antibacterial activities.

## Materials and Methods

### Plant Materials

The leaves of *R. fraxinifolius* were collected from Cibodas Botanical Garden (latitude: -6°44' 19.19" and longitude: 107°00' 12.00" E) on November 2017. *R. fraxinifolius* has compound leaves, with peduncle, rachis, pedicel, and leaflets. Leaf shoots were taken from the 1<sup>st</sup> to 3<sup>rd</sup> leaves in each particular branch. Leaflets had not properly opened and they were green and glowing on the upper surface but were reddish on the lower surface. The texture was very soft, so they could be easily cut by hand. Young leaves were taken from below shoot leaves av. 4<sup>th</sup> – 9<sup>th</sup> leaf (depending on the size of the branch) towards the base of the branch, leaflets were completely open, green and glowing, with a flush of red in some parts (lower surface and midrib of the leaflets, upper part of the rachis and pedicels), and the branch where the leaves attached was very firm. Mature leaves are located close to the base of the branch. They were green but did not glow; only the spines and a very small portion of the rachis were reddish, the texture was hard and the spines were sharp. Leaves were prepared in three different ways: fresh leaf, dried at room temperature (air drying), and dried at 50°C (oven drying). The dried sample was placed in an airtight container until further use.

### Chemical Reagents

Antioxidant reagent: DPPH (*2,2-diphenyl-1-picrylhydrazyl*), Follin-Ciocalteu Reagent, gallic acid, and quercetin were purchased from Sigma-Aldrich (St. Louis, USA). Vanillin, Na<sub>2</sub>CO<sub>3</sub>, AlCl<sub>3</sub>, NaNO<sub>2</sub>, NaOH, and H<sub>2</sub>SO<sub>4</sub>, were obtained from Merck (Darmstadt, Germany).  $\alpha$ -Glucosidase assay:  $\alpha$ -glucosidase [(EC 3.2.1.20)] type I from *S. cerevisiae* was purchased from Wako (Tokyo, Japan) and 4-nitrophenyl-Dglucopyranoside (PNPG) was purchased from Sigma (St. Louis, USA). Other commercially available chemicals were used as received and solvents were distilled before being used.

### Extraction Method

The sample leaves of *R. fraxinifolius* (20 g) were macerated with methanol (250 mL x 3) at room temperature, and the solvent was evaporated *in vacuo*. The dried methanol extract was further used for biological activities assay and phytochemistry identification.

### Qualitative Phytochemicals Screening Content of *R. fraxinifolius* Extracts

Qualitative phytochemical screening was carried by colorimetric methods to detect the presence of secondary metabolites in the extract using standard methods.<sup>9,10</sup> Whereas for The total phenolic content was estimated according to Folin-Ciocalteu method<sup>11,12</sup> and total flavonoid was determined according to aluminium trichloride method<sup>12,13</sup> using quercetin as the reference compound.

### Biological Activities Assay of *R. fraxinifolius* Extracts

The experiment methods for antioxidant was conducted by DPPH free radical scavenging effect and antidiabetes assay was determined based on  $\alpha$ -glucosidase inhibitory activity ( $\alpha$ -GIs) were the same as described in our previous report.<sup>13</sup>

### Determination of Antibacterial Activity

The antibacterial activities of the extracts were evaluated by disc diffusion assay<sup>14</sup> with slight modification. The antibacterial activity test was carried out by diffusion method of using 6 mm diameter paper discs with test bacteria (*Staphylococcus aureus* (InaCC-B4), *Bacillus subtilis* (InaCC-B334), and *Escherichia coli* (InaCC-B5)). The antibacterial

activity test was carried out with three repetitions. 10  $\mu$ L of each sample extract (5%) was pipetted into the filter disc on NA media that had been inoculated with test bacteria, then placed incubation was carried out at 37°C overnight. Streptomycin was used as a positive control, whereas DMSO was used as a negative control. Observations were made on the formation of inhibition zones around the paper disc.

### Liquid Chromatography-Mass Spectrometry-Mass Spectrometry (LCMS-MS)

Secondary metabolites from methanol extracts were determined using a LCMS-MS analysis, Xevo G2-XS QToF Waters MS Technologies (USA) equipped with an electrospray ionisation (ESI) source was coupled to an UPLC analysis was performed using a Waters Acquity Ultra Performance LC system. The analysis procedure was carried out in accordance with previously reported.<sup>15</sup>

### Statistical Analysis

Data were analyzed using Microsoft Excel and reported as mean  $\pm$  standard deviation of triplicate determination.

### Results and Discussion

Drying is an important process in postharvest management and sales of herbs. Herbal drying is intended to inhibit microbial growth and retardation of enzymatic reactions that can prevent the stability of active compounds. However, it can also give rise to other changes that might affect the herb quality due to the loss of bioactive compounds, although some of the phytochemicals are more thermostable than others.<sup>7</sup> The effect of the fresh leaf (FL), air-dried (AD) and oven-dried (OD) samples of *R. fraxinifolius* Poir. young leaves were observed on qualitative, quantitative, and biological activities and they results were summarised in Table 1, 2 and 3. Although the phytochemical investigation revealed the presence of flavonoids, tannin, phytosterol, and phenolic compounds in all plant extracts (Table 1), quantitative analysis showed different amount of total phenol and flavonoid in all samples (Table 2). In our study, FL showed the highest phenolic and flavonoid contents of 25.35  $\pm$  1.06 mg GAE/g and 28.71  $\pm$  2.07 mg QE/g extract, respectively, whereas AD and OD had lower concentration of phenolic and flavonoid compounds (Table 2). It might be because drying caused reduction in phenolic and flavonoid contents

in leaves. This finding was consistent with several research reports<sup>6</sup> that oven drying could degrade some phytochemicals, such phenolic compounds from plants. This could mean that these compounds were sensitive to drying treatment.

Previous studies have indicated that the drying temperature led to a notable decline in phytochemicals.<sup>6</sup> This could be due to the intense and prolonged drying that resulted in enzymatic

degradation of the phytochemicals.<sup>16</sup> Both total phenolic and flavonoid contents obtained in this study were relatively lower than those of leaf extract of *R. fraxinifolius* tested in previous studies<sup>4</sup>, which was extracted using Soxhlet apparatus by n-hexane, ethyl acetate, and methanol. However, it was slightly higher than the total phenolic and flavonoid contents of *R. moluccanus* L., *R. fraxinifolius* Poir., and *R. alpestris* Blume.<sup>7</sup>

**Table 1: Yield and qualitative phytochemical screening of extracts**

Extract	Yield of extract (%)	Metabolites						
		Flavonoid	Alkaloid	Steroid	Terpen	Tanin	Saponin	Quinon
FL	10.96	+	-	+	-	+	+	+
AD	15.56	+	-	+	-	+	+	+
OD	25.00	+	-	+	-	+	+	+

+: present –: absent

FL: fresh young leaves

AD: young leaves dry wind

OD: young leaves dry oven

The antioxidant activity in all extracts was determined by DPPH free radical scavenger assay. The highest percentage inhibition was found in the FL extract, while the OD extract had the lowest antioxidant activity (Table 3). It seems that the antioxidant activity has positive correlation with the total phenolic and flavonoid contents in fresh leaves,

which is strengthened by the low  $IC_{50}$  value. Phenolic constituents, such as flavonoids, phenolic acids and tannins, were ubiquitous secondary metabolites in plants. They were known to have antioxidant activity and it was likely that the activity of these extracts was due to these compounds.<sup>17</sup>

**Table 2: Quantitative phytochemical analysis**

Extract	Total Phenolic Content (mg GAE/g extract)	Total Flavonoid content (mg QE/g extract)
FL	25.35 ± 1.06	28.71 ± 2.07
AD	21.21 ± 1.29	20.04 ± 2.61
OD	7.54 ± 2.54	8.62 ± 3.43

Polyphenolic compounds in plants were reported to exert various biological effects, including antioxidant activity and carbohydrate hydrolyzing enzyme, due to their ability to bind with protein.<sup>18</sup> Various *in vitro* assay showed that many plant polyphenols in fact possessed carbohydrate hydrolyzing

enzyme inhibitory activities that contributed to lower postprandial hyperglycemia in the management of diabetes. In this study, the hypoglycemic potential of *R. fraxinifolius* leaves was evaluated by the  $\alpha$ -glucosidase inhibition assay. The AD extract demonstrated high inhibitory activity on

$\alpha$ -glucosidase (Table 3). The quality of the enzymatic inhibition in these extracts was determined by calculating IC<sub>50</sub> value, with the low number indicating high enzymatic inhibition quality. The order of the *R. fraxinifolius* leaf extract starting from the highest

inhibitory activity by IC<sub>50</sub> values could be established as followed: air drying (8.86  $\mu$ g/mL) > fresh (12.35  $\mu$ g/mL) > oven drying (27.55  $\mu$ g/mL); this suggested that extract might potentially be used as anti-diabetic remedy.

**Table 3: Biological activities of extract**

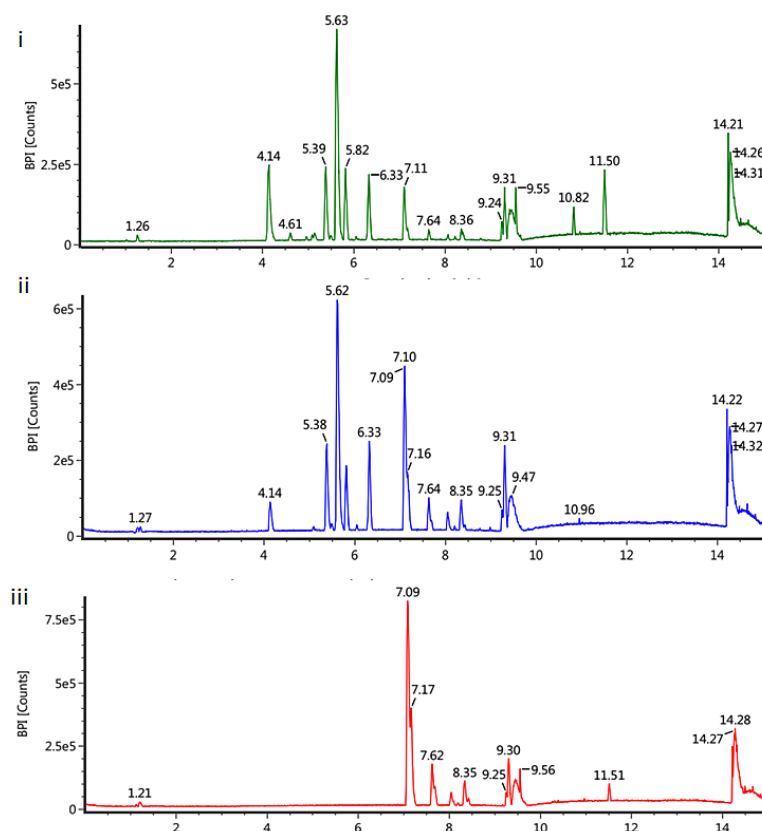
Extract	DPPH (IC <sub>50</sub> $\mu$ g/mL)	$\alpha$ -Gls activity (IC <sub>50</sub> $\mu$ g/mL)	Antibacterial activity (mm)		
			<i>E. coli</i>	<i>S. aureus</i>	<i>B. subtilis</i>
FL	45.51 $\pm$ 5.03	12.35 $\pm$ 1.48	11	12	12
AD	52.22 $\pm$ 2.88	8.86 $\pm$ 1.41	12	10	13
OD	144.43 $\pm$ 6.19	27.55 $\pm$ 2.23	10	9	9
Quercetin	14.17 $\pm$ 4.72	6.04 $\pm$ 2.14	-	-	-
Streptomycin Sulfat	-	-	23	20	27

The study of  $\alpha$ -glucosidase inhibitory activity of *Rubus* leaf extract was rarely reported compared to that of the fruit of *Rubus* sp extract.<sup>19</sup> However, this finding supported the previous report that aqueous extract of *Rubus* sp leaves showed possible

anti-diabetic activity in rats, and daily administration of 5g/kg leaves of the infusion decreased 50% glucose-induced hyperglycemia in alloxan-diabetic rabbits.<sup>17</sup>

**Table 4: Chemical composition of fresh and dried leaf extract**

Sample	Component name	Observed m/z	Observed RT (min)
FL	Abrusoside A	647.4794	5.63
	Epianhydrobelachinal	469.3307	5.83
	Epianhydrobelachinal	469.3304	6.33
	Luteolin-7-O-glucuronide	463.0865	5.39
	Poricoric acid B	485.3255	7.12
AD	Abrusoside A	647.4794	5.63
	Epianhydrobelachinal	469.3307	5.83
	Lucialdehyde B	453.3353	9.32
	Luteolin-7-O-glucuronide	463.0866	5.39
	Poricoric acid B	485.3255	7.12
OD	Ephedradine C	537.3033	9.56
	Epianhydrobelachinal	469.3305	8.34
	Epianhydrobelachinal	469.3308	7.63
	Epianhydrobelachinal	469.3305	8.05
	Lucialdehyde B	453.3353	9.31
	Poricoric acid B	485.3255	7.10



**Fig. 2: LCMS-MS chromatogram of the chemical constituents of *Rubus* leaf extract. (i) fresh leaves (FL), (ii) air dried (AD), and (iii) oven dried (OD) of young leaves**

*Rubus* species are known to contain secondary metabolites which active against some common pathogenic bacteria. Therefore, in this study, the extract was investigated on antibacterial activity against *E. coli* and *S. aureus*. The result of our current research demonstrated that all leaf extracts of *R. fraxinifolius* were moderate against Gram-positive and Gram-negative bacteria (Table 3) when compared to standard. Phenolic compounds such as flavone, quercetin, and naringenin potentially contributed to the antibacterial activities against *S. aureus*, *B. subtilis*, and *E. coli*.<sup>7</sup> Our result showed that fresh and dried leaves had no noticeable difference on antibacterial activities. It might be that other active compounds, besides polyphenolic, contributed to antibacterial activity. This study was only a preliminary attempt to assess drying effect on bioactivity potential of *R. fraxinifolius* leaf extract. Hence, further detailed bioassay would need to be applied for assessing the antibacterial activity.

Profiling of LCMS-MS was conducted to identify bioactive compounds present in the *R. fraxinifolius* leaf extract (Figure 2). The chromatogram of methanol extract showed that oven drying could cause more degradation of several compounds in leaf extract than in both dried and fresh leaves. As shown in table 4, Luteolin-7-O-glucuronide, an active antioxidant flavonoid glycoside, was detected in fresh and air-dried extracts, while it was not found in the oven-dried leaf extract. There was no dry extract at the oven (Table 4).

Based on the results above, air drying could be chosen as a suitable technique for *R. fraxinifolius* leaf extract because it could still preserve the composition/content of the active compounds (antioxidants and lower hyperglycemia).

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leaf extract because it could still preserve the composition/content of the active compounds (antioxidants and lower hyperglycemia).

### Conclusion

Preliminary chemical examination indicated the presence of polyphenols and flavonoids, which might be responsible for antioxidant and antidiabetic ( $\alpha$ -glucosidase inhibitory) activities. The presence of the phytochemical compounds in *R. fraxinifolius* leaf extract indicated that fresh or dried leaves of *Rubus* have medicinal potential and might function as antioxidant, anti-hyperglycemic, and anti-microbial. However, oven dried leaves had the least biological activities, implying that oven drying might not be an efficient method for drying *Rubus* leaves. Further studies on the isolation of active constituent(s)

along with further *in vitro* studies, need to be investigated in detail to explore the pharmaceutical and nutraceutical use of *R. fraxinifolius* leaf extract.

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### Conflict of Interest

We have no conflict of interest to declare.

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