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Different leaf maturities and withering durations affect the antioxidant potential and aroma compound of Indonesian bay leaf [*Syzygium polyanthum* (Wight) Walp.]

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Abstract

Indonesian bay leaves [IBL; *Syzygium polyanthum* (Wight) Walp.] are frequently used as a seasoning in various Indonesian cuisines. The potential of IBL leaves extract as an antioxidant and aroma source based on the leaf maturities and withering durations is not yet known. Therefore, the present work aimed to study the effect of IBL leaf maturities and withering durations on the changes in antioxidant potentials and aroma compound. Results showed that the total phenolic, flavonoid, and tannin contents of old IBL leaves extract yielded the highest values (89.08 gallic acid equivalent (GAE)/g sample, 37.11 mg quercetin equivalent (QE)/g sample, and 2.72 mg tannic acid equivalent (TAE)/g sample, respectively) as compared to half-old and young leaves extracts. Similarly, the antioxidant activities of old IBL leaves extract were also the highest at 77.06 mg TEAC/g sample (DPPH assay), 7.92 mg TEAC/g sample (FRAP assay), and 83.19 mg TEAC/g sample (ABTS assay) as compared to half-old and young leaves extracts. The yields of essential oil and total β-ocimene (key aroma compound) from old IBL leaves were also the highest as compared to half-old and young leaves. Nevertheless, all these parameters significantly decreased after two days of withering treatment. Therefore, in order to maximise the antioxidant capacity and aroma compound, it is henceforth suggested that IBL leaves should not be used more than two days after harvest.

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Introduction

Indonesia is a tropical country with various types of medicinal plants. Around 30,000 out of 40,000 species of medicinal plants worldwide can be found in Indonesia (Roosita *et al.,* 2008). Many Indonesians traditionally use plants as medicines. One such plant is Indonesian bay leaf [IBL; *Syzygium polyanthum* (Wight) Walp.] also known as Indian baywatch and Japanese baywatch, and locally known as *salam* leaf. The IBL is widely used by the locals to reduce cholesterol, diabetes, hypertension, gastritis, and diarrhoea. The IBL contains phenols, flavonoids, tannins, and some terpenoids that function as antioxidants (Hamad *et al.,* 2017). As a flavouring agent, IBL contains β-ocimene, which is a terpenoid compound responsible for its floral aroma and green character (Farré-Armengol *et al.,* 2017). Previous IBL studies relating to antioxidants and β-ocimene were more focused towards the extraction conditions, while the leaf maturity and withering duration have not much been discussed.

Antioxidants are compounds that can inhibit or prevent oxidation of a substrate by free radicals. It has been found that the leaf maturity could affect the antioxidant potentials of the leaves and their extracts. Ethanol extract of younger *Camellia sinensis* (tea) leaves had higher percentage of flavonoids (Dorkbuakaew *et al.,* 2016), while the phenolic and flavonoid compounds in older *Aquilaria beccariana* (agarwood) leaves were higher as compared to those in younger leaves (Anwar *et al.,* 2017).

The general method to determine antioxidant activities in plants and foods is the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, by examining the capability of various antioxidants to distribute their hydrogen to make a stable DPPH (reduced form) (Prior *et al.,* 2005). Additionally, the ferric reducing antioxidant power (FRAP) assay can compare multiple antioxidants for their capabilities to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) through electron distribution (Kurniawati *et al.,*

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2017). The 2'-azino-bis(3-ethyl-benzo-thiazoline-6-sulphonic acid (ABTS) assay compares various antioxidants based on the capability to stabilise free radical compounds by distributing proton radicals (Thaipong *et al.,* 2006). These different assays give different results, which are caused by the influence of chemical structures of the antioxidants, sources of free radical, and different physicochemical properties of the samples (Kazemi *et al.,* 2011).

It is a common practice in Indonesia to wither the IBL as a pre-treatment prior to use. Withering has been proven to cause enzymatic and non-enzymatic changes in plant metabolism, thus affecting the phytochemical compounds in *Carica papaya* L. (papaya) (Raja *et al.,* 2019) and *Persea americana* Mill. (avocado) (Rajan *et al.,* 2011). Withering also resulted in the change in terpenoid and aroma compounds in *Nicotiana tabacum* L. (tobacco) (Raju, 2013). Therefore, the present work aimed at comparing the varying leaf maturities and withering durations on the phytochemical compound profiles (total phenolic, total flavonoid, and total tannin contents) and antioxidant activities (by DPPH, FRAP, and ABTS assays) of IBL extract, as well as the yield of essential oil and yield of β-ocimene from the essential oil of IBL.

Materials and methods

Materials

Fresh IBL were obtained from Bina Agro Mandiri, Kasihan, Bantul, Special Region of Yogyakarta, Indonesia (length of 9 to 12 cm and width of 4 to 6 cm). The selected leaves were free of diseases and damages. The maturities of IBL were young (shoot+1; light green), half-old (shoot+4; medium-dark green), and old (dark green; shoot+7). The chemicals and reagents used were purchased from Merck & Co. (New Jersey, USA). All three maturity levels were withered for four days with day 0 considered as fresh to a total of 15 treatments (young, half-old, old × 0 d, 1 d, 2 d, 3 d, 4 d).

Preparation of IBL extracts

To prepare the extract, 10 g of sample (for each treatment) was placed in a blender with 50 mL of 96% absolute ethanol, and macerated. The resulting mixture was put into 250 mL Erlenmeyer flask, and stirred using a hot plate stirrer (24 h, 50°C, 300 rpm). The mixture was then centrifuged (30 min, 3,000 rpm). The supernatant was retrieved and concentrated using a rotary vacuum evaporator (3 h, 175 mBar, 40°C). The extracts were stored at 4°C until further analyses.

Determination of total phenolic content of IBL extracts

The total phenolic content (TPC) of IBL extracts was determined using a gallic acid calibration curve with a series of concentrations (20, 40, 60, 80, 100 µg/mL in a mixture of ethanol:aquadest 1:1 v/v). Each solution (both IBL extract and gallic acid standard) was pipetted (0.5 mL) and added to 5 mL of 10% Folin-Ciocalteu reagent. The solution was added to 4 mL of 1 M sodium carbonate solution, and left for 15 min. The absorbance was measured at 751.5 nm using a Thermo Fisher Scientific GENESYS™ 10S UV-vis spectrophotometer. Standard solution concentration (x) and its absorbance (y) were used to construct the calibration curve (Singleton and Rossi, 1965). Results were expressed as mg gallic acid equivalent (GAE)/g sample.

Determination of total flavonoid content of IBL extracts

The total flavonoid content (TFC) of IBL extracts was determined following Chang and Jeon (2003) using quercetin as the standard. Each solution (both IBL extract and quercetin standard) was pipetted (25 µL) and added to 75 µL of 96% ethanol, 5 µL of 10% of aluminium chloride, 5 µL of 1 M potassium acetate, and 140 µL of aquadest. Then, the mixture was incubated for 30 min at room temperature after which absorbance was measured using a UV-vis spectrophotometer at 440 nm. Five standard concentrations (25, 50, 75, 100, 125 µg/mL solution in 96% ethanol) were used to construct a calibration curve. Results were expressed as mg quercetin equivalent (QE)/g sample.

Determination of total tannin content of IBL extracts

The total tannin content (TTC) of IBL extracts was determined following Chanwitheesuk *et al.* (2005). Briefly, 1 mL of IBL extract was added to 99 mL aquadest. Next, 0.1 mL of the solution was added to 0.1 mL of Folin-Ciocalteu reagent, homogenised using a vortex, mixed with 2 mL of sodium carbonate, and homogenised again using a vortex. Absorbance was then measured using a UV-vis spectrophotometer at 760 nm following 30 min incubation at room temperature. Tannic acid at five concentrations (20, 40, 60, 80, 100 µg/mL) were used to construct a calibration curve. Results were expressed as mg of tannic acid equivalent (TAE)/g sample.

Antioxidant activity of IBL extracts with DPPH assay

The DPPH assay was performed with Trolox as a standard solution. Five standard concentrations (400, 500, 600, 700, 800 µg/mL in 96% ethanol) were used to construct a calibration curve. Each solution (both IBL extract and Trolox standard) was added to 1 mL of 0.4 mM DPPH solution, and the volume brought to 5 mL by 96% ethanol. The mixture was homogenised and left for 30 min, and absorbance was measured using a UV-vis spectrophotometer at 515 nm. The percentage of free radical inhibition was calculated using Eq. 1:

$$\% \text{ inhibition} = \frac{\text{Control Abs.} - \text{Sample Abs.}}{\text{Control Abs.}} \text{ x}100\% \quad \text{(Eq. 1)}$$

where, Control Abs. = absorbance of the control solution without antioxidant, and Sample Abs. = absorbance of sample/Trolox.

Results were expressed as mg Trolox equivalent antioxidant capacity (TEAC) per mg sample (Prior *et al.,* 2005).

Antioxidant activity of IBL extracts with FRAP assay

The FRAP assay was performed by taking either 1 mL of IBL extract or Trolox standard solution in various concentrations of 5, 10, 15, 20, 25 mg/L and placing them in a test tube. The sample or standard solution was then added with 1.5 mL phosphate buffer (pH 6.6) and 1.5 mL of 1% potassium ferricyanide solution. The mixture was homogenised, incubated (50°C, 20 min), and then added to 1.5 mL of 10% trichloroacetic acid solution. The mixture was then centrifuged (3,000 rpm, 30 min). The top layer (2.5 mL) was taken and added with 2.5 mL of aquadest, and 0.5 mL of 0.1% ferric chloride was added. The mixture was then incubated (25°C, 10 min). The absorbance of the solution was then measured using a UV-vis spectrophotometer at 711 nm. The antioxidant activity of the sample was calculated using the calibration curve, and expressed as mg Trolox equivalent antioxidant capacity (TEAC) per mg sample unit (Kurniawati *et al.,* 2017).

Antioxidant activity of IBL extracts with ABTS assay

The ABTS assay was performed following Thaipong *et al.* (2006). Standard solution calibration curve was constructed using Trolox standard solutions (50, 100, 150, 200, 250 µg/mL). Next, 1 mL of IBL extract or Trolox standard solution was added to 1 mL of ABTS solution, and the volume was brought up to 5 mL with absolute ethanol. The absorbance of the solution was measured using a UV-vis spectrophotometer at 750 nm. The antioxidant power was calculated using Eq. 2:

$$\% \text{ inhibition} = \frac{\text{Control Abs.} - \text{Sample Abs.}}{\text{Control Abs.}} \text{ x}100\% \quad \text{(Eq. 2)}$$

Results were expressed as mg Trolox equivalent antioxidant capacity (TEAC) per mg sample.

Determination of β-ocimene from IBL essential oil by GC-MS

IBL essential oil was obtained by water distillation with a water to sample ratio of 5 mL/g. The yield of IBL essential oil was calculated based on the ratio of the volume of essential oil per weight of distilled IBL. The water content of IBL was first obtained to provide a sample equivalent to 100 g of dried IBL. The IBL essential oil was then analysed using the gas chromatography-mass spectrometry (GC-MS) analysis to get the profile of its volatile compounds (Kassahun, 2019). Then, the yield of β-ocimene from IBL essential oil was quantified using a calibration curve of ocimene standard concentrations (50, 100, 150, 200, and 250 ppm in 1 mL hexane) and their percent relative area in GC-MS chromatogram. For GC-MS, 1 µL of the sample was injected into a Shimadzu GC-17A GC-MS with QP5000 MS, 30 m CP Sil 5 CB column dimethylpolysiloxane under the following condition: 60°C initial column temperature, 250°C final column temperatures (10°C/min temperature rise), 270°C injector temperature, 280°C detector temperature, helium as carrier gas, electron impact ionising, and 70 eV ionizing energy (Vrhovsek *et al.,* 2014).

Statistical analysis

Experiment was performed by a completely randomised design. The data were then tabulated with Microsoft Excel 2007. If the test results in the analysis of variance (ANOVA) table showed different effects ($F_{calculated} > F_{table}$), additional tests were performed using Duncan's multiple range test (DMRT) with a significance level of 95%.

Results and discussion

Bioactive compounds of IBL extracts

The bioactive compound profile of IBL extracts is presented in Table 1. In young, half-old, and old IBL extracts, the TPC increased significantly at 62.18 ± 0.23, 80.99 ± 1.10, and 89.08 ± 1.23 mg GAE/g sample, respectively, for extracts without any withering treatment (fresh; day 0). This indicated that

older leaves had higher TPC as compared to younger leaves. This is in agreement with Anwar *et al.* (2017) who reported that phenolic content increased with maturity. Among different withering durations, it was found that withering from 0 to 1 day could still maintain the TPC, and the levels did not change significantly. As withering progressed, the TPC significantly decreased. Similar trend was observed for all maturity levels. During withering, the polyphenol oxidase enzyme activity could be the main factor that caused the TPC to decrease (Omiadze *et al.,* 2014).

For TFC, similar trend to TPC was also observed such that old leaves had the highest TFC followed by half-old, and young leaves (37.11 ± 3.15, 33.28 ± 0.10, and 30.18 ± 2.09 mg QE/g sample, respectively, on day 0). Among different withering durations, it was found that withering from 0 to 1 day could still maintain the TFC, and the levels did not change significantly. As withering progressed, the TFC significantly decreased. Similar trend was observed for all maturity levels. The biosynthesis of flavonoids originates from merging the shikimate (aromatic compound synthetic pathway) and acetate-malonate pathways. The combined pathways produce secondary metabolites. Therefore, the flavonoid content is affected during plant development and enzymatic reactions during withering (Hua *et al.,* 2016).

For TTC, similar trend to TPC and TFC was observed such that old leaves had the highest TTC followed by half-old, and young leaves (2.72 ± 0.01, 1.66 ± 0.09, and 0.66 ± 0.19 mg TAE/g sample, respectively, on day 0). Among different withering durations, it was found that withering from 0 to 1 day could still maintain the TTC, and the levels did not change significantly. As withering progressed, the TTC significantly decreased. Similar trend was

Table 1. Bioactive compounds of Indonesian bay leaves extracts at different maturities and withering durations.

Maturity	**Withering durations (days)**	**Bioactive compound**		
		Total phenolic content (mg GAE/g sample)	**Total flavonoid content (mg QE/g sample)**	**Total tannin content (mg TAE/g sample)**
Young leaves	0	62.18 ± 0.23^{a}	30.18 ± 2.09^{a}	0.66 ± 0.19^{a}
	1	61.28 ± 1.10^{a}	30.03 ± 0.04^{a}	0.53 ± 2.04^{b}
	2	55.04 ± 3.07^{b}	28.59 ± 0.85^{ab}	0.20 ± 1.11^{c}
	3	53.51 ± 0.03^{bc}	26.44 ± 1.06^{b}	0.09 ± 1.01^{c}
	4	48.10 ± 0.27^{c}	24.90 ± 0.12^{c}	0.05 ± 0.01^{c}
Half-old leaves	0	80.99 ± 1.10^{a}	33.28 ± 0.10^{a}	1.66 ± 0.09^{a}
	1	79.15 ± 0.35^{a}	33.67 ± 0.51^{a}	1.61 ± 0.11^{a}
	2	72.46 ± 3.62^{b}	30.07 ± 0.15^{b}	1.27 ± 1.03^{b}
	3	69.14 ± 2.15^{bc}	29.05 ± 2.01^{b}	1.25 ± 0.30^{bc}
	4	62.11 ± 0.28^{c}	27.41 ± 1.21^{c}	1.07 ± 0.07^{c}
Old leaves	0	89.08 ± 1.23^{a}	37.11 ± 3.15^{a}	2.72 ± 0.01^{a}
	1	88.01 ± 0.17^{a}	37.40 ± 0.15^{a}	2.67 ± 0.07^{a}
	2	81.24 ± 1.62^{ab}	35.98 ± 1.98^{ab}	2.01 ± 2.00^{b}
	3	78.55 ± 0.61^{b}	33.16 ± 0.23^{b}	2.06 ± 0.22^{bc}
	4	68.89 ± 0.38^{c}	30.21 ± 1.40^{c}	1.87 ± 0.05^{c}

GAE: gallic acid equivalent; QE: quercetin equivalent; and TAE: tannic acid equivalent. Means within a column followed by different lowercase superscripts are significantly different ($p < 0.05$).

observed for all maturity levels. Tannin is also a secondary metabolite that is affected by plant development. The decrease in tannin that occurred during withering could be due to tannin oxidation to quinone, which produced a brown colour in the leaves (Kusuma *et al.,* 2011).

Antioxidant activities of IBL extracts

The antioxidant activities of IBL extracts are shown in Table 2. For the DPPH assay, old leaves extract had higher antioxidant activity (77.06 ± 0.07 mg TEAC/g sample) as compared to half-old leaves extract (67.45 ± 0.05 mg TEAC/g sample) and young leaves extract (30.19 ± 0.09 mg TEAC/g sample). The total antioxidant activity of IBL extracts could be maintained until day 1 of withering after which it decreased across all maturity levels. The decrease in the antioxidant activity could be caused by the oxidation of the bioactive compounds throughout withering (Othman *et al.,* 2014).

For the FRAP assay, the antioxidant activities were quite different from those of the DPPH assay; the FRAP values were relatively small when compared with the DPPH values. However, the trend was not much different. The old leaves extract had higher antioxidant activity (7.92 ± 0.03 mg TEAC/g sample) as compared to half-old leaves extract (6.41 ± 0.13 mg TEAC/g sample) and young leaves extract (4.36 ± 0.25 mg TEAC/g sample). Similar to DPPH assay, the total antioxidant activity of IBL extracts could be maintained until day 1 of withering after which it decreased across all maturity levels.

For the ABTS assay, the antioxidant activities were not much different from those of the DPPH assay. However, it showed that free radical

Table 2. Antioxidant activities of Indonesian bay leaves extracts at different maturities and withering durations.

Maturity	**Withering durations (days)**	**Antioxidant activity**		
		DPPH assay (mg TEAC/g sample)	**FRAP assay (mg TEAC/g sample)**	**ABTS assay (mg TEAC/g sample)**
Young leaves	0	30.19 ± 0.09[a]	4.36 ± 0.25[a]	59.22 ± 0.14[a]
	1	30.03 ± 0.04[a]	4.22 ± 0.28[a]	57.25 ± 0.03[a]
	2	27.59 ± 0.56[b]	3.71 ± 1.01[b]	55.56 ± 3.16[ab]
	3	25.44 ± 1.48[bc]	3.22 ± 0.13[b]	49.71 ± 1.86[b]
	4	24.90 ± 2.12[c]	2.51 ± 0.56[c]	46.35 ± 0.45[c]
Half-old leaves	0	67.45 ± 0.05[a]	6.41 ± 0.13[a]	72.06 ± 0.06[a]
	1	66.17 ± 0.20[a]	6.13 ± 0.17[a]	70.50 ± 0.74[a]
	2	64.04 ± 0.19[b]	5.70 ± 0.01[b]	65.65 ± 2.04[b]
	3	61.06 ± 1.49[bc]	5.19 ± 0.02[bc]	64.80 ± 1.15[b]
	4	59.98 ± 1.20[c]	4.82 ± 0.27[c]	60.51 ± 0.55[c]
Old leaves	0	77.06 ± 0.07[a]	7.92 ± 0.03[a]	83.19 ± 0.22[a]
	1	77.28 ± 0.23[a]	7.82 ± 0.03[a]	83.88 ± 0.73[a]
	2	73.16 ± 0.06[b]	7.20 ± 2.07[b]	81.79 ± 2.29[ab]
	3	71.42 ± 0.01[b]	7.12 ± 0.01[bc]	76.04 ± 0.02[b]
	4	70.51 ± 1.32[c]	6.90 ± 0.13[c]	72.02 ± 0.02[c]

DPPH: 2,2-diphenyl-1-picrylhydrazyl; FRAP: ferric reducing antioxidant power; ABTS: 2,2-azino-bis (3-ethylbenzothiazodine-6-sulfonic acid) diammonium salt; and TAEC: Trolox equivalent antioxidant capacity. Means within a column followed by different lowercase superscripts are significantly different ($p < 0.05$).

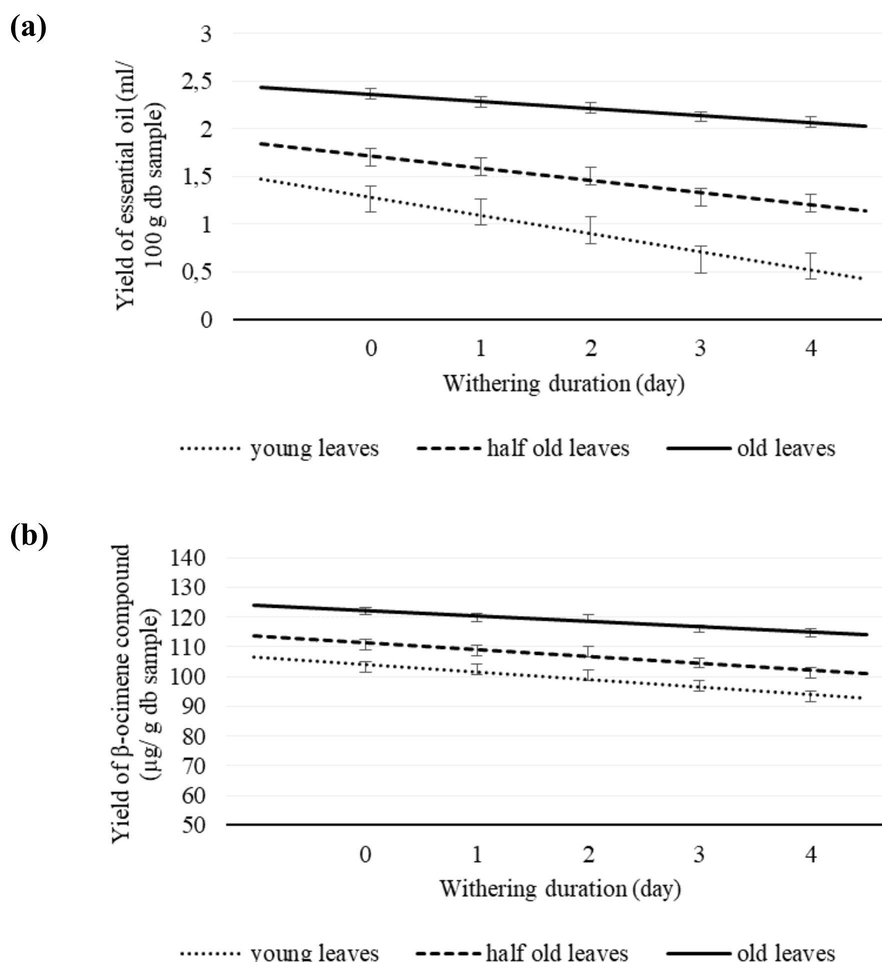

Figure 1. Yield of Indonesian bay leaves essential oil (a), and yield of β-ocimene in the Indonesian bay leaves essential oil (b), at different maturities and withering durations.

inhibitory activity was higher in ABTS assay than the other assays. Old leaves extract had higher antioxidant activity (83.19 ± 0.22 mg TEAC/g sample) as compared to half-old leaves extract (72.06 ± 0.06 TEAC/g sample) and young leaves extracts (59.22 ± 0.14 mg TEAC/g sample). It was also observed that the antioxidant activity could be maintained until day 2 of withering, and then decreased across all maturity levels.

The FRAP assay compares samples' antioxidant activities based on their capabilities to reduce ferric ions to ferrous ions. The FRAP assay works better at low pH values. So, this assay is more suitable if the sample has high organic acids. As for the ABTS assay, it is almost the same as the DPPH assay in which it depends on the phenolic group antioxidants. However, the DPPH assay is commonly carried out in 50% ethanol/water, while the ABTS assay is commonly carried out in solvents with more water (80%) as described by Oh *et al.* (2013).

Yield of β-ocimene from IBL essential oil

The yields of essential oil from IBL, and the yields of β-ocimene from IBL essential oil at different maturity levels and withering durations are shown in Figures 1a and 1b, respectively. It appears that old IBL had significantly higher essential oil yields (2.37 ± 0.03 mL/100 g dry weight of IBL) as compared to half-old and young IBL (1.70 ± 0.10 and 1.27 ± 0.12 mL/100 g dry weight of IBL, respectively). Among withering durations, the yield of essential oil from young leaves was only maintained until day 1 of withering, and significantly decreased from day 2 to day 4. Essential oil tended to evaporate during withering; so, a longer withering duration could cause a reduction in the yield of essential oil (Yoswathana, 2013). This was also true for the yield of β-ocimene from IBL essential oil. β-ocimene was found highest from the essential oil of older leaves (122.04 ± 0.43 µg/mL oil) as compared to those from half-old and young leaves (110.83 ± 0.30 and 103.19 ± 0.42 µg/mL oil, respectively). The presence of a high yield of β-ocimene in old leaves

essential oil can be explained by the fact that β-ocimene, as one of monoterpenoid hydrocarbons, would increase during plant development (Gershenzon *et al.,* 2000). As withering progressed, the yield of β-ocimene from IBL essential oils decreased across all maturity levels tested. Withering could influence the yield of β-ocimene due to oxidation which convert β-ocimene to its oxidised monoterpenoid form, thus decreasing its yield as withering progressed (Farré-Armengol *et al.,* 2017).

Conclusion

Old Indonesian bay leaves extract presented higher level of bioactive compounds as compared to half-old and young leaves. The presence of higher level bioactive compounds affected the antioxidant activities based on the DPPH, FRAP, and ABTS assays; old leaves yielded higher antioxidant activities as compared to half-old and young leaves. For essential oil yield, old leaves yielded the highest as compared to half-old and young leaves. For β-ocimene yield, old leaves similarly yielded the highest as compared to half-old and young leaves. Almost all variables could only be maintained until the first day or at the most to the second day of the withering duration treatments. Therefore, it is henceforth suggested for Indonesian bay leaves to not be used more than two days after harvesting to keep the quality of their aroma and antioxidant potential.

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