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Journal of Functional Food and Nutraceutical

ANTIHYPERGLYCEMIC ACTIVITIES OF ULI BANANA LEAVES ON ORAL SUGAR TOLERANCE

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ABSTRACT

Banana has been widely cultivated. This study aimed to determine the antihyperglycemic activity of Uli banana leaves infusion. The antihyperglycemic activity was evaluated by oral glucose and sucrose tolerance test. A bolus of sugar was given after Uli banana leaves infusion and blood was sampled at 0, 15, 30, 60, 90 and 120 minutes for glucose analyses. The trapezoidal rule was used to determine the area under the curve (AUC) blood glucose. Infusion of Uli banana leaves 3.3 g/kg showed a significant decrease AUC ($p<0.05$) in the glucose tolerance test, while that of dose 0.8 g/kg reduced significantly ($p<0.05$) in the sucrose tolerance test. The results showed that Uli banana leaves infusion possesses antihyperglycemic effect in mice.

Keywords: *Antihyperglycemic, glucose, leaves; sucrose, uli banana*

ABSTRAK

Buah pisang telah dibudidayakan secara luas. Penelitian ini bertujuan untuk mengetahui aktivitas antihiperglikemik dari infusa daun pisang Uli. Uji aktivitas antihiperglikemik dievaluasi dengan menggunakan uji toleransi glukosa dan sukrosa oral. Bolus gula diberikan setelah infusa daun pisang Uli dan dilakukan sampling darah pada menit ke-0, 15, 30, 60, 90 dan 120 untuk dilakukan analisis glukosa darah. Nilai *Area under the Curve* (AUC) glukosa darah dihitungkan menggunakan metode trapezoid. Infusa daun pisang Uli dosis 3,3 g/kg menunjukkan penurunan AUC signifikan ($p<0,05$) pada uji toleransi glukosa, sedangkan dosis 0,8 g/kg memberikan penurunan AUC signifikan ($p<0,05$) pada uji toleransi sukrosa. Hasil penelitian menunjukkan bahwa infusa daun pisang Uli mempunyai efek antihiperglikemik pada mencit.

Kata kunci: *Antihiperglikemik, daun, glukosa, pisang uli, sukrosa*

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INTRODUCTION

The usage of traditional medicine and medicinal plants to maintain health is widely applied in developing countries. Therefore, efforts are needed to properly identify and recognize the importance of medicinal plants to implement these strategies (Sofowora et al., 2013).

The use of natural antioxidants provides an alternative prevention strategy as well as to treat many chronic and degenerative diseases. Banana, a member of family Musaceae, is a tree cultivated in many tropical regions around the world, including in Indonesia. The total number of cultivars of bananas has been estimated to be around 300 to more than 1,000. Various parts of banana, such as fruits, peel, leaves, roots, and pseudostem have shown their medicinal potential, like antiulcerogenic, antioxidant, antimicrobial, antipyretic, and antihyperglycemic activities (Karadi et al., 2011; Pannangpetch et al., 2001; Eleazu et al., 2010; Maya et al., 2015; Kappel et al., 2013). Based on these findings, we designed the present study with an objective to evaluate antihyperglycemic activity of Uli banana leaves (*Musa paradisiaca* L. Uli) infusion in glucose tolerance by using oral sugar tolerance test.

MATERIALS AND METHOD

Materials

Fresh Uli banana leaves were collected in the same season and from the same area in Kebun Plasma Nutfah Pisang Yogyakarta. Glucose and sucrose were acquired from Merck Millipore, Germany. Male mice (weight 18-23 g; age, 8 to 10 week) were purchased from the Imono Laboratory, Sanata Dharma University, Indonesia. Animals were acclimated for one week before experiments and kept in the housing facilities. They were fed standard pelleted diet and drink ad libitum and maintained at 22±2 ºC with a fixed 12h artificial light period. The study protocol was approved by the Medical and Health Research Ethics Committee Faculty of Medicine Gadjah Mada University-Dr. Sardjito General Hospital Yogyakarta Indonesia with approval number KE/FK/0510/EC/2019 and KE/FK/0833/EC/2019.

Preparation of infusion of Uli banana leaves (IBU)

Collected Uli banana leaves were washed and minced to pieces at ± 2 mm thickness. The leaves were dried using an oven at 50 ºC and powdered using a powdering machine and sifted using a sieve with mesh number 50. Ten grams of Uli banana leaves were weighed and added with 100 mL of distilled water, which then heated over a water bath at 90 °C for 15 minutes.

Oral glucose tolerance test

Test animals fasted overnight. Mice were randomly divided into 5 groups (n= 5/group) and received treatment orally: Group I was control group. Group II was given 2 g/kg glucose solution (Rathod *et al*., 2011; Ali *et al*., 2013; Wahyuningsih *et al*., 2018; Gunawan-Puteri, *et al.*, 2018). Group III-V was given IBU dose of 0.8, 1.67, 3.3 g/kg. Thirty minutes after IBU administration, all mice group III-V were consecutively given glucose solution (Yusoff *et al*., 2015; Pattanayak *et al*., 2009; James *et al*., 2009; Gunawan-Puteri *et al*., 2018). Blood was collected from the lateral tail vein, and blood glucose was measured using GlucoDr®auto glucometer (All Medicus Co. Ltd) at 0 (before treatment), 15, 30, 60, 90 and 120 min after the sugar challenge (Wulandari, 2016; Yeo *et al*., 2011). The trapezoidal rule was used to determine the area under the curve (AUC) blood glucose (Wongnawa *et al*., 2014; Eyesin *et al*., 2010; Jo *et al*., 2011; Fransisca *et al*., 2018).

Oral sucrose tolerance test (OSucTT)

Overnight-fasted mice rats were divided into 4 groups and the procedure mentioned in Oral Glucose Tolerance Test was applied to a similar set of animals of group II-V. However, sucrose, at a dose of 4 g/kg B.W. was administered in place of glucose (Ali *et al*. 2013, Gunawan-Puteri *et al*., 2018; Fransisca *et al.*, 2018).

Statistical analysis

Statistical significance was evaluated using SPSS 22 software with variance (ANOVA) and subsequently by Scheffe test. A p-value <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

This study evaluated the antihyperglycemic activity of IBU in glucose tolerance by using oral sugar tolerance test. The oral sugar tolerance model is a widely used experimental procedure for metabolic studies in mice. Glucose and sucrose are the most commonly consumed sugars. Excessive consumption of sugar stimulates type 2 diabetes mellitus, which is associated with obesity and insulin resistance. In this present study, the postprandial blood glucose levels will increase after glucose or sucrose was given. The AUCs of glucose levels of mice given both glucose and sucrose were significantly higher ($p<0.05$) than that of control mice, 1316.18 and 1309.36 vs 1020.98 mmol.min/L, respectively. Sakamoto *et al*. (2012) reported hyperglycemia in the early phase after oral glucose administration in mice, and the abnormal glucose metabolism in the liver can cause glucose intolerance observed in the mice.

Tabel 1 shows the effect of Uli banana leaves after oral glucose administration on mice. The administration IBU 3.3 g/kg showed a smaller AUC area and was statistically significant compared to glucose group ($p<0.05$). IBU 0.8 and 1.67 g/kg failed to exert any reduction on the tolerance level following oral glucose loading. Results of the glucose tolerance test revealed that IBU dose 3.3 g/kg was the most effective in lowering AUCs of glucose levels in mice (121.7%), whereas IBU 0.88 and 1.67 g/kg did not exhibit any significant antihyperglycemic effect in mice.

As shown in Table 2, IBU 0.8 g/kg of IBU caused a significant reduction in AUCs glucose levels compared to sucrose group ($p<0,05$). On the other hand, IBU 1.67 and 3.3 g/kg did not show significant differences, when compared to sucrose group.

Although, in this study, we provided evidence that IBU had antihyperglycemic activity, significant difference effect of IBU was found between glucose and sucrose administration. Considering that glucose as monosaccharide and sucrose as disaccharide, it could be speculated that the action mechanism of IBU is different from that of sugar. The exact potency of IBU on postprandial hyperglycemia in a mice model is not yet clear and the potential antihyperglycemic component remains to be investigated.

Table 1. Percentage of reduction of AUC of Uli banana leaves after oral glucose administration on mice

Treatment	AUC (mmol.min /L)	% reduction of AUC
Control	1020.98 ± 176.64^{b}	-
Glucose	1316.18 ± 176.64^{a}	-
IBU 0.8 g/kg + glucose	1243.51 ± 71.46	24.6
IBU 1.67 g/kg + glucose	1355.31 ± 56.12^{a}	-13.3
IBU 3.3 g/kg + glucose	957.05 ± 74.36^{b}	121.7

Values are expressed as mean ± SD of five animals in each group. a: $p<0.05$ vs control; b: $p<0.05$ vs glucose. IBU: infusion of Uli banana leaves.

Table 2. Percentage of reduction of AUC of Uli banana leaves after oral sucrose administration on mice

Treatment	AUC (mmol.min /L)	% reduction of AUC
Control	1020.98 ± 176.64^{b}	-
Sucrose	1309.36 ± 92.72^{a}	-
IBU 0.8 g/kg + sucrose	1090.50 ± 89.33^{b}	75.9
IBU 1.67 g/kg + sucrose	1210.54 ± 120.76	34.3
IBU 3.3 g/kg + sucrose	1235.27 ± 105.60	25.7

Values are expressed as mean ± SD of five animals in each group. a: $p<0.05$ vs control; b: $p<0.05$ vs sucrose. IBU: infusion of Uli banana leaves.

These results may demonstrate the positive effect of oral administration of Uli banana leaves infusion (IBU) against hyperglycemia resulting in sugar tolerance test. Hypoglycemic and/or antihyperglycemic activity of banana have been reported by some references. Kappel *et al*. (2013) reported the significant antihyperglycemic effect of banana leaves on the regulation of glucose homeostasis. More recently, Adewoye & Ige (2016) demonstrated that the methanol extract of banana leaves has hypoglycemic properties in alloxan-induced diabetic rats. Beidokhti & Jäger, (2017) reported the hypoglycemic effects of banana due to their natural ability of pancreatic β-cells for insulin secretion, or bioactive compounds such as flavonoids, alkaloids and anthocyanins, which act as insulin-like molecules or insulin secretagogues. Based on previous study, the antihyperglycemic

activity of IBU might be occurs through inhibition of insulin secretion. Further molecular research is needed to find out the mechanisms of active components contained in banana leaves.

CONCLUSION

Infusion of Uli banana leaves 3.3 g/kg showed an 121.7% reduction in AUC glucose levels in the glucose tolerance test, while a 75.9% reduction of dose 0.8 g/kg in the sucrose tolerance test. Overall, these results show that Uli banana leaves infusion possesses antihyperglycemic activity in glucose tolerance by using oral sugar tolerance test. These pharmacological activities provide pharmacological evidence of the potential use of banana leaves as an antihyperglycemic agent. Therefore, IBU can be developed to prevent diabetes through controlling insulin secretion which can further control blood glucose levels.

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Congratulations

JFFN Chief Editor, **Prof. C. Hanny Wijaya**, has been re-elected as **the Chairwoman of Indonesian Society for Functional Food and Nutraceutical (P3FNI)** for the period of 2020 - 2023, with unison consensus on P3FNI Congress on 20 December 2020.

JFFN wishes **Prof. C. Hanny Wijaya** and the whole of **P3FNI Council congratulation.**

May you have many more successful milestones in the future endeavors.

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ANTI-ALLERGY POTENTIAL OF *AVERRHOA BILIMBI* LINN. FRUIT WATER EXTRACT SHOWN BY ITS SUPPRESSIVE EFFECT ON THE DEGRANULATION OF RBL-2H3 CELLS

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ABSTRACT

Allergy rhinitis (AR), as reported by the World Allergy Organization (WAO), is one of the highest prevalence allergies affecting 10-30% of all adults and up to 40% of children. In Indonesia, current evidence showed that the prevalence of AR is increasing. *Averrhoa bilimbi* Linn. fruit (AF), or locally known as *belimbing wuluh* has potentials to treat many diseases due to the abundant of polyphenol content, including to the treatment of allergies. Therefore, this study was aimed to investigate the anti-allergy potential of AF *in vitro*. The anti-allergy effect of *Averrhoa bilimbi* Linn. fruit water extract (AFWE) was examined using RBL-2H3 cells. At first, the cytotoxicity effect of AFWE was determined by WST-8 assay. The release of β-hexosaminidase by RBL-2H3 cells was also measured to evaluate degranulation suppression activity of AFWE. Lastly, calcium assay was employed to investigate the intracellular calcium concentration ($[Ca^{2+}]i$). Results demonstrated that AFWE does not show any cytotoxicity at any given concentration. In addition, AFWE at 1.25 mg/mL showed sufficient inhibitory effect towards degranulation by RBL-2H3 cells. Moreover, the degranulation-suppressing activity of AFWE was resulted from the inhibition of calcium-dependent signaling pathways. Unfortunately, the properties of active substances from AFWE have not been investigated. To conclude, this study indicated that AFWE has potential as an alternative treatment for allergic diseases.

Keywords: *Allergic rhinitis, anti-allergy, averrhoa bilimbi, beta-hexosaminidase, RBL-2H3 cells*

ABSTRAK

Rinitis alergi (AR), sebagaimana dilaporkan oleh *World Allergy Organization* (WAO), merupakan salah satu alergi dengan prevalensi tertinggi yang mempengaruhi 10-30% orang dewasa dan 40% anak-anak. Di Indonesia, bukti terkini menunjukan terjadinya peningkatan prevalensi AR. Buah *Averrhoa bilimbi* Linn. (AF), atau yang dikenal sebagai belimbing wuluh, berpotensi untuk mencegah atau mereduksi risiko beberapa penyakit, termasuk pada penyakit alergi, karena melimpahnya kandungan polifenol. Oleh sebab itu, studi ini bertujuan untuk menginvestigasi potensi anti-alergi AF secara *in vitro*. Pengujian efek anti-alergi dari ekstrak air buah *Averrhoa bilimbi* Linn. (AFWE) dilakukan pada sel RBL-2H3. Efek sitotoksisitas dari AFWE diuji menggunakan metode WST-8. Lalu, pelepasan β-hexosaminidase dari sel RBL-2H3 diukur untuk mengevaluasi aktivitas penekanan degranulasi dari AFWE. Terakhir, uji kalsium dilakukan untuk menginvestigasi konsentrasi kalsium intraselular ($[Ca^{2+}]i$). Hasil studi menunjukan bahwa AFWE tidak memiliki efek sitotoksik di setiap konsentrasi yang diujikan. Selain itu, AFWE pada konsentrasi 1,25 mg/mL menunjukan efek penghambatan yang cukup terhadap degranulasi sel RBL-2H3. Aktivitas penekanan degranulasi oleh AFWE dihasilkan dari adanya penghambatan pada *calcium-dependent signaling pathway*. Akan tetapi, properti kandungan aktif dari AFWE belum dapat terinvestigasi. Sebagai kesimpulan, studi ini mengindikasikan bahwa AFWE memiliki potensi sebagai sumber pangan alternatif untuk memperbaiki kondisi pasien dengan penyakit alergi.

Kata kunci: *Anti-alergi, averrhoa bilimbi, beta-hexosaminidase, rinitis alergi, sel RBL-2H3*

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INTRODUCTION

Allergic rhinitis (AR) is defined as an inflammation of the membrane lining in the nasal cavity which is indicated by one or more symptoms such as sneezing, nasal itching, nasal congestion, and nasal discharge (Bousquet *et al*., 2008). As reported by Word Allergy Organization (WAO), AR affected 10-30% of the total population and up to 40% of children worldwide (Pawankar *et al*., 2011). In Indonesia, the latest national report by International Study of Asthma and Allergies in Childhood (ISAAC) found that the prevalence of AR was less than 5% (Mallol *et al*., 2013). However, current evidence showed that the prevalence is increasing, particularly in the big cities, including Bandung and Surabaya to 38% and 23%, respectively (Fauzi *et al*., 2015; Soegiarto *et al*., 2019).

AR is stimulated by the cross-link of antigen to immunoglobulin E (IgE) bound on high-affinity IgE receptors, known as FcεRI (Ishida *et al*., 2013). This action stimulates the calcium dependent signaling pathway (Lyn-Syk-LAT-PLCγ) in FcεRI receptor expressing-cells, such as mast cells and basophils, which leads to Ca^{2+} liberation from endoplasmic reticulum (ER) (Sun *et al*., 2014). As the results, the cells are degranulated and chemical mediators are released such as β-hexosaminidase, histamine, and inflammatory cytokines, inducing acute allergic responses (Metcalfe *et al*., 2009).

Averrhoa bilimbi Linn. is a Southeast Asian endemic plant species, which is underutilized. It is used as one of food ingredients in Indonesian traditional dishes, such as *garang asam*, *sayur asem*, and *asem-asem*. *Averrhoa bilimbi* Linn. fruit (AF) is also used as ethnomedicine for skin care (Ahmed and Alhassan, 2016), to treat syphilis (Samuel *et al*., 2010), whooping cough, obesity, hypertension, and diabetes (Ahmed and Alhassan, 2016). Besides, scientific studies revealed the capability of AF as antihypertensive (Lestari *et al*., 2018), antihyperlipidemic (John and Pta, 2019), antidiabetic (Kurup and Mini, 2014), antimicrobial (Mokhtar and Aziz, 2016), anti-inflammatory (Suluvoy *et al*., 2017), and anticancer (Nair *et al*., 2016). However, assessment of AF for its anti-allergic potentials has not been done to date.

In phytochemical studies conducted by Hasanuzzaman *et al*. (2013) and Yan *et al*. (2013), AF was reported to be a promising source of polyphenols, which include phenolic acids, flavonoids, and tannins. Hasanuzzaman and colleagues also found that the polyphenols are present in aqueous extract of AF. Polyphenols, particularly flavonoids, have been strongly associated with the alleviation and prevention of IgE-mediated allergic diseases. Polyphenol-induced alleviation of allergic reactions is done through the reduction of expression of MHC-II on dendritic cells, causing the reduction the antigen presentation from dendritic cells to T_H2 cells. This action leads to the suppression of inflammatory cytokines (IL-4 and IL-13) released by T_H2 cells, resulting in the reduction of B cells recruitment and the reduction of antigen specific IgE production (Singh *et al*., 2011; Tanaka and Takahashi, 2013).

To analyze anti-allergy potential of AF water extract (AFWE), Rat Basophilic Leukemia (RBL)-2H3 cells were used in this *in vitro* study. RBL-2H3 cells mimic the properties of mast cells and express high levels of FcεRI on the surface of the cells when activated by IgE-allergen complex (Fu *et al*., 2019).

MATERIALS AND METHOD

This study was performed at Animal Cell Technology Laboratory, Ehime University, Japan and at Indonesia International Institute for Life Sciences (i3L), Jakarta, Indonesia. The cytotoxicity effect, suppression of β-Hexosaminidase release, and suppression of intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) were investigated. In this study, unnoted materials were purchased from either Nacalai Tesque (Kyoto, Japan) or Fujifilm Wako Pure Chemical (Osaka, Japan).

Sample preparation

Averrhoa bilimbi Linn. fruits were from Tangerang, Indonesia, and randomly sampled. The fruits were washed and freeze-dried at Pilot Plant, i3L. The freeze-dried fruits (the whole fruit, including flesh, peel and seeds) were made into powder and soaked in distilled water (DW) at concentration of 2 g/mL overnight at room

temperature. The extract was centrifuged twice at 4,000 rpm for 3 min and 15,000 rpm for 20 min, sequentially. pH of the supernatant was adjusted to 7.4 using 0.1 M of NaOH and the supernatant was filtered. The solution was freeze-dried again to obtain the water-soluble substances in the extract. The dried form of the water-soluble extract was rehydrated with DW at 20 mg/mL. The extract solution was sterilized using a 0.22 µm filter syringe in sterile condition and stored at -35°C until further used. Prior to use, the extract was further diluted into 10.00, 5.00, 2.50, and 1.25 mg/mL. The processed extract was then brought to Japan for further analysis.

RBL-2H3 cells seeding and sample treatment

RBL-2H3 cells seeding was performed according to Ishida *et al*. (2013) with minor modification. Cells were seeded into 96-well plate in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing 100 µg/mL of streptomycin, 100 U/mL of penicillin, and 5% of fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS, USA) at 4×10^5 cells/well. The cells were sensitized with DNP-IgE (Sigma-Aldrich, St. Louis, MO, USA) and incubated overnight at 37°C in a humidified 5% of CO_2 incubator. The cells were washed using modified Tyrode's buffer (1.8 mM $CaCl_2$, 5.6 mM glucose, 20 mM HEPES, 5mM KCl, 1 mM $MgCl_2$, 135 mM NaCl, and 0.05% BSA, pH 7.4) twice and treated with 120 µL of AFWE at different concentration for 10 min. Cells degranulation was induced by adding 10 µL of DNP-HSA diluted in modified Tyrode's Buffer, and the cells were incubated for 30 min.

Cytotoxicity assay

Cytotoxicity of AFWE on RBL-2H3 cells were measured using WST-8 assay kit (Kishida Chemical, Osaka, Japan) according to the manufacturer's instruction. In brief, after RBL-2H3 cells were seeded in a 96 well plate, they were treated with AFWE and were degranulated (as induced by DNP-HSA). The cells were then washed once with 200 µL of phosphate buffered saline (PBS). Then, 110 µL of 5% FBS-DMEM containing 5% of WST-8 solution was added to each well. After that, the cells were incubated for 25 min at 37°C. Cells viability was measured through absorbance measurement using microplate reader (Model 680; Bio-Rad Laboratories, Hercules, CA, USA) at 450 nm.

β-Hexosaminidase assay

β-Hexosaminidase assay was performed according to Ishida *et al*. (2013) with minor modification. In brief, after 30-min of incubation following the degranulation of the cells, the degranulation process was terminated by incubating the cells on ice for 10 min. Supernatant was collected, and the cells were lysed by using a hand sonicator in a 0.1% Triton X-100 Tyrode's buffer for 5 s. The cell lysate and supernatant were transferred into a new 96-well plate, and 100 µL of 0.1 mM citrate buffer (pH 4.5) containing 3.3 mM p-nitrophenyl-2-acetoamid-2-deoxy-β-d-glucopyranoside (Wako Pure Chemical Industries) was added to each well. The microplate was incubated at 37°C for 25 min, and the reaction was terminated by adding 100 µL of 2 M glycine buffer (pH 10.4). β-Hexosaminidase release was measured using microplate reader (Model 550; Bio-Rad Laboratories) at 405 nm and the rate of release was calculated according to the formula: β-Hexosaminidase Release (%) = $\{A_{supernatant} - A_{blank\ of\ supernatant}\} / \{(A_{cell\ lysate} - A_{blank\ of\ cell\ lysate}) + (A_{supernatant} - A_{blank\ of\ supernatant})\}$.

Calcium assay

Intracellular Ca^{2+} concentration was measured using Calcium Kit-Fluo 3 AM (Dojindo Laboratories, Kumamoto, Japan). Following RBL-2H3 cells sensitization with DNP-IgE, the cells were washed twice with PBS and were labeled using 100 µL of Fluo-3 AM fluorescence dye. The cells were incubated for 1 h. Labeled cells were rinsed with PBS and treated with AFWE at different concentrations. Then, the cells were degranulated by adding DNP-HSA and the $[Ca^{2+}]i$ was measured by using fluorescence microplate reader (SH-8000 Lab; Corona Electric, Ibaraki, Japan) at λ_{ex} = 480 nm, λ_{em} = 530 nm for 2 min, in which fluorescent intensity was monitored every 10 s.

Statistical analysis

All experiments in this study were conducted in two batches with triplication in each batch. The data were presented as mean ± standard error mean (SEM). One-way analysis of variance (ANOVA) and Tukey's test were conducted to obtain the statistical significance between AFWE and control.

RESULTS

In this study, the effect of AFWE on viability of RBL-2H3 cells was first to be examined by treating the cells with different concentrations AFWE ranging from 1.25 to 10.00 mg/mL. As shown in **Figure 1**, AFWE did not show any cytotoxicity at any given concentration on RBL-2H3 cells. These results indicated that all concentrations of AFWE can be further analyzed for its anti-allergy effect.

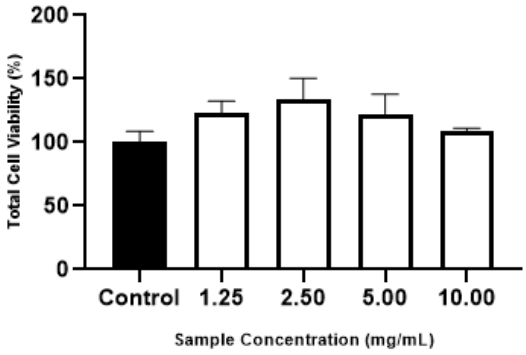

Figure 1. RBL-2H3 cells viability. Results were presented as the mean ± SEM (n = 6). Data were collected in two batches (independent measurement) with three replications in each batch.

The anti-allergy effect of AFWE was examined through β-Hexosaminidase assay to check its capability to suppress the release of β-Hexosaminidase by the degranulated cells (**Figure 2**). The suppression effect of AFWE towards β-Hexosaminidase release was reported as the ratio of β-Hexosaminidase that was released-to-supernatant to the total of β-Hexosaminidase presence in both supernatant and lysed cells. The results showed that AFWE significantly suppresses β-Hexosaminidase release in a dose dependent manner, suggesting that AFWE has a degranulation-suppressing activity. Moreover, the results demonstrated that 1.25 mg/mL of AFWE was sufficient to inhibit β-Hexosaminidase release from RBL-2H3 cells. However, 2.50 mg/mL of AFWE showed more potent inhibition.

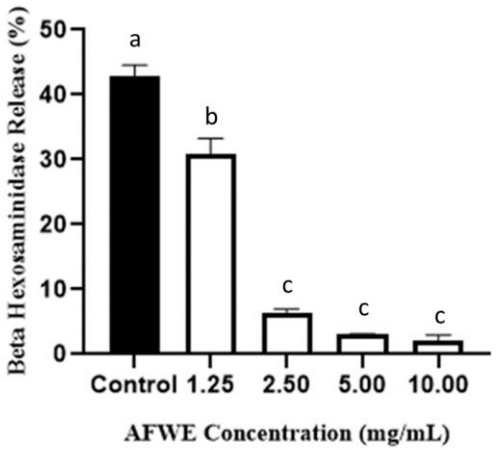

Figure 2. β-Hexosaminidase release. Results were presented as the mean ± SEM (n = 6). Data were collected in two batches (independent measurement) with three replications in each batch. Statistically significant differences between samples are represented by different alphabets ($p < 0.05$).

In order to investigate the effect of AFWE to calcium dependent signaling pathway, $[Ca^{2+}]i$ was examined. As shown in **Figure 3**, $[Ca^{2+}]i$ is rapidly elevated in non-treated RBL-2H3 cells, while the elevation of $[Ca^{2+}]i$ was suppressed by the addition of AFWE. These results suggested that the degranulation-suppressing activity of AFWE was resulted from the inhibition of calcium dependent signaling pathways.

DISCUSSION

Averrhoa bilimbi Linn. is an underutilized natural source that is commonly found in Indonesia and other tropical countries, such as Malaysia and Philippines. Its leaves and fruits are used as food ingredients and ethnomedicine (Ahmed and Alhassan, 2016), and recently, scientific studies

showed that *Averrhoa bilimbi* Linn. fruit (AF) has high potentials to be a functional food, because it was reported to exert several beneficial health effects, such as antihypertensive (Lestari *et al*., 2018), antihyperlipidemic (John and Pta, 2019), antidiabetic (Kurup and Mini, 2014), antimicrobial (Mokhtar and Aziz, 2016), anti-inflammatory (Suluvoy *et al*., 2017), and anticancer (Nair *et al*., 2016). Phytochemical studies also revealed the abundance content of polyphenols in AF, especially flavonoids, tannins, and phenolic compounds. Previously, numerous studies revealed the association of polyphenols with anti-allergy (Juríková *et al*., 2015; Magarone and Jirillo, 2012; Tanaka *et al*., 2019). Therefore, the potential benefits of AFWE as an anti-allergy is important to be investigated.

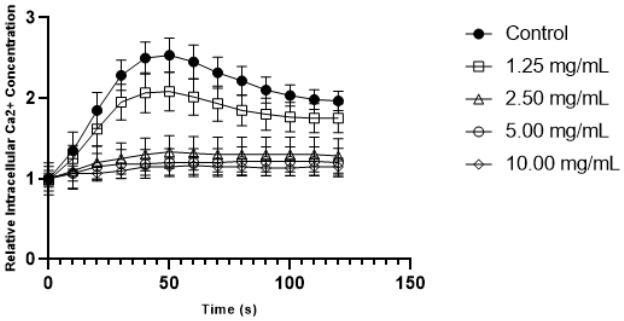

Figure 3. [Ca^{2+}]i level in RBL-2H3 cells following the treatment by control and AFWE. Results were presented as the mean ± SEM (n = 6). Data were collected in two batches (independent measurement) with three replications in each batch

In this study, the cytotoxic effect of AFWE was first examined and results showed that AFWE does not possess cytotoxic effect toward RBL-2H3 cells. After the safety of AFWE towards RBL-2H3 cells was confirmed, the potential of AFWE to inhibit degranulation of RBL-2H3 cells was examined. The results demonstrated that AFWE successfully suppresses degranulation on RBL-2H3 cells in a dose dependent manner, denoted by the suppression of β-Hexosaminidase release (**Figure 2**). Moreover, as shown in **Figure 3**, the suppression of β-Hexosaminidase release by AFWE was caused by the suppression of intracellular Ca^{2+} concentration ([Ca^{2+}]i). These results suggested that AFWE has a good potential as an anti-allergy.

The anti-allergy effect of AFWE might be because of the presence of polyphenols, particularly flavonoids. Previous studies revealed the mode of action of polyphenols as anti-allergy is done through the reduction of expression of MHC-II on dendritic cells (Singh *et al*., 2011). This action leads to the reduction of antigen presented from dendritic cells to T_H2 cells, causing suppression of inflammatory cytokines released by T_H2 cells. This results in the suppression of IgE production from B cells (Tanaka and Takahashi, 2013). In study by Yoo *et al.* (2014), aged black garlic extract fraction rich in polyphenols and flavonoids were reported to have potent inhibitory effect towards β-Hexosaminidase release. In addition, polyphenols from apple such as apple condensed tannins was also reported to have strong inhibitory effect toward β-Hexosaminidase release from RBL-2H3 cells (Kanda *et al.*, 1998). The polyphenols content in AF have not been fully identified yet. However, study by Muhamad *et al.* (2015) mentioned the presence of catechin in AF. Previously, it has been reported that the consumption of tea containing catechin has successfully alleviated the symptoms of mouse with Japanese cedar pollinosis (Maeda-Yamamoto *et al.*, 2007). The consumption of catechin and its derivatives was previously reported to have significant effects on inhibiting IgE-mediated allergy through the prevention of tyrosine phosphorylation (Maeda-Yamamoto *et al.*, 2004) and the production of IL-4 and IL-13 released by T_H2 cells (Singh *et al*., 2011). Besides that, ethyl acetate fraction of AF also reported to contain quercetin (Kurup and Mini, 2017). Previously, quercetin has been elaborated for its potential to inhibit histamine release from FcεRI receptor expressing-cells (Scheller *et al.*, 2011), and to inhibit the activation of MHC-II in APCs (Gong and Chen, 2003). A study by Kempuraj *et al.* (2005) showed that quercetin and kaempferol suppress [Ca^{2+}]i in the mast cells. However, the presence of kaempferol and other flavonoids such as chrysin and apigenin in AF have not been identified yet, especially in aqueous extract.

Unfortunately, the molecular mechanism on how AFWE suppresses degranulation of RBL-2H3 cells is still unknown. Referring to the previous study, Lyn and Syk are frequently utilized as protein biomarkers in immunoblotting analysis as both of this proteins are responsible for $[Ca^{2+}]i$ liberation, the activation of mitogen-activated protein kinases (MAPKs), and also the degranulation of the mast cells (Yoo *et al.*, 2014). Since the Lyn and Syk pathway induce the activation of MAPKs pathway, then the utilization of MAPKs, including p38 mitogen activated protein kinase, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated protein kinase (ERK), can also be alternative biomarkers (Lian *et al.*, 2015). In a study by Ishida *et al.* in 2013, PLCγ was also one of the biomarkers in the immunoblotting test. PLCγ has an important role in Ca^{2+} signaling (Putney and Tomita, 2012). In addition, Fyn and Gab2 are also candidates for protein biomarkers. Besides its responsibility for accumulation of PI3K, study by Nishida *et al.* (2005) showed that Fyn and Gab2 are also responsible for the formation of microtubules in mast cells. In this case, the microtubule leads to the translocation of granules to the plasma membrane and results in the degranulation of the cells. Therefore, further investigations are required to clarify the mechanism underlying the inhibitory activity of AFWE on degranulation of RBL-2H3 cells.

CONCLUSION

AFWE successfully demonstrated its anti-allergy potential through *in vitro* experiment using RBL-2H3 cells. Results showed that AFWE inhibit the release of β-Hexosaminidase from RBL-2H3 cells through the suppression of intracellular Ca^{2+} ion $[Ca^{2+}]i$ concentration. These findings indicate the potential of *Averrhoa bilimbi* fruit as an alternative for patients with allergic rhinitis and/or other IgE-mediated allergies. Further investigation is needed to identify the active substance in AFWE and to elucidate its mode of actions.

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DETECTION OF SECONDARY METABOLITE OF *MYCENA PELIANTHINA* GROWTH IN VARIOUS LIQUID MEDIUM

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ABSTRACT

Mushrooms which is mostly belong to the phylum Basidiomycota have been recognized as functional foods and a source for the nutraceuticals. Genus Mycena is one of mushrooms, which has been investigated for its secondary metabolites. *Mycena pelianthina* produces pelianthinarubins A, pelianthinarubins B, muscarin, and epimuscarin from alkaloid group. The purposes of this research were to determine the effects of composition of medium and incubation time on production of mycelial biomass of *M. pelianthina* in liquid medium and to know the group of secondary metabolite compounds produced. This research was done experimentally using a Factorial Completely Randomized Design consisted of 12 treatments and three replications. The first factor was medium type, which were Mushroom Complete Medium (MCM), Potato Dextrose Yeast Broth (PDYB), and Yeast Malt Extract Medium (YM). The second factor was the incubation time consisting of 15, 20, 25, and 30 days. The observed main parameters were the dry weight of mycelial biomass and the secondary metabolite groups. The dried weights of mycelia were analyzed using analysis of variance (ANOVA), continued with Duncan test at a 95% confidence level. The best growth of *M. pelianthina* mycelium was produced on PDYB medium at incubation time of 20 days with an average mycelial dry weight of 0.92 g/100 mL. *M. pelianthina* contained secondary metabolite compound groups of alkaloids, terpenoids, and flavonoids.

Keywords: *Alkaloid, flavonoids, liquid medium, mycena pelianthina, secondary metabolite, terpenoids*

ABSTRAK

Jamur pangan merupakan salah satu bahan pangan yang dikenal sebagai pangan fungsional dan sebagai sumber nutrasetikal. Salah satu jamur pangan yang dikenal menghasilkan senyawa aktif sebagai metabolit sekunder adalah jamur *Mycena pelianthina*. Jamur ini dikenal menghasilkan senyawa metabolit sekunder pelianthinarubins A, pelianthinarubins B, muscarin, and epimuscarin dari kelompok alkaloid. Tujuan penelitian ini adalah untuk mengetahui pengaruh medium pertumbuhan dan waktu inkubasi terhadap bobot miselia *M. pelianthina* serta mengetahui golongan senyawa metabolit sekunder yang dihasilkannya. Penelitian ini dilakukan secara eksperimental menggunakan Rancangan Acak Lengkap Faktorial, dengan 12 perlakuan dan tiga kali ulangan. Faktor pertama meliputi jenis medium, yaitu *Mushroom Complete Medium* (MCM), *Potato Dextrose Yeast Broth* (PDYB), dan *Yeast Malt Extract Medium* (YM). Faktor kedua meliputi waktu inkubasi, yaitu 15, 20, 25, dan 30 hari. Bobot kering miselia tertinggi (0,92 g/100 mL) dihasilkan pada medium PDYB dengan waktu inkubasi 20 hari. *M. pelianthina* mengandung senyawa metabolit sekunder golongan alkaloid, terpenoid, dan flavonoid.

Kata kunci: *Alkaloid, flavonoid, medium cair, metabolit sekunder, mycena pelianthina, terpenoid*

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INTRODUCTION

The potential of mushrooms as a functional food and as a source of nutraceutical has been widely studied and developed in several countries. Mushrooms are considered to produce various types of bioactive metabolites. These bioactive metabolites can be either primary metabolites or secondary metabolites (Ekowati *et al*., 2016). Various kinds of bioactive compounds have been isolated and identified, including those from the groups of polysaccharides, proteins, phenols, vitamin B, tocopherols, organic acids and terpenoids (Khatua *et al*., 2013). Some of them have been approved for clinical uses (Ren *et al*., 2014).

Mycena is a large group of Basidiomycetes. Several species belong to this genus have been studied for their bioactivity and the structures of metabolite compounds have been identified. Strobilurin bioactive compounds isolated from *M. galopoda*, *M. atromarginata*, *M. rosella* and *M. vitilis* have antifungal and antibacterial activity (Bäurle & Anke, 1980). Leaianafulvene isolated from *M. leaiana* and tintinnadiol from *M. tintinnabulum* is cytotoxic (Engler *et al.*, 1998). However, research on bioactive compounds produced by *M. pelianthina* has not been widely carried out.

Mushroom growth can be influenced by the composition of medium and incubation time. Liquid medium is widely used for production of mycelial biomass since it has advantages such as a short incubation period, low possibility of contamination, and easy to take of mycelia for further analysis (Ekowati *et al.*, 2011). The composition of the medium can affect microorganisms for producing secondary metabolites. According to Suciatmih (2010), potato dextrose yeast broth is a fermentation medium commonly used for testing secondary metabolites. According to Behera and Gupta (2015), production of mycelial biomass in mushroom complete medium is higher than that of yeast malt extract medium. Mushroom complete medium is a complete medium that is very suitable for mushroom growth because it contains a source of carbon, nitrogen, vitamins, and minerals (Kim *et al*., 2002).

Mushrooms need time to hydrolyze available nutrient sources for growth and secondary metabolite production. The longer incubation time, the mycelium growth increases to a certain extent so that the production of metabolites is also high (Saputra, 2010). Bäurle *et al.* (1982) reported that the best growth of *M. viridimarginata* mycelium is at 25 days incubation on YMG medium.

Secondary metabolite compounds can be detected using TLC (Thin Layer Chromatography). It can separate chemical components, based on the principle of adsorption and partitioning of compounds by the stationary phase (adsorbent) and the mobile phase (eluent). The chemical compounds move up based on the mobile phase because the adsorption of adsorbent is different so that the chemical components can move at different distances based on the level of polarity. The displacement process causes the separation of chemical components (Alen *et al*., 2017). The purpose of this study were to elucidate the best media for production of mycelial biomass of *M. pelianthina* and to identify the compound group of secondary metabolites they produced.

MATERIALS AND METHOD

Pure culture of *M. pelianthina* was obtained from the Laboratory of Mycology and Phytopathology, Faculty of Biology, Jenderal Soedirman University. It was cultured on PDA medium on areas of 90 mm petri dish diameter. A piece of mycelium of 5 mm in diameter was inoculated using a cork borer in that various liquid media. The experimental design used in this research was a Factorial Completely Randomized Design (CRD) consisting of 12 treatments and three replications. The first factor was medium type i.e. Mushroom Complete Medium (MCM), Potato Dextrose Yeast Broth (PDYB), and Yeast Malt Extract Medium (YM). The second factor was the incubation time, namely, 15, 20, 25, and 30 days. The main observed parameters were the dry weight of mycelia and the secondary metabolite groups.

The composition of liquid medium.

The MCM was comprised of 20 g of glucose, 0.46 g of KH_2PO_4, 1 g of K_2HPO_4, 0.5 g of $MgSO_4.7H_2O$, 2 of g peptone, and 2 g of yeast extract (Kim *et al*., 2002). The PDYB was comprised of 200 g of potato, 3 g of yeast extract, 20 g of dextrose, and 1,000 mL of distilled water (Maharani *et al*., 2014). The YM was comprised of 10 g of glucose, 3 g of malt extract, 5 g of peptone, and 5 g of yeast extract (Kim *et al*., 2002).

Cultivation of M. pelianthina mushroom (Elfita *et al*., 2014)

Mycena pelianthina mycelium was inoculated by transferring 5 plugs, respectively (5 mm diameter) from rejuvenating medium into a 250 mL Erlenmeyer flask containing 100 mL of PDYB, MCM, and YM, respectively. Each Erlenmeyer flask was closed with cotton and glued with plastic wrap. The inoculated media were then incubated for 15, 20, 25, 30 days at room temperature using a rotary incubator.

Weight of M. pelianthina mycelia (Irianto *et al*., 2008)

The mycelial cultures that had been incubated for 15, 20, 25, or 30 days were filtered using Whatman papers no. 41, and a vacuum pump was used to accelerate the filtration. The mycelia from each filtered sample were weighed and then dried in an oven at a temperature of 60°C until a constant weight was obtained and the dry weight was recorded.

Determination of pH medium

The pH of medium was determined using Horiba Laqua pH meter (Wagestu et al., 2016).

Extraction of M. pelianthina mycelium (Vamanu, 2013)

Extraction of *M. pelianthina* mycelium was conducted following the procedures shown in the diagram Figure 1.

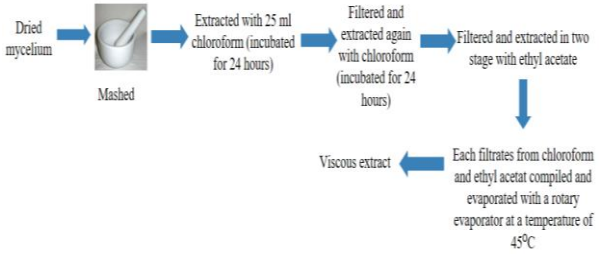

Figure 1. Extraction of *M. pelianthina* mycelia

Production of M. pelianthina filtrate extract (Ekowati *et al*., 2011)

Production of *M. pelianthina* filtrate extract was conducted following the procedures shown in the diagram Figure 2.

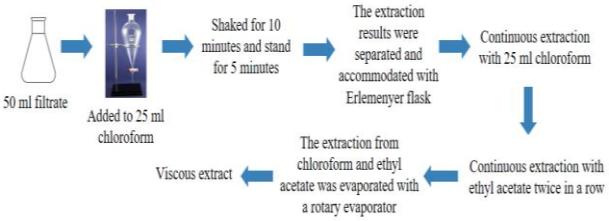

Figure 2. Production of *M. pelianthina* extract mixtures

Identification of secondary metabolite group compounds (Wagner *et al*., 1984)

Identification of alkaloid, terpenoid, and flavonoid were carried out using a silica gel GF254 plate with the mobile phase of dichloromethane: methanol (10:1) mixture. Each eluted spot was calculated by the value of Retardation Factor (Rf) with the following formula.

$$Rf\ (cm) = \frac{\text{Distance moved by the compound}}{\text{Distance moved by the surface of the solvent}} \quad (1)$$

Statistical analysis

Mycelial dry weight data obtained were analyzed using ANOVA at p = 5% and were continued with the Duncan's Multiple Range Test (DMRT) at the p

values. The detection of secondary metabolites was performed descriptively.

RESULTS AND DISCUSSION

Mycena pelianthina mycelial biomass

The growth of *M. pelianthina* mushroom on MCM and YM media continued to increase until 30 days of incubation. The growth of *M. pelianthina* mushroom on PDYB medium increased until 20 days and then decreased during 25 and 30 days (Figure 3). The interaction between the types of media and the incubation time significantly affected the dry weight of mycelia (Table 1).

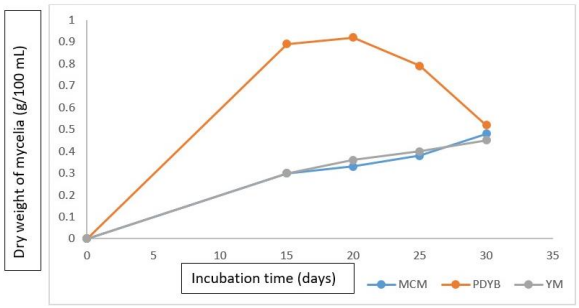

Figure 3. The growth of *M. pelianthina* in mushroom complete medium (MCM), potato dextrose yeast broth (PDYB), and yeast malt extract medium (YM), respectively.

The growths of *M. pelianthina* on MCM and YM, respectively, were in the exponential phase from 15 to 30 days of incubation. The growth of *M. pelianthina* in the MCM and YM, respectively, were not in either the stationary phase nor the death phase because the mushroom was still utilizing nutrients for growth so that mycelial dry weight continued to increase. The growth of *M. pelianthina* on PDYB medium entered the death phase subsequently, during the 25 to 30 days of incubation. According to Setyati *et al.* (2015), the death phase was characterized by a decreasing rate of growth caused by a lack of growth material such as vitamins and mineral elements

PDYB was the best medium for the growth of *M. pelianthina* mycelium regarding the production of mycelial dry weight. According to Teoh & Don (2012), yeast extract, glucose, $MgSO_4.7H_2O$, KH_2PO_4, K_2HPO_4 contained in MCM can increase the mycelial growth. On the other hand, Laurie et al. (2015) reported that potatoes in PDYB medium contain more complete minerals and vitamins. Potatoes contain minerals such as calcium, iron, magnesium, phosphorus, potassium, and zinc as well as vitamins such as thiamine, niacin, riboflavin, and vitamin B6. Based on the research by Behera & Gupta (2015), YM produced a lower mycelial dry weight compared to MCM for the mushroom *Rusula* spp, *Pleurotus sajor-caju*, *Lentinus tuberregium* and *Calocybe indica* because YM has limited mineral elements.

Table 1. Statistical analysis of dry weight mycelium of *M. pelianthina* in MCM, PDYB, YM medium and incubation time

Media	Incubation Time (days)	Dry Weight of Mycelia (g/100 mL)
MCM	15	0.30 [a]
	20	0.33 [a]
	25	0.38 [a]
	30	0.48 [a]
PDYB	15	0.89 [b]
	20	0.92 [b]
	25	0.79 [b]
	30	0.52 [a]
YM	15	0.30 [a]
	20	0.36 [a]
	25	0.40 [a]
	30	0.45 [a]

Note: Numbers with different notation show significantly different at p = 5%

Based on the ANOVA results (Table 2), the interaction between the type of medium and the incubation time had a significant effect on the dry weight gain of mycelia. Mycelial growth can be influenced by several factors such as growth medium, incubation time, pH, temperature, nutrition and several environmental factors (Muthu & Shanmugasundaram, 2015). The growth medium is the most important factor because it contains nutrients needed for mycelial growth. The nutrients needed by microorganisms for growth include carbon, nitrogen, non-metallic elements such as sulfur and phosphorus, metal elements such as Ca, Zn, Na, K, Cu, Mn, Mg, and Fe, vitamins, water,

and energy. Mushrooms require a certain amount of time to break the sources of nutrients down that are available in the medium and then use it up for growing and production of secondary metabolites (Cappucino & Sherman, 2014).

PDYB is the main medium for mushrooms growth. Mushrooms can metabolize starch in potatoes resulting in dissolved sugars that act as sources of carbon and energy as well (Laurie *et al*., 2015). Potato is a complex medium that provides nitrogen, enzymes, vitamins and mineral elements for mushrooms growth. In addition, the yeast extract in the PDYB contained not only organic nitrogen; but also vitamins, minerals, sugar and cofactors. High carbon sources and yeast contain various supporting compounds for growth, enabling efficient mushroom growth (Suciatmih, 2010).

Every type of mushroom required a different suitable medium to grow optimally (Smith & Onions, 1994). Each medium has different nutrient compositions to support the mushroom's needs for growing, but the essential goal is to provide balanced nutrients and at adequate concentrations that can enable the mushroom growing well.

Table 2. Anova of dry weight of *M.pelianthina* mycelia in different growth media and incubation times

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F	Sig
Treatment	11	1.703(a)	0.155	10.314	0.000
Time	3	0.017	0.006	0.380	0.768
Medium	2	1.318	0.659	43.910	0.000
Time × Medium	6	0.368	0.061	4.083	0.006
Error	24	0.360	0.015		
Total	35	2.063			

Note : Sig value < 0.05 indicates that the factorial variables have a significant effect on dry weight gain of mycelia

Based on the results of the study, mushrooms grown on MCM decreased the pH (Table 3). According to Angelia *et al*. (2013), the increase of incubation time lowered the medium pH since more sugars were converted into acids. The organic acids produced by the fungus included pyruvic acid and citric acid. The PDYB and YM, respectively, showed pH increase due to mushroom growth (Table 3). According to Sari (2011), the increased pH was caused by the production of ammonia from nitrogen containing compounds in the growth medium.

Table 3. pH Values of the growth media before and after incubation periods

Medium	Incubation Time (days)	Average	
		Initial pH	Final pH
MCM	15	6	5
	20	6	5
	25	6	5
	30	6	5
PDYB	15	6	7
	20	6	7
	25	6	7
	30	6	7
YM	15	6	6
	20	6	7
	25	6	7
	30	6	7

Identification of secondary metabolite group

To elucidate the class of secondary metabolites the mycelial extracts of *M. pelianthina* obtained from chloroform and ethyl acetate, respectively, were then mixed and tentatively determined through TLC method. The results of the active compounds characterized in *M. pelianthina* mycelial concentrated extracts are presented in Figure 4. Alkaloid, terpenoid and flavonoid groups were detected in each medium and the distribution was different for each extract.

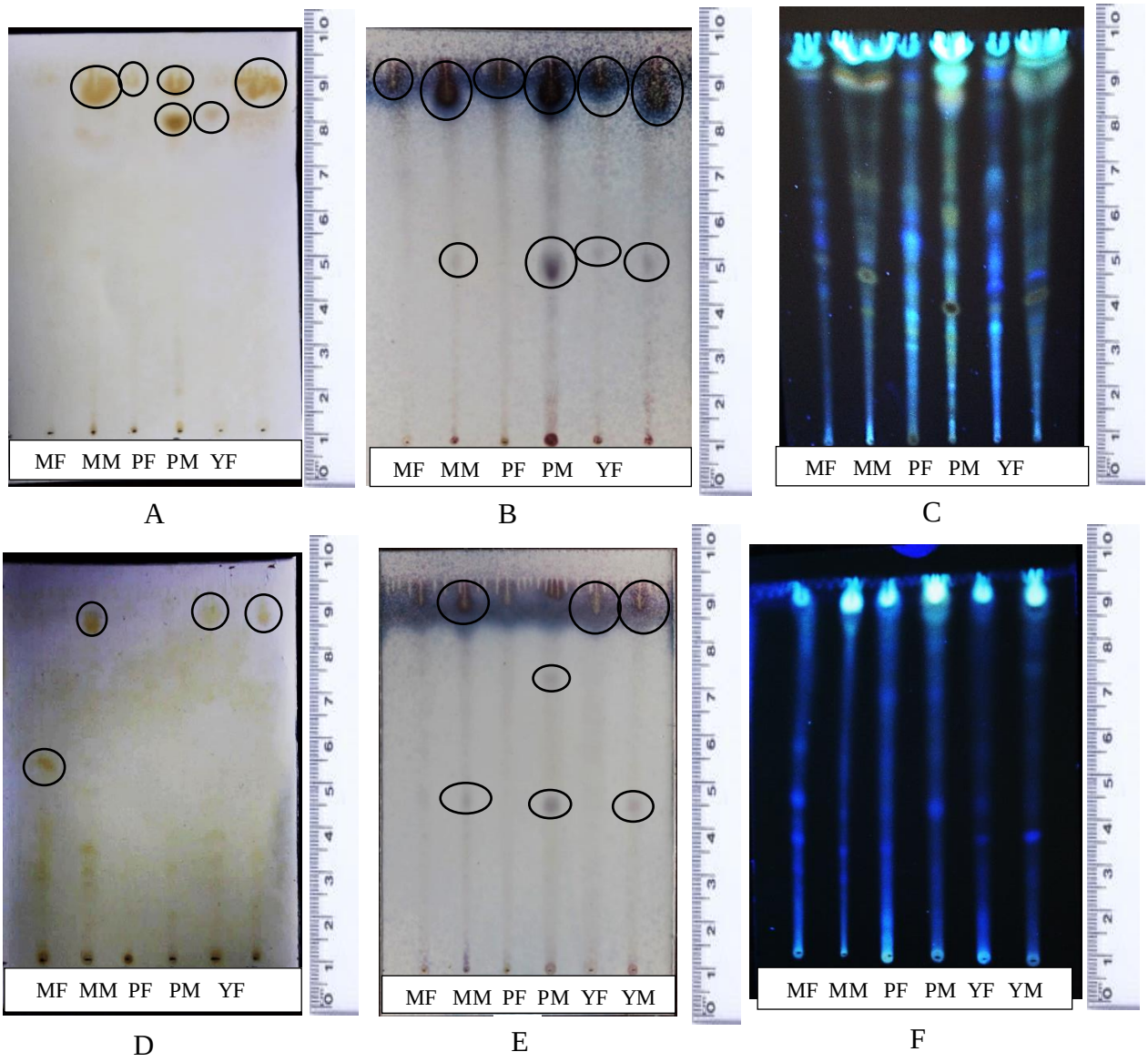

Figure 4. Chloroform extract chromatogram: (A) alkaloid, (B) terpenoid, and (C) flavonoid; ethyl acetate extract chromatogram: (D) alkaloid (E) terpenoid (F) flavonoid

Brownish-yellow spots were formed on the GF254 silica gel plate after being sprayed with Dragendorff reagents (Figure 4.A and 4.D). The Rf values ranged from 0.81 to 0.92 in chloroform elucidation and 0.51 to 0.91 in ethyl acetate eluent. Furthermore, blackish purple spots were formed on the GF254 silica gel plate after sprayed with Vanillin-sulfuric acid reagent (Figure 4.B and 4.E). The Rf values ranged from 0.44 to 0.92 in chloroform mobile phase and 0.41 to 0.91 in ethyl acetate elution. On the other hand, Figures 4.C and 4.F show blue and green spots after sprayed with

$AlCl_3$ reagents observed under 366 nm UV light. The Rf values ranged from 0.12-0.95 when elucidated using chloroform and 0.20-0.94 for ethyl acetate.

The production of bioactive metabolites from microorganisms depends on special adaptations to the environment (Padmavathi *et al*., 2012). The growth medium and incubation conditions play an important role in the production of secondary metabolites. Each observed medium had different compositions. MCM contains the main carbon source of glucose which is more easily metabolized by mushroom. Micronutrients KH_2PO_4, K_2HPO_4, $Na_2HPO_4.12H_2O$, $MgSO_4.7H_2O$, $(NH_4)_2SO_4$ and KCl are important elements for the production of secondary metabolites (Teoh & Don, 2012). PDYB contains a complex carbon source derived from potatoes. YM also contains complex carbon sources derived from maltose. Alkaloids, terpenoids, and flavonoids were detected in PDYB and YM. This was not in accordance with the statement Purwantini *et al*. (2015), that complex carbon sources such as malt extract are more difficult to be metabolized by microorganisms and can result in inhibition of biosynthesis of secondary metabolites. Ramakhrisna & Ravishankar (2011) reported that the production of secondary metabolites as self-defense will increase under stress.

Figure 4 shows trends in accordance with the statement of Suhaenah & Nuryanti (2017), the potential results of the presence of alkaloids are marked with yellow to orange spots after being sprayed with Dragendorff reagent. According to Eva (2007), Rf values for alkaloids ranged from 0.69 to 0.9. Saxena *et al*. (2013) stated that alkaloids have many pharmacological activities including anti-hypertensive, anti-arrhythmic, anti-malaria, and anti-cancer effects.

Vanillin-sulfuric acid reagent is used to detect terpenoid compounds, steroids and essential oil components (Sulistijowati & Gunawan, 2001). Terpenoid compounds on the TLC will change to blue-purple colored spots after sprayed with vanillin-sulfuric acid reagent. Terpenoids are bioactive compounds that can be used as antifungal compounds (Lutfiyanti *et al*., 2012). The toxic nature of terpenoids is known to have the ability to inhibit fungal growth by damaging cell membranes.

Rahayu *et al*. (2015) reported, in similar elution system, spots with Rf values between 0.2–0.75 indicate spots containing flavonoids. On the other hand, Firdiyani *et al*. (2015) stated that flavonoid compounds are non-polar compounds. Flavonoids however have a sugar group which makes it dissolve easily in either polar or semi-polar solvent. According to Prameswari & Widjanarko (2014), flavonoids are polyphenol compounds which have various pharmacological activities such as antioxidants, anti-inflammatory, and anticancer. Overall, type of medium, incubation time and their interactions process are the key factors in increasing mycelia dry weights simultaneously extraction amplified the secondary metabolites' existences.

CONCLUSION

Potato dextrose yeast broth was the best medium for the growth of *Mycena pelianthina*. The highest production of biomass mycelium was 0.92 g/100 mL of the media for 20 days of incubation at room temperature. The extract of *Mycena pelianthina* mycelia contained secondary metabolite compound groups of alkaloid, terpenoid, and flavonoid.

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THE ROLE OF DECAFFEINATED COFFEE IN REDUCING THE RISK OF HYPERTENSION: A SYSTEMATIC REVIEW

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ABSTRACT

Hypertension or increased blood pressure is a degenerative disease with a high prevalence, as well as the biggest cause of premature death in the world. Meanwhile, coffee is a popular and most consumed beverage around the world. Coffee can harm some people with certain health problems including hypertension due to its caffeine content. Decaffeinated coffee is known to be an alternative for people with hypertension with reduced caffeine content up to 97%. However, studies on the role of decaffeinated coffee in reducing the risk of hypertension are still varied indicating that a systematic review is needed. Hence, this study summarizes the evidence related to the role of decaffeinated coffee in reducing the risk of hypertension by using Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA). The inclusion criteria for scientific journals were determined based on Participants, Intervention, Control, and Outcome (PICO). The search using boolean operation resulted in 13 research articles for data extraction. The result revealed that decaffeinated coffee could decrease or did not give any effect on blood pressure or another biological hypertension marker compared to regular coffee.

Keywords: *Coffee, decaffeinated coffee, hypertension, regular coffee, systematic review*

ABSTRAK

Hipertensi atau peningkatan tekanan darah merupakan penyakit degeneratif dengan prevalensi tinggi, sekaligus penyebab kematian dini terbesar di dunia. Sedangkan kopi merupakan minuman yang populer dan paling banyak dikonsumsi di seluruh dunia. Kopi dapat membahayakan sebagian orang dengan masalah kesehatan tertentu termasuk hipertensi karena kandungan kafeinnya. Kopi tanpa kafein diketahui bisa menjadi alternatif bagi penderita hipertensi dengan kandungan kafein yang berkurang hingga 97%. Namun, penelitian tentang pengaruh kopi dekafeinasi dalam menurunkan risiko hipertensi masih bervariasi, mengindikasikan perlu dilakukan tinjauan sistematis. Penelitian ini menggunakan metode tinjauan sistematis untuk meringkas hasil penelitian terkait efek kopi tanpa kafein dan kopi biasa terhadap risiko hipertensi menggunakan Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA). Kriteria inklusi untuk jurnal ilmiah ditentukan berdasarkan Peserta, Intervensi, Pengendalian, Hasil (PICO). Pencarian menggunakan operasi boolean menghasilkan 13 artikel penelitian untuk ekstraksi data. Hasil penelitian menunjukkan bahwa kopi tanpa kafein memberikan efek yang bervariasi antara dapat menurunkan atau tidak berpengaruh pada tekanan darah atau penanda hipertensi biologis lainnya dibandingkan kopi biasa.

Kata kunci: *Hipertensi, kopi, kopi biasa, kopi tanpa kafein, tinjauan sistematis*

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INTRODUCTION

Coffee is a widely consumed beverage and is popular throughout the world (Butt *et al.,* 2011). Coffee is known as a regular beverage with extensive health benefits, such as increasing the physical and mental performance of its regular drinkers (Zhang *et al.,* 2011). Besides, consumption at the right dose per day indicates a reduction in risk from various health risks (Poole *et al.,* 2017). However, coffee can harm some people with certain health problems, such as hypertension. This is caused by the high caffeine content in coffee (Geleijnse, 2008). A method called decaffeination is developed in order to reduce the risk of increased blood pressure and other negative effects due to caffeine. This method is aimed to to reduce the caffeine content in coffee (Butt *et al.,* 2011).

Decaffeinated coffee is known to be an alternative for people with hypertension, or people who have a low tolerance for caffeine because of the reduced caffeine content of up to 97% (Kumar and Ravishankar, 2009). At present, an updated study in the form of a systematic review needs to be conducted to evaluate the differences in the impact of decaffeinated and regular coffee on the risk of hypertension, given that coffee is considered a lifestyle trend.

Coffee and health

Coffee is known to provide health benefits, including an anti-inflammatory, antifibrotic, and anticancer (Poole *et al.,* 2017). Roasted coffee beans which are subsequently consumed as drinks have various bioactive components, including caffeine, chlorogenic acid, diterpenes, cafestol, and kahweol (Poole *et al.,* 2017).

The coffee processing starting from postharvest handling (fermented or natural), roasting level, and brewing methods affect the content of bioactive compounds in coffee (Hameed *et al.,* 2018). In addition to these factors, the human body's response to bioactive components in coffee also influences the bioavailability and health effects of coffee (Yang *et al.,* 2010).

Coffee contains approximately 43% carbohydrates (70-85% polysaccharides, arabinogalactans, mannans, glucans, sucrose, reducing sugars, lignins, and pectins), 10-15% lipids (75% triacylglycerols, 18,5% esters of diterpenes, free sterols, sterol glucosides, waxes, tocopherols, and phosphatides), 7,5-10% protein, another nitrogenous compound (1% caffeine, 0,7-1% trigonelline, and 0,01-0,04% nicotinic acid), 25% melanoidins, 3,7-5% minerals and organic and inorganic acids, and esters (1-4% chlorogenic acids and other phenolic compounds, 1,4-2,5% aliphatic acids and quinic acid and <0,3% inorganic acids). Those reported data regarding the amounts of nutrients could be varied due to variability in the terms of raw material production, processing, and brewing, which lead to the final product (the brew) (Farah, 2018).

Caffeine and hypertension

Hypertension is a factor of many chronic diseases such as coronary artery disease, stroke, heart failure, and kidney disease. In patients with hypertension, an increase or decrease in blood pressure can have a significant impact on various health conditions (Rhee *et al.,* 2016). Also, several physiological and biological markers are referred to as indicators for incidents of hypertension, namely urinary catecholamines, and serum cholesterol. Lifestyle factors are factors that greatly influence the high prevalence of hypertension in the world. One lifestyle that is quite highlighted is the consumption of caffeine, both naturally or synthetically (Turnbull *et al.,* 2017). Coffee as a major caffeine beverage consumed around the world contains some bioactive compounds, including caffeine. Caffeine can increase blood pressure and hypertension risk because it has a pressure effect and the ability to increase vascular resistance (Lovallo *et al.,* 2004). However, the effect of caffeine can vary in each person due to genetic factors, and the tolerance of body to caffeine (Rhee *et al.,* 2016).

Caffeine consumption is a concern for authorities and regulatory bodies, given the impact that can vary between groups. Caffeine is thought to harm pregnant and lactating women, children and adolescents, young adults, and people with mental

health and cardiovascular problems (Temple *et al.*, 2017).

Decaffeinated coffee

Decaffeinated coffee is coffee beans that have undergone a process of caffeine reduction. The decaffeination process can be carried out using solvents such as water, organic solvents, or carbon dioxide (De Paula and Farah, 2019). Decaffeinated coffee aims to reduce caffeine levels and as an alternative for people with certain health problems such as hypertension, and low caffeine tolerance. The decaffeination process is done before the coffee beans are roasted and ground, so that the nutrients from decaffeinated coffee are almost similar to regular coffee, except for the caffeine content. The difference that arises after decaffeinated coffee is the aroma and taste of coffee beans become milder, and a slight color change can occur depending on the process method used (De Paula and Farah, 2019).

The caffeine content in decaffeinated coffee is around 0-7 mg/cup (180 mL), while that in regular coffee is 70-140 mg/180 mL (Ramalakshmi and Raghavan, 1999). Decaffeinated coffee contains antioxidants which can be lower by around 15%. This can be due to the decaffeination process which dissolves polar or nonpolar bioactive compounds in coffee beans. The dominant bioactive compounds in decaffeinated coffee are hydrocinnamic acids and polyphenols. Besides, decaffeinated coffee also has several micronutrients such as vitamins and minerals (Ramalakshmi and Raghavan, 1999). Mineral such potassium found in decaffeinated coffee has a modest blood pressure-lowering effect in normotensive person with low dietary intake (Frank *et al.*, 1998).

Decaffeination process

Decaffeination aimed to minimize the negative physiological effect of caffeine and still keep the desirable flavor and aroma attributes of coffee (Heilmann, 2008). The decaffeination procedures nowadays are divided into decaffeination with the use of chemical solvent for extraction a chemical solvent, water, and supercritical CO_2 (Heilmann, 2008).

Decaffeination with chemical solvent requires a solvent that is virtually immiscible with water to maintain the water-soluble components from the bean, especially the flavor precursor. There are several considerations of using a chemical solvent, such as relatively low investment and operating cost, and high quality. More than 50 % of the worldwide capacity of decaffeination is based on the chemical solvent (Heilmann, 2008). A variety of organic solvents are suitable for this type of extraction, but methylene chloride (CH_2Cl_2) and ethyl acetate are mostly used. With organic solvent decaffeination, the beans are firstly contacted with steam and water to increase their moisture content up to 25 or even 40 wt%. After that moisture content is reached, the extraction process begun within the fixed beds (e.g percolation, column batteries, carousel extractors) or in an agitated system (e.g rotating drums). The rate-controlling or intense bean agitation does not necessary since the diffusion of caffeine in the beans is rather slow (Pietsch, 2017).

The water decaffeination process developed as an alternative to chemical solvent extraction due to anticipation of DCM being banned by the food authorities. However, DCM is still approved by the relevant organization as a food solvent. Water decaffeination is relatively much more expensive in investment but still used by around 22 % of the world coffee decaffeination process since it resulted in clearer and naturally decaffeinated coffee (Pietsch, 2017). The lack of water decaffeination is including the limited selectivity of water, which could lead to the extraction of the non-caffeine compound like sugar. To solve this problem, there are two principal ways, either to hinder the water from extracting non-caffeine coffee soluble compound or to incorporate the extracted soluble into the green beans (Heilmann, 2008).

The process of decaffeination using supercritical CO_2 is generally like the other process, starting from the swelling of the beans with water and then extraction in the percolation columns with high-pressure vessels commonly at 30 MPa. Heilman (2008) proposed three methods could be used for decaffeination using supercritical CO_2. The first one is the moistened green beans are mixed with

the CO_2 stream. The mixture will pass into a washing tower where the caffeine is absorbed in water. This process will be recycled up to 10 hours long, or until almost all of the caffeine is dissolved in the water.

METHOD

This study uses a systematic review method to summarize the evidence related to the effect of decaffeinated and regular coffee on the risk of hypertension. This research will use Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA), which are the recent guidelines to select literature included in the systematic review. The process of article selection was shown in Figure 1.

Inclusion and exclusion criteria

The inclusion criteria for scientific journals were determined based on Participants, Intervention, Control, Outcome (PICO). Meanwhile, the exclusion criteria will be nonhuman study, use none of the regular and decaffeinated coffee, did not use the control, and measures none of the physiological and biological markers of hypertension. The PICO for this review is shown in Table 1.

Table 1. PICO of the systematic review

Inclusion Factors	Criteria
Participants (P)	Human
Intervention (I)	Administration of decaffeinated and regular coffee, or one of them
Control (C)	Nonhabitual coffee drinkers, regular coffee, or non-coffee beverages
Outcomes (O)	Physiological and biological hypertension marker after administration of coffee

Databases and search terms

In this systematic review, the relevant scientific articles were searched in several databases such as PubMed, Science Direct, Cochrane, Embase, and Google Scholar since those databases are the most common to find publications regarding the topic. Also, in this review, the relevant article will be searched using the boolean search. Boolean search is a structured search where users can include several operations (AND, OR, NOT) for specific search results. This method aims to find the relevant subject content in journal articles. In search of the references, the boolean operation used is “Decaffeinated coffee” AND "regular coffee" AND ("hypertension" OR "blood pressure"). Besides, all studies of humans published up to 20 August 2020 were eligible due to the lack of articles with the desired topic in the early 10 years.

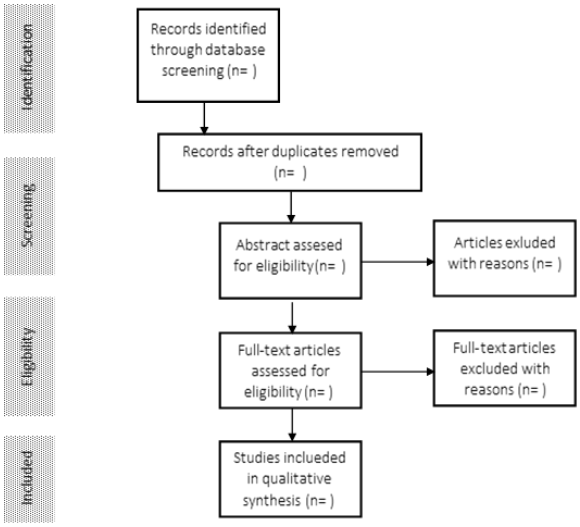

Figure 1. PRISMA guideline for systematic review

Data extraction

Data extraction is a process to gather the core information from the scientific articles which pass the screening process. Data extraction was conducted after the screening of duplication and abstract relevance. The core information extracted referred to Cochrane Guidelines for Systematic Review 2019. The data extraction process

including the proofreading of the full-text article, and summarize the information with a systematic review software management. The systematic review management tools used in this review was Ms. Excel with a customized workbook with the rows and columns designed according to the information being extracted. The type of information was determined based on relevance with the study topic and scientific field of the author.

RESULTS AND DISCUSSION

Entering the boolean operation with terms consist of “decaffeinated coffee” AND "regular coffee" AND ("hypertension" OR "blood pressure") in databases such as PubMed, Cochrane, Embase, and Google Scholar resulted in 47 published articles as shown in Figure 2. These articles then screened for duplicate entries. This process resulted 46 articles. The abstract of these articles was then screened for specific contexts including the type of study, interventions, participants, time, and outcome. This step then removed 12 articles. The remaining (typo error, remaining) 34 articles were then screened based on full-text proofreading for specific context including the clarity of methodology. The clarity of the methodology means the research articles give a well chronological aspect started from the selection of participants (ethical clearance, health condition parameters), source of materials (regular and decaffeinated coffee), and outcome of studies (should be for hypertension parameters; blood pressure, heart rate, pulse rate, catecholamines). These screening steps then removed 12 articles. The remaining (the remaining, typo error) 13 articles are further included for qualitative synthesis. The result of the qualitative synthesis is shown in Table 2.

DISCUSSION

Search results and study characteristics

After passing the screening process to exclude the duplication, and abstract relevance, thirteen scientific articles were obtained for further qualitative analysis. The thirteen scientific articles were published between 1983 and 2019. The study type of thirteen scientific articles consists of five double-blind trials, one single-blind study, two cohort studies, one prospective study, and one experimental analytic research. Total 109,036 participants were included in this systematic review with varied research duration, from one day up to 10 years. The health condition of participants also varied but was limited to smoking habits, coffee drinking habits, and hypertension diagnoses history. Figure 3 showing the profile of 13 scientific articles included in this review.

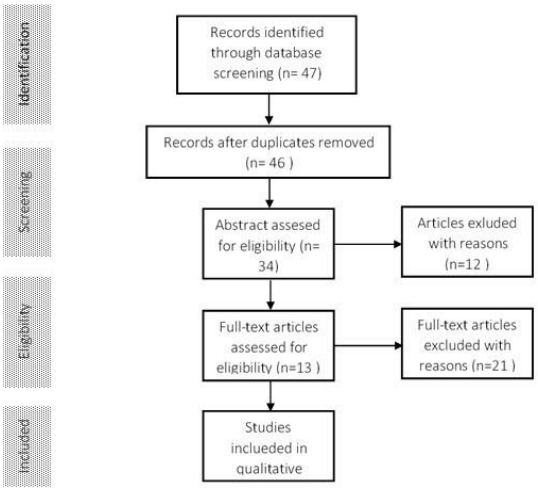

Figure 2. The results of articles based on boolean search from several databases

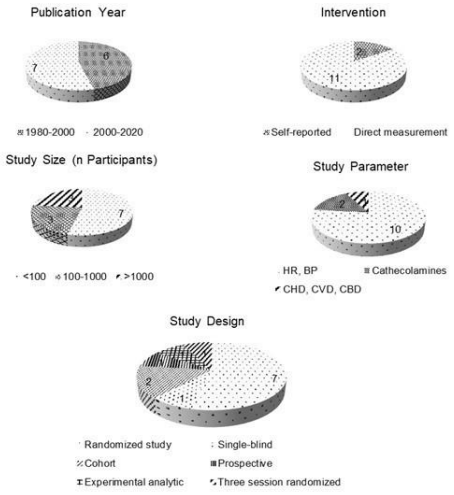

Figure 3. The profile of scientific articles included in qualitative analysis

Table 2. The results of data extraction from 13 studies included in the systematic review

Number	Year	Author	Subjects (n)	Intervention	Caffeine Content	Study type	Main Outcomes
1	1983	Ammon *et.al*	10 M	8 cups/day (1,8 g regular coffee, and 1,8 decaffeinated coffee)	Total caffeine 504 mg in 8 cups of regular coffee. The caffeine content of decaffeinated coffee was not mentioned	Double-blind crossover trial	Heavy coffee ingestion (eight cups/day = 504 mg) caffeine/day only slightly raises blood pressure. Groups who ingested decaffeinated coffee first, experience an increase in blood pressure.
2	1985	Smits *et.al*	12 (6 M, 6 F)	Two single-blind tests (one with regular coffee, one with decaffeinated coffee)	Total caffeine 280 mg in 2 cups of regular coffee. The caffeine content of decaffeinated coffee was not mentioned	Single-blind study	Rise in SBP (Systolic Blood Pressure) and DBP (Diastolic *Blood Pressure)* after caffeine-abstinence of only 17 hours

Number	Year	Author	Subjects (n)	Intervention	Caffeine Content	Study type	Main Outcomes
3	1988	Prakash *et.al*	9 (7 M, 2 F)	2 cups in fasting state	Total caffeine 175 mg in 2 cups of regular coffee, and 6 mg in 2 cups of decaffeinated coffee	Double-blind randomized study	Two cups of decaffeinated coffee had no adverse cardiovascular effect in the healthy subject
4	1989	Dusseldorp *et.al*	45 (22 M, 23 F)	5 cups/day of regular coffee for 6 weeks, and 5 cups/day of decaffeinated coffee for 6 weeks	Total caffeine in the regular coffee period was 445 mg, and 40 mg during the decaffeinated coffee period	Double-blind trial	Chronic consumption of 5 cups decaffeinated coffee/day in comparison with regular coffee causes a significant but small fall in ambulant blood pressure in men and women
5	1991	Grobbee *et.al*	69	4-6 140 mL cups/day of filtered decaffeinated coffee, and 75 mg pills/day of caffeine for 9 weeks	Total caffeine 75 mg/day for group 1	The double-blind, randomized trial	The abstinence from caffeine for 9 weeks trial does not affect blood serum or lipid serum

Number	Year	Author	Subjects (n)	Intervention	Caffeine Content	Study type	Main Outcomes
6	1994	Superko *et.al*	186 M	A cup/day coffee ingestion	Not specified	Randomized trial	Statistically no significant difference in blood pressure between three group
7	2005	Karatzis *et.al*	16 (8 M, 8 F)	A cup of warm instant coffee containing 80 mg caffeine, or a cup of decaffeinated coffee within 10 minutes, on 2 different days, a week apart	80 mg caffeine/cup for regular coffee. Caffeine coffee of decaffeinated coffee was not mentioned.	Randomized, double-blind, crossover	No change of aortic blood pressure was observed following consumption of decaffeinated coffee
8	2008	Greenberg *et.al*	1354	Subjects asked how many cups each day of regular coffee, decaffeinated coffee, regular tea, and decaffeinated tea intake	Not specified	Cohort study	Regular coffee consumption showed a significant negative association with CHD, but not CVD pf CBD. All decaffeinated beverages were not significantly associated with CVD, CHD, or CBD mortality

Number	Year	Author	Subjects (n)	Intervention	Caffeine Content	Study type	Main Outcomes
9	2012	Renda *et.al*	110 M	Administration of 40 mL of either decaffeinated coffee preparation plus 3 mg, or decaffeinated coffee alone	Not specified	The double-blind, randomized trial	Compared with decaffeinated coffee, regular coffee was associated with a mean significant increase in SBP
10	2013	Azuamah *et.al*	103	Administration of 1 cup coffee (3,6 g decaffeinated coffee in 200 mL of water)	Not specified	Experimental analytic research	The mean systolic pressure reduced after decaffeinated coffee ingestion
11	2015	Zimmermann-Viehoff *et.al*	77 (74% F)	Three laboratory session in randomized order (espresso, decaffeinated espresso, and warm water)	The caffeine content of regular espresso is 256,8 mg/cup. The caffeine content of decaffeinated espresso is 36 mg/cup	Randomized three-session crossover design	In habitual coffee consumers, the vagally mediated HRV was significantly lower after consumption of decaffeinated espresso compared to a regular espresso. Increases of SBP were only found in the non-habitual consumers

Number	Year	Author	Subjects (n)	Intervention	Caffeine Content	Study type	Main Outcomes
12	2016	Rhee *et.al*	93 676 person-years follow up	Self-reported questionnaires of type, amount, and total caffeine consumption	Not specified	Prospective study	Regular coffee, decaffeinated coffee, and caffeine are not risk factors for hypertension in menopausal women
13	2019	Navarro *et.al*	13 369	Validated semi-quantitative food frequency questionnaire	Not specified	Cohort study	Coffee consumption (either regular or decaffeinated) was not significantly associated with the risk of hypertension

The effect of regular and decaffeinated coffee on the risk of hypertension

Observational studies

A cohort study was conducted by Greenberg *et al.* (2008) to evaluate the relationship between regular coffee consumption, cardiovascular disease, and heart valve disease in the elderly. The study carried out 1,354 subject participants. The participants were taken from a previous study in 1948 by Framingham. The subject participants were all asked how many cups each day of regular coffee, decaffeinated coffee, regular tea, and decaffeinated tea intake during the 10.1 average years of follow up. Subjects with any missing data were excluded from the analysis and resulted in 1,354 subjects aged between 65.4 and 96.6 years. Beverage intake, either regular or decaffeinated coffee, was determined by Food-Frequency Question (FFQ). The subjects were asked how many cups of regular and decaffeinated coffee intake each day. Besides, the dosage of beverages intake was categorized as any and no cups each day. The findings suggested that the association between regular coffee consumption and decreased CHD (Coronary Heart Disease) mortality risk may have been related to the effect against the development of heart valve disease. In subjects with BP (Blood Pressure) less than the Stage 1 hypertension level and no heart valve disease diagnoses at baseline, consumption of any regular coffee was associated with a 71% decreased risk of CHD mortality and 56% decreased risk of valve disease. Therefore, generally, the study concluded that regular coffee consumption showed a significant negative association with CHD, but not CVD (Cardiovascular Disease) and CBD (Cerebrovascular Disease).

A prospective study by Rhee *et al.* (2016) was done in the United States with 93,676 person-years follow-up. This study aimed to investigate the relation between coffee and caffeine consumption and the risk of hypertension in postmenopausal women. The subject participants of this study were postmenopausal women aged 50-79 years who enrolled in the study at 40 clinical centers nationwide from September 1993 to December 1998. However, this study excluded women with hypertension at baseline (SBP or DBP ≥140 mmHg or ≥90 mmHg respectively), and women being treated for hypertension. The participants were able to select several categories for the dosage namely 1 cup/day, 2-3 cups/day, and ≥6 cups/day for regular or decaffeinated coffee. The data revealed that no clinically meaningful differences in baseline SBP (Systolic Blood Pressure) and DBP (Diastolic *Blood Pressure)* across regular coffee intake categories. This study also revealed that higher total caffeine intake was associated with a lower mean SBP. In general, this study also suggested that no consistent association was found between regular and decaffeinated coffee intake and the risk of hypertension.

Navarro *et al.* (2019) conducted a cohort study with 13,369 healthy subject participants. All of the participants were asked to fill the validated semi-quantitative Food Frequency Questionaire (FFQ) and allowed to choose coffee consumption categories by following options; never or seldom, <1 cup/day and, >2 cups/day. Also, the FFQ asked the participants included several factors such as socio-demographic factors (sex, age, marital and employment status, university degree), anthropometric variables (weight, height), health-related habits (smoking status, alcohol consumption, physical activity), and clinical variables (use of medication, CHD history of personal and family, cancer, and other diseases). Hazard analysis was conducted to obtain the relation between the three coffee drinking habits with those variables. The results revealed a significant interaction between regular coffee consumption and sex, where women who consumed at least 2 cups of regular coffee per day showed a Hazard Ratio of 0.74 compared to women who never or seldom drinking regular coffee. The researcher found that no significant association was found between regular coffee consumption with those with higher adherence to the Mediterranean diet and among men. The analysis also showed that consumption of decaffeinated coffee did not significantly associate with sex, age, smoking, or adherence Mediterranean diet, as well as the incidence of hypertension.

Experimental studies

Smits (1985) conducted a single-blind study with 12 subject participants, which consist of 6 males and 6 females. The subject participants included in the study were all used to consume coffee with daily intake ranged between 3 up to 10 cups/day. During the treatment period, 2 cups/day of regular or decaffeinated coffee were administered. Two cups of regular coffee were equal to 280 mg caffeine, while the total caffeine in decaffeinated coffee was not mentioned. All of the subjects underwent two single-blind tests, one with regular coffee, and one with decaffeinated coffee in a random sequence. The parameter related to the risk of hypertension used in this research consist of SBP, DBP, Heart Rate (HR), and Forearm Blood Flow (FBF).

In this research, the subjects were asked to abstain from caffeine for 17 hours and to smoke for 4 hours before start the treatment. The treatment started after 20 minutes rest period. After that, in the next 20 minutes, SBP, DBP, HR, and FBF were measured every 5 minutes. The results found that there are differences in the SBP, DBP, HR, and FBF before and after coffee ingestion. There was a significant rise of SBP and DBP after coffee ingestion of 4.4 ± 1.4% and 11.8 ± 2.5%. On the other hand, HR decreased within 8.4 ± 3.1%. After decaffeinated coffee ingestion, the DBP showed a rise of 4 ± 1.4 mmHg, but SBP and HR did not alter significantly. Besides, statistical analysis showed that the coffee induced rise of DBP was significantly higher than the increase of DBP after decaffeinated coffee ($P<0,05$). Meanwhile, the mean FBF remains unchanged in both tests. Catecholamines were also measured in this research and it shows that regular coffee induces a rise of plasma catecholamines significantly higher than the changes after decaffeinated coffee.

Ammon *et al.* (1983) conducted a double-blind cross-over study with ten male participants. The participants included in the research were aged between 20 and 30 years old, nonsmokers, and used to coffee. The participants in this study were divided into two main groups. The first group was the participants firstly treated with 8 cups/day of decaffeinated coffee during a half period of research (2 weeks), then the rest half period was treated with 8 cups/day of regular coffee. The second group was the participants firstly treated with 8 cups/day of regular coffee for a half period of research (2 weeks), then the rest half period was treated with 8 cups/day of decaffeinated coffee. Therefore, the total duration of this research is 5 weeks, which is divided into a week of the control period, and four weeks of the treatment period. The total caffeine content for a daily consumption is 504 mg for regular coffee. The total caffeine content for decaffeinated coffee is not mentioned.

Blood pressure, heart rate, and urinary catecholamines were measured as parameters in this study. This study shows that the ingestion of regular coffee led to an immediate change in mean blood pressure by 6 mmHg between control and treatment periods in the second group. Besides, it also shows the change in mean blood pressure by 4 mmHg between control and treatment periods in the first group. Besides, the significant differences between the first and second groups were observed only during the first 3 or 5 days after switching to regular coffee. The participants who digest decaffeinated coffee first followed by regular coffee showed a significant increase in blood pressure. Meanwhile, the participants who digest regular coffee first followed by decaffeinated coffee did not show a difference in blood pressure.

Other parameters measured in this article are heart rate and urinary catecholamines. The initial heart rate of the participants is 74 ± 3 beats/min for the first group and 65 ± 5 beats/min for the second group. These results are considered as not statistically different ($P > 0,1$). A significant increase in heart rate was observed in neither group. The heart rate increase was obtained after switching to regular coffee. These findings indicated that regular coffee consumption might increase the heart rate compared to decaffeinated coffee. Meanwhile, the urinary catecholamines level measured in this research was neither observed with significant difference in the first or second group.

Prakash and Kaushik (1988) conducted a double-blind randomized study to evaluate the acute effect of decaffeinated coffee on heart rate, blood

pressure and exercise performance in healthy subjects. This study included 9 subject participants, which consist of 7 males and 2 females. Five of the subject participants included in the study drank no coffee normally, while the remaining four drank 3-5 cups/day. Also, 6 of 9 participants were nonsmokers, two smoke a half pack a day, and one smoked one pack a day. There is no group specification in this study. Each of the study participants must drink two cups of coffee in a fasting state. The fasting state in this study refers to the resting time before the participants start the exercise. The caffeine content of regular coffee given to the participants was 175 mg, while two cups of decaffeinated coffee contain 6 mg of caffeine. After ingestion of the coffee, each of the participants exercised on a treadmill.

The parameter used in this study was blood pressure value which was measured with each increase in the workload. This measurement was conducted immediately after recovery, and at 1, 3, 5, 10, and 14 minutes after the conclusion of the exercise. The result of this study found that the heart rate/min before, after 30 minutes, and the peak of the exercise was different between regular and decaffeinated coffee treatment.

Before coffee ingestion, the mean heart rate/min value of regular coffee drinkers was slightly higher compared to decaffeinated coffee drinkers (74.7 ± 10.5 and 72.3 ± 7.2). Thirty minutes of exercise and after coffee ingestion, the mean heart rate/min value of regular coffee was higher compared to decaffeinated coffee drinkers 72.3 ± 14.5 and 68.8 ± 7.8).

At the peak of exercise, the mean heart rate/min value of regular coffee drinkers is also higher compared to decaffeinated coffee drinkers (174 ± 14.6 and 173 ± 15.5). The mean SBP value before coffee ingestion, the regular coffee drinkers showed a lower mean SBP value compared to decaffeinated coffee drinkers. However, after 30 minutes of coffee ingestion, the mean SBP value of regular coffee drinkers were higher compared to decaffeinated coffee drinkers, and the same for the mean SBP value at the peak of exercise. The mean DBP value of regular coffee drinkers at measurement before, after 30 minutes, and the peak of exercises were all lower compared to decaffeinated coffee drinkers. In conclusion, this study generally suggested that regular or decaffeinated coffee give a similar cardiovascular effect in healthy subjects even though there was no medical basis to claim the better option between regular or decaffeinated coffee.

Van Dusseldorp *et al.* (1989) published a double-blind study to evaluate the effect of decaffeinated and regular coffee on blood pressure. This study carried out 45 participants which consist of 22 males, and 23 females. All of the participants included in the study were aged between 17 and 45 years. The participants apparently healthy, and were not smoking; use any medication, on a prescribed diet, pregnant, and working at night shifts.

Each of the participants should be habitual coffee consumers of 4-6 cups/day. The 45 participants in this study were divided into two groups, namely Group 1 for the participants who were treated firstly with regular coffee, then followed by decaffeinated coffee, and Group 2 for the participants who were treated firstly with decaffeinated coffee followed by regular coffee. The amount of regular coffee consumption in this research was 5 cups/day for 6 weeks, and the same goes for decaffeinated coffee. The total caffeine consumed during the regular and decaffeinated coffee period was 445 mg and 40 mg consecutively.

The SBP (Systolic Blood Pressure), DBP (Diastolic Blood Pressure), and HR (heart rate) values were the parameters used in this study. Participants measured their ambulant blood pressure and heart rate at 7:30 and 10:00 AM and 1:00, 5:30, and 10:30 PM in a day per week by random order. The results showed that the SBP and DBP values of regular coffee drinkers were higher compared to decaffeinated coffee drinkers (SBP value 110,9 mmHg and 109,4 mmHg), (DBP value 69,6 mmHg and 68,6 mmHg). The result goes the same for the mean arterial value (83,4 mmHg and 82,2 mmHg). This study showed that 30 of 45 subjects had a lower SBP and DBP when consuming decaffeinated coffee compared to when consuming regular coffee. The researcher stated that there is

clear evidence that caffeine could elevate the blood pressure in subjects who had abstained from caffeine for at least 1 week (Dusseldorp *et al.*, 1989). Besides, caffeine users who abstained from caffeine for 12-24 hours could produce a significant increase in blood pressure and a decrease in heart rate (Dusseldorp *et al.*, 1989).

Grobbee (1991) conducted a 12 weeks double-blind randomized study to evaluate the relation between caffeine, blood pressure, and serum lipids. This study carried out 69 participants with unspecified gender and characteristic. These participants were divided into two groups named Group 1 for participants who were treated with filtered decaffeinated coffee and an equal number of placebo tablets containing 75 mg caffeine. Group 2 was placebo or control which was treated by decaffeinated coffee and an equal number of placebo tablets and did not contain caffeine. The amount of coffee that was given to the participants in Group 1 was 4-6 cups/day. The SBP, DBP, HR, and serum cholesterol values were the parameters used in this study. The results showed that the changes in SBP, DBP, and HR were not significantly different between the two groups. The SBP value net change between the regular and decaffeinated coffee groups was found higher in the sixth week (2.2 mmHg).

The effect of caffeine through the administration of decaffeinated coffee and caffeine tablets was assessed by comparing the change value from the 3, 6, and 9 weeks respectively. The total, HDL, and LDL serum cholesterol were not affected significantly by caffeine. The same goes for apolipoprotein A-1 and apolipoprotein B. The researcher stated that this result was in line with their previous findings in a randomized trial which focused on the brewing method of coffee given in the intervention. Meanwhile, the blood pressure (SBP and DBP) and heart rate value also found not significantly different during 9 weeks of intervention. In conclusion, caffeine has no adverse effect on cardiovascular observed from the change in serum lipids, blood pressure, and heart rate in this study.

Superko *et al.* (1994) conduct 8 weeks randomized trial to investigate the effects of cessation of regular coffee consumption on ambulatory and resting blood pressure in men. This study carried out 186 men in middle age, and they were nonsmoker, normotensive, and habitual regular coffee consumers. The subject participants were divided into three groups, which are for regular coffee (n=52), decaffeinated coffee (n=51), and no coffee (n=47). All groups were administered with a cup of coffee per day with unspecified caffeine content. The parameter of this study was the BP value in the morning after abstention from food, coffee, and vigorous physical activity.

The SBP, DBP, and HR values were measured in three periods, which are 900-1200, 1200-1500, and 1500-1800 hours. The statistical analysis among all 3 groups indicated a significant difference in SBP changes at 900 to 1200, 1200 to 1500, and 1500 to 1800 hours. Meanwhile, the significant differences in DBP were found from 1200 to 1500, and 1500 to 1800 hours. In comparison, two groups that switched to decaffeinated coffee or discontinue regular coffee showed significant decreases in SBP at 3 time periods; 900 to 1200, 1200 to 1500, and 1500 to 1800 hours. Besides, the DBP value showed significant decreases for the non coffee group from 1200 to 1500, and 1500 to 1800 hours period. Meanwhile, the DBP decrease in the decaffeinated coffee group was found from 1200 to 1500, and 1500 to 1800 hours period. In conclusion, based on statistical analysis, there was no significant difference in SBP, DBP, and heart rate between the three groups.

Karatzis *et al.* (2005) conducted a randomized, double-blind crossover study with 16 subject participants which consist of 8 males and 8 females. The participants included in this study should be healthy, normotensive, not obese, and did not suffer from diabetes, kidney, liver, or other metabolic disorder. The participants consumed a cup of warm instant coffee containing 80 mg caffeine, or a cup of decaffeinated coffee within 10 minutes on 2 different days for 1 week apart. For these periods, participants were not allowed to consume regular products such as tea, chocolate, and soft drinks for at least 12 hours before the study. Hemodynamic measurements which consist of peripheral blood pressure, central blood pressure, wave reflection, and arterial stiffness,

augmentation index, and arrival time were used as parameters in this study. The data showed that peripheral SBP did not change significantly after regular or decaffeinated coffee consumption, even though the baseline characteristics at the two sessions of the study did not differ significantly. The peripheral DBP significantly increased after regular administration (4 mmHg in different), while no change was observed after decaffeinated coffee consumption. Central SBP increased by almost 4 mmHg after regular coffee consumption, and it was not significantly altered following decaffeinated coffee consumption.

Renda *et al.* (2012) reported a 2 days double-blind randomized trial using 110 subject participants. All of the participants included in the study were moderate coffee drinkers in health conditions. The participants were divided into two groups, one for participants who received 40 mL decaffeinated coffee added with 3 mg/kg caffeine, and the control group who received only decaffeinated coffee. The BP and HR measurements were conducted within 6 minutes intervals for 2 hours after coffee administration. The study was run early in the afternoon within 2 consecutive days. The parameters used in this study were HR, BP, plasma caffeine, and plasma catecholamines value. The baseline HR, as well as SBP and DBP were similar before regular or decaffeinated coffee administration (SBP: 132,6 ± 12 compared to 131,6 ± 11 mmHg; DBP: 79,6 ± 10 mmHg compared with 78,6 ± 9 mmHg). The SBP and DBP values were both significantly higher only 18 minutes after regular coffee intake compared with decaffeinated coffee. This condition persisted until the end of the study. Most subjects who administered regular coffee experiencing an increase in BP (but some experiencing a decrease in BP) compared to subjects who administered decaffeinated coffee as a control. This result confirmed a wide intersubject variability in the BP response to coffee.

Azuamah and Esenwah (2013) evaluate the effect of decaffeinated coffee on the systolic blood pressure and pulse rate. This study designed as experimental analytic research with 103 subject participants aged between 15 and 30 years, and Body Mass Index (BMI) between 20 and 25. All of the subject participants were administrated with 1 cup of coffee (3,6 f decaffeinated coffee in 200 mL of water). The blood pressure was measured at 45 minutes before and after decaffeinated coffee administration. The mean SBP before the ingestion of decaffeinated coffee was 108,66 mmHg, which later this value decreased by 2,62% within 45 minutes after decaffeinated coffee ingestion to 105,81 mmHg. The mean pulse rate between before and after the ingestion of decaffeinated also showed the same results (75,28 to 73,07 bpm; reduction of 2,94%). From these data, the null hypothesis was rejected since statistical analysis showed a significant difference in the SBP before and after decaffeinated coffee ingestion. Also, this study concluded that the ingestion of decaffeinated coffee produced a reduction in SBP and pulse rate.

Meera *et al.* (2015) conducted a randomized three-session crossover design the study to evaluate the short-term effects of espresso coffee on heart rate variability and blood pressure in habitual and non-habitual coffee consumers. These 5 days study carried out 77 subject participants which consist of 38 habitual and 39 non-habitual coffee drinkers aged between 18 and 50 years. All of the subject participants took part in three laboratory sessions in randomized order (regular espresso, decaffeinated espresso, and warm water). The caffeine content of regular espresso was 256,8 mg/cup, while the caffeine content of decaffeinated espresso was 36 mg/cup. As a parameter, HR and BP value during the rest period before and after ingestion coffee was measured.

All subjects were to drank the respective beverages maximum of 10 minutes. After that, subjects were allowed to rest, and continue to drank other beverages 50 minutes later. The HR, SBP, and DBP were measured at 30 minutes after espresso ingestion.

The HR decrease was higher after regular espresso ingestion compared with decaffeinated espresso in habitual consumers. The same goes for HR decrease after regular espresso and decaffeinated espresso in non-habitual coffee consumers. The SBP decrease was found higher after decaffeinated espresso ingestion compared to regular espresso in habitual coffee consumers. The same result goes

for SBP decrease in non-habitual coffee consumers. The DBP decrease was found higher after decaffeinated coffee ingestion in habitual coffee consumers, and so on with the decrease of DBP in non-habitual coffee consumers. Meanwhile, the DBP increased higher after regular espresso compared to decaffeinated espresso in non-habitual coffee consumers. However, statistical analysis from a total of 77 participants revealed that no significant differences regarding any of the physiological variables including HR, SBP, and DBP before and after the consumption of regular espresso, decaffeinated espresso, and warm water. Besides, the study suggests that no evidence is found for specific short-term effects of regular espresso on vagal activity (the baseline activity of vagus nerve to maintain several body functions at rest, including heart rate, lungs, and digestion) in healthy subjects.

This review is aimed to observe the relation between caffeinated and decaffeinated coffee and the risk of hypertension. However, the research article reviewed in this study can have several limitations. The first one is that the observation of the acute effect of caffeine either in the form of coffee (regular or decaffeinated) or capsules may not reflect long-term effects since the tolerance of caffeine can change in every participant (Van Dam *et al.,* 2020). Second, although longer-term randomized trials are desirable, such studies are sometimes not feasible due to cost and practical consideration. However, such studies using self-report in measures the coffee consumption frequency are highly accurate and reproducible. Several parameters that lead to error or bias in the draw the conclusion including the variation in cup size, brew strength, type of coffee bean, and the number of other components added to the coffee (e.g sugar, creamer, milk) (Van Dam *et al.,* 2020).

Concerns regarding coffee and caffeine intake that may increase the risk of hypertension and cardiovascular disease have existed for years, but recently, the evidence health benefit of coffee also appeared. Coffee is known to contain hundreds of other biologically active phytochemicals despite caffeine, such as chlorogenic acid and ligands, alkaloid trigonelline, melanoidins (formed during roasting), and a modest amount of minerals such magnesium, potassium, and vitamin (Colombo and Papetti, 2020). These compounds may contribute to health improvement in humans, including the intestinal, cardiovascular, cognitive, and other physiological properties. Besides, most of the previously mentioned chemical compound of coffee has antioxidant properties and may reduce oxidative stress which leads to the various health problems. Contrarily, a biological compound such as diterpene cafestol which presents in unfiltered coffee could increase serum cholesterol. This suggests that the incidence of a health problem such as hypertension could be caused by other compounds or factors and not caffeine itself (Van Dam *et al.*, 2020).

CONCLUSION

Based on clinical studies in general or unspecified subjects, consumption of regular coffee tends to increase the DBP and SBP more compared to decaffeinated coffee. The DBP and SBP increased after 18, 20 and 30 minutes after coffee consumption.

Meanwhile, consumption of decaffeinated coffee could reduce the SBP from the initial value. Besides, it was found that regular coffee induces a rise of plasma catecholamines significantly higher than the changes after decaffeinated coffee. In the non-smoker subjects, regular coffee might increase the heart rate compared to decaffeinated coffee. Besides, the subjects tend to have a lower SBP and DBP when consuming decaffeinated coffee compared to when consuming regular coffee.

Meanwhile, the catecholamines were found not significantly different in both type coffee consumption. However, there is study which conclude that regular coffee increase catecholamines. Based on coffee drinking habits, it was found that regular coffee intake might increase the heart rate compared to decaffeinated coffee while decaffeinated coffee could to significant decreases in SBP compared to regular coffee in habitual coffee drinkers. In the moderate coffee drinkers, regular coffee leads to both an increase and a decrease in BP from the initial value compared to decaffeinated coffee. In the non-habitual coffee drinkers, the heart rate was found

decreased and DBP was found increased from the baseline after regular coffee consumption compared to decaffeinated coffee.

In addition, on subjects with high activity (exercise), it was found that regular or decaffeinated coffee gives a similar cardiovascular effect in healthy subjects compared to placebo.

From several cohort studies with a different population (two stages of hypertension, postmenopausal women, and Mediterranean subjects) that are reviewed in this study, it was found that consumption of regular coffee is negatively associated with the incidence of hypertension in the subject with lower blood pressure (less than < 160 and DBP <100 mmHg). In the postmenopausal subjects, no consistent association was found between regular and decaffeinated coffee intake and the risk of hypertension. In a more specific cohort, which is the Mediterranean cohort, it was found that neither regular nor decaffeinated coffee consumption was significantly associated with the incidence of hypertension.

A systematic review regarding the effect of regular and decaffeinated coffee could be improved by integrating more factors such as the relationship between decaffeination technology, amount of caffeine, and other hypertension markers despite blood pressure, heart rate, and catecholamines. Several parameters of hypertension that can be used for further research include C-reactive protein, fibrinogen, plasminogen activator inhibitor-1, aldosterone, renin, b-type natriuretic peptide, and N-terminal pro atrial natriuretic peptide, homocysteine, and urinary albumin/creatinine ratio to evaluate the effect of regular or decaffeinated coffee.

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ANTIOXIDANT ACTIVITIES OF LEMONGRASS WITH SOLVENT MULTI-STEP EXTRACTION MICROWAVE-ASSISTED EXTRACTION AS NATURAL FOOD PRESERVATIVE

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ABSTRACT

The use of synthetic preservatives is considered to have an adverse effect (carcinogenic) upon prolong consumption. Lemongrass *(Cymbopogon citratus)* is a plant that has bioactive components to act as antioxidants and potential to use as a natural food preservative. Bioactive components can be non-polar, semi-polar and polar; therefore, to determine the dominant bioactive components, a solvent multi-step extraction carried out. This study aimed to determine the specific bioactive components of lemongrass (antioxidant activity, total phenolic content and total flavonoids) suitable of polarity in the leaves and stem of lemongrass extract obtained from solvent multi-step extraction with Microwave-Assisted Extraction. The solvent used is ethanol (polar), ethyl acetate (semi-polar) and n-hexane (non-polar). The result showed that the highest bioactive components obtained from the polar stem lemongrass with total phenolic content of 19.31 mg GAE/g, flavonoids of 3.31 mg GAE/g. This result related to antioxidant activity of the extract of 79.96 %. The high antioxidant activity showed that lemongrass has potential to be used as a natural food preservative, especially in high fat food products.

Keywords: *Antioxidants, lemongrass, multi-step extraction, natural food preservative*

ABSTRAK

Penggunaan pengawet sintesis dinilai dapat memberikan efek buruk (karsinogenik) jika dikosumsi dalam jangka panjang. Serai dapur (*Cymbopogon citratus*) merupakan tanaman yang memiliki komponen bioaktif yang berperan sebagai antioksidan berpotensi digunakan sebagai pengawet alami pangan. Komponen bioaktif dapat bersifat non polar, semi-polar dan polar sehingga untuk mengetahui komponen bioaktif dominan pada serai dapur dilakukan ekstraksi bertingkat. Penelitian ini bertujuan untuk mengetahui komponen bioaktif spesifik serai dapur (aktivitas antioksidan, total fenol dan total flavonoid) sesuai tingkat kepolarannya pada bagian tanaman daun dan batang serai dapur hasil ekstraksi pelarut bertingkat dengan metode Microwave-Assisted Extraction (MAE). Pelarut yang digunakan pada penelitian ini adalah polar (etanol), semi polar (etil asetat), dan non polar (n-hexana). Metode penelitian antara lain: 1) ekstraksi pelarut bertingkat; 2) pengujian meliputi penangkapan radikal radikal 2,2-diphenyl-1-picrylhydrazyl (DPPH), pengukuran total fenolik (Follin Ciocalteu) dan total flavonoid. Hasil penelitian menunjukan bahwa komponen bioaktif tertinggi pada bagian batang serai dapur bersifat polar meliputi total fenolik (19,31 mg GAE/g), flavonoid (3,31 mg GAE/g) hasil ini berbanding lurus dengan kapasitas penangkapan radikal (DPPH) sebesar (79,96%). Tingginya antioksidan pada serai dapur membuktikan bahwa serai dapur potesial digunakan sebagai pengawet alami pangan terutama pada produk tinggi lemak.

Kata kunci: *Antioksidan, ekstraksi pelarut bertingkat, pengawet alami pangan, serai dapur*

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INTRODUCTION

Antioxidants is important in preventing changes or damage in foods containing lipids (Naufalin, 2019). Synthetic antioxidants such as Butylated Hydroxytoluen (BHT), Tertbutylhydroxyquinone (TBHQ) and Butylated Hydroxyanisole (BHA), is commonly used in compliance to regulations (Sukardi, 2001). However, the use of synthetic preservatives was suggested to possess risk to the body if consumed for prolonged time. According to Namiki (1990), BHT utilisation is limited due to the carcinogenic properties. Research conducted by Aprilia *et al.* (2018) showed that the use of BHT can significantly damage the kidneys in Wistar rats. The finding has led into efforts in the uses of natural food preservatives in a yearning concern for healthier ingredient.

Several common compounds from natural ingredients possessing antioxidant activities are vitamin C, vitamin E, carotenoids, phenolic compounds, and polyphenols. There are about eight thousand plants that contain phenolic compounds and half of them are in the class of flavonoids. Phenolic compounds are included in the largest phytochemical group in plants that have antioxidant activity (Sulaeman *et al.*, 2011). The flavonoids that have antioxidant activity include flavones, flavonols, isoflavones, catechins, flavonols, and chalcones. This is because the -OH group and the double bond (> C = C) are owned by these compounds.

Cholifah *et al* (2017) observed that addition of garlic and pandan leaves was able to increase the shelf life of tofu products, compared to those without. The research suggested the flavonoid content in garlic and pandan leaves work as antioxidant that suppress oxidation reactions and also as antibacterial agent. The components of thymol, carvarol, zingerone, gingerol, hydroxytyrosol can inhibit peroxidation in capturing peroxyl radicals. Another study by Susilowati and Harningsih (2015) showed that flavonols in shallots act as a strong antioxidant and was able to inhibit the process of rancidity of coconut oil. Ginger extract in soybean oil and sunflower oil has also shown protection against auto-oxidation in oil (Sukandi, 2001).

The use of antioxidants as natural preservatives is still minimal, especially in the food industry. Despite of the abundant availability, they are considered to have low effectiveness and practicality, unstable in processing conditions and, quite importantly, affect sensory properties in food products. Appropriate method is required to produce effective and efficient natural preservatives for several utilization in food, such as edible coating on fresh meat or high-fat products, as well as BHA, BHQ substitute.

Indonesia, that was long known for its wealthiness of biodiversity, is also known as a herb and spices producer, including *Cymbopogan citratus*. *C. citratus* not only enrich the taste of food, but may also act as antioxidants and antimicrobials; putting the herb into potential ingredient to be utilised as a natural preservative in food. Lemongrass or *C. citratus* is easy to find because it is easy to cultivate on various types of soil. Research by Hamza *et al.* (2009) stated that lemongrass extract contains antioxidants in the form of phytochemical compounds, including; saponins, tannins, alkaloids, flavonoids. Lemongrass leaves has bioactive compounds, such as; phenols, flavonoid, tannins, and compounds which has many sulfide and alkaloid groups. Kanopa *et al*. (2012) states that the presence of these compounds is potential as antioxidants. Furthermore, Pujawati *et al.* (2009) stated that the simplicia extract of lemongrass inhibit the growth of *Candida albicans* at concentration of 0.4 %. Lemongrass essential oil inhibit the growth of *Eshericia coli*, *Staphylococcus aureus* (Apriliani *et al.*, 2014) and inhibit the growth of caterpillars on cabbage leaves (*Plutella xylostella*) at a concentration of 1.5 % (Prasetyo *et al.*, 2013). The antioxidant activities that are complemented with antimicrobial activities supported further exploration of lemongrass as potential natural preservative.

Extraction is a common method in acquiring bioactive components (Erminawati *et al.*, 2019). Multi-step extraction is an extraction that can carried out in stages to produce certain compounds that specifically extracted in each solvent used by two or more solvents. The multi-step extraction can be carried out starting by dissolving the material in the solvent with the lowest polarity level, namely

non-polar, then semi-polar and finally the polar solvent. Various bioactive components in lemongrass might have different level of polarities (non-polar, semi-polar or polar), and therefore multi-step extraction could be carried out to determine the dominant and specific bioactive components according to the polarity level.

Solvent is an important consideration in extraction, as it highly correlates to the affinity of the compound targeted, polarity and polar groups. A material will dissolve in a solvent of the same polarity (Sudarmadji *et al.*, 1989; Septiana and Asnani, 2012). N-hexane is a solvent that can extract semi-polar components such as fat and oil; which is volatile, with boiling point between 65-70 ℃ (Susanti, 2012). Its volatile properties, is practical in preventing n-hexane to come with the extract. One of the semi-polar solvents that are often used in the extraction of essential oils is ethyl acetate, the boiling point is almost the same as n-hexane, which is 77 ℃. Ethanol solvent is a relatively harmless polar solvent with high absorption of electromagnetic energy (Yulianti, 2014). Research conducted by Aliyudin (2019) stated that 70% ethanol with ingredients to solvents ratio of 1:10 w/v, produced the highest total phenolic content and antioxidants in lemongrass stem concentrate.

The principle of multi-step extraction is to dissolve materials in several solvents. One of the methods that can optimally produce bioactive components according to polarity is the microwave-assisted extraction (MAE) method. The study of Romadanu et al., (2018), used multi step extraction of non-polar semi-polar and polar solvents, which is specific bioactive components of the lotus flower; obtained that the polar solvent (methanol) produced higher antioxidants compare to other solvents. Harianingsih (2018), states that MAE method application resulted in higher yield of citronellal bioactive component, compared to other extraction methods observed in the study. MAE advantages come from its short extraction time, less solvent and electrical energy requirement. In MAE, temperature control can be easily done and therefore may benefit extraction of thermos-labile materials or compounds.

Based on the above background; there is currently no research that combines these two methods a multi-step extraction research carried out using the microwave-assisted extraction of lemongrass to identify specific bioactive components, namely; total flavonoids, total phenolic content and antioxidant activity according to the polarity level in order to increase the functional and economic value of lemongrass.

MATERIALS AND METHOD

Materials

Materials used in this research are; stems and leaves of lemongrass obtained from Sumbang, Banyumas, Central Java (with specification: fresh, light green to dark green color, age 8-10 months), aquades, Folin-Ciocalteu, Sodium carbonate (Na_2CO_3) (2 %), HCL, Methanol (PA), DPPH, Ethanol (90 %), N-Hexane, Ethanol (70%), Ethyl Acetate, $AlCl_3$, Potassium acetate (CH_3COOK).

Method

Sample preparation

The lemongrass plant is cut as follows; 15 cm from the rot considered as the stem and the rest considered as the leaves. Lemongrass leaves and stems sorted, washed using clean running water. Furthermore, reduced the size to ± 0.5 cm. Then arranged on a stainless steel pan and dried using a cabinet dryer at 55 °C. The dry lemongrass blended for ± 2-5 min. to powder.

Multi-step extraction using the Microwave-Assisted Extraction (MAE)

In this research, the type of extraction carried out is a multi-step extraction. The process of making lemongrass extract based on modified in previous studies (Erminawati *et al.*, 2019). The Lemongrass (150 g) is put into 500 mL erlenmeyer then dissolved using a multi-step starting with non-polar n-hexane (1: 6 w/v), the sample is extracted using a microwave at 225 watts for 5 min. The filtrate is filtered using filter paper, evaporated to remove the solvent using a vacuum rotary evaporator at 70 °C to form lemongrass filtrate. Meanwhile, the pulp

extracted using semi-polar solvent ethyl acetate (1: 4 w/v) using microwave. The same operation the polar ethanol (70 %) solvent (1:10 w/v). A summary of the multi-step extraction showed in Figure 1.

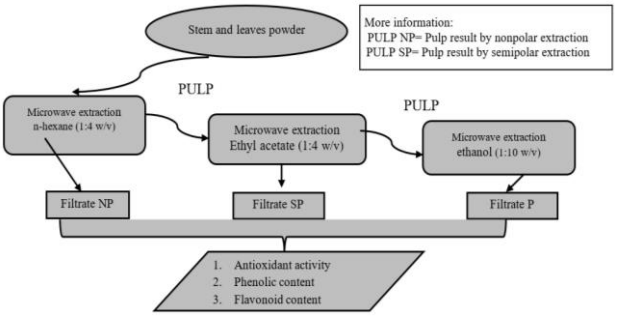

Figure 1. Summary of the multi-step extraction lemongrass

Antioxidant activity (Gadow *et al.*, 1997)

The total antioxidant activity determined based on the difference absorbance of the solution. A 0.0063 g DPPH powder diluted in 100 ml of pure methanol. The samples were made in a 20.000 ppm dilution series. A total of 2 ml extract solution mixed with 2 ml of 0.16 mM DPPH solution, then vortex for 1 min, left for 30 min, measured the absorbance using a UV-VIS spectrophotometer at 517 nm. Calculate the corrected absorbance using the formula:

$$\% \text{ inhibition} = \frac{Ao - As}{Ao} \times 100\%$$

Description: A_0: blank absorbance, As : sample absorbance

Total phenol content (Chew *et al.*, 2009 modified)

Natrium carbonate (Na_2CO_3) (2 g) diluted in 100 ml of distilled water. Dilute 0.1 ml of Follin by adding 0.1 ml of distilled water (1:1 v/v). The sample diluted 100 x with ethanol. Add 2 ml Na_2CO_3 to 0.1 ml sample solution, then let stand for 2 min, then add 0.1 ml Follin reagent. Vortex for 1 min, incubated for 30 min. at room temperature and dark room. Measure the absorbance using a UV-VIS spectrophotometer at 765 nm. Gallic acid used as standard.

Total flavonoid content (Ipandi *et al.*, 2016).

A total of 1 ml sample was reacted with 0.1 ml $AlCl_3$ 2%, vortex for 1 min. Add 1 ml of potassium acetate (CH_3COOK) (1.76 %); Votex again for 1 min, then incubated for 60 min in the dark. Measure the absorbance using a UV-VIS spectrophotometer at 435 nm.

Data analysis

Parametric variable data analyzed using variance test or ANOVA (Analysis of Variance) at the 5% level and if it shows a real effect on the treatment then followed by the DMRT (Duncan's Multiple Range Test).

RESULTS AND DISCUSSION

Total phenolic content

Total phenolic analysis determines the total phenolic content in the extract from the standard curve equation for gallic acid. The compounds were determined spectrophotometrically using the method of Chew *et al.* (2009) with Follin-Ciocalteu reagent. This reagent oxidizes the phenolics (alkaline salts) and reduces to a molybdenum-tungsten complex reaction. Where phenolics are found in alkaline solutions, while the Follin-Ciocalteu reagent and its products are unstable in alkaline conditions to form a blue complex so that it detected by a spectrophotometer. The resulting color is highly dependent on the hydroxyl group and the location of these groups in the molecular structure. The increase in color indicates in the concentration of phenolic compounds.

The total phenolic content is calculated by entering the absorbance value data sample into the linear regression line equation y = ax + b, which is obtained from the gallic acid calibration curve. The

results of the total phenolic content in GAE (gallic acid equivalent) units, mg of extract concentration per gram of sample (mg GAE/g sample) (Lestari *et al.*, 2018). The results of phenol analysis presented in Figure 2.

Solvent polarity determined by the dielectric constant, along with the dielectric constant of hexane, ethyl acetate, ethanol, methanol and water, were 1.89; 6.02; 24.30; 33.60; and 80.40, respectively. The greater dielectric constant the more polar the solvent (Sudarmadji *et al.*, 1989). As shown in Figure 3, higher total phenolic content was observed in the lemongrass extracted with polar solvent (ethanol), with three highest content observed in the lemongrass stem extracted using ethanol (19.13 mg GAE/g), followed by lemongrass leaves extracted using ethanol (15.80 mg GAE/g) and then lemongrass stem extracted using ethyl acetate (14.74 mg GAE/g). This data indicates that the phenolic components found in lemongrass distributed in the polar to semi-polar range. The highest total phenolic in stem with ethanol is thought to be due to the phenol structure found in lemongrass stem which more polar (containing many hydroxyl groups) than semi-polar leaves so that they will dissolve more easily if dissolved by a polar solvent with a polarity degree of 33.60 (Sanga and Katja, 2011). Ethanol considered very appropriate for extracting materials that contain polar components.

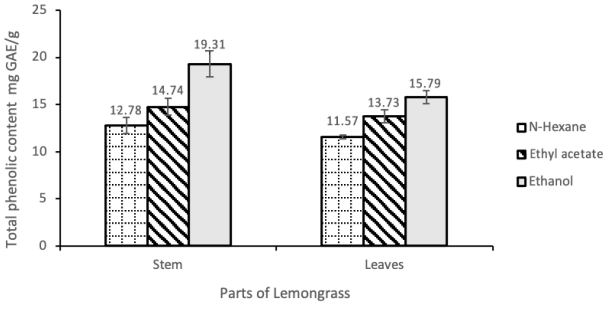

Figure 2. Total phenolic content of lemongrass stem and leaves

Previous research, conducted by Aliyudin (2019), found that phenol content of lemongrass stem with ethanol at the same concentration and extraction method was 12.07 mg GAE / g, smaller than the results of this study. This proves that multi-step extraction can maximize extract bioactive components such as total phenol by using the right solvent, ethanol. In conjunction, Septiana and Asnani (2012) reported that the polyphenol content obtained in multi-step extraction was higher than single extraction. In accordance with the purpose of multi-step combine with microwave assisted obtain a more specific or pure component. Extraction process employing microwave energy, which was absorbed by the compound within the cell. The absorbed energy increased the cell temperature and triggered pressure from within, allowing cell lysis and extraction process due to damage of the cell structure. Research by Kusnady et al. (2017) showed that the optimal time of microwave extraction to produce the total phenolic was five minutes, as yield for the phenolic content decrease after 5 minutes.

Phenolic compounds have a structural diversity ranging from simple phenols to complexes components. The potential of phenolic compounds as antioxidants caused by the presence of hydroxyl groups in phenol compounds. The hydroxyl group can function as a hydrogen atom contributor when it reacts with radical compounds via electrons, which can prevent the oxidation process. Phenolic compounds can react with peroxyl radicals to end the chain reaction (Wright *et al.*, 2001; Budhiyanti, 2013). Phenolic antioxidant activity depends on the number and position of the hydroxyl group in relation to the carboxyl functional group in the aromatic ring. Andiana, et al. (2019) found that encapsulated extract of rice husks has a total phenolic content of 3,125 mg GAE/g that may act as antioxidant, and also shown to inhibit the activity of gram-negative and gram-positive bacteria in tofu, therefore had the potential to be utilized a natural food preservative.

Not only act in food preservation of easily oxidized food , phenolic compounds also play role in health maintenance and disease prevention. Alshikh *et al.* (2015) reported that the phenolic compounds present in nuts not only contribute to organoleptic

properties but also benefit health. (Yusmiati *et al.*, 2012) reported that red tea extract or green tea at the given doses (150 mg and 300 mg) can reduce the risk of atherosclerosis in mice on an atherogenic diet as evidenced by a significant reduction in the number of foam cells. Antioxidant activity from the polyphenol compounds of red and green teas reduced the oxidation stresses, especially the lipoprotein components and reducing the reactivity of lipid peroxides which have behaved as free radicals. The antioxidant action was associated with the existence of double bonds and hydroxyl groups in these polyphenol compounds that play role as oxidants and free radicals scavenger (Yusmiati *et al.*, 2012). The oxidation stress is commonly associated with atherosclerosis and following coronary heart disease, a blockage of the arteries due to the build-up of cholesterol plaque which blocks blood flow to the body's organs.

Total flavonoids

Determination of flavonoid levels was using Chang (2002); this method compared with the quercetin standard at maximum wavelength at 435 nm. During the test, the flavonoids in the sample will react with $AlCl_3$ and form a yellow complex. Flavonoids are one of the bioactive components that almost found in all plants, based on Figure 3. The flavonoid framework consists of one aromatic ring A, one aromatic ring B, and a heterocyclic middle ring containing oxygen and this ring oxidized form is used as the basis for dividing flavonoids into its sub-groups (Dewi, 2018). Flavonoids are classified based on the addition of the oxygen chain and distribution of the hydroxyl group between flavonols, isoflavones, flavanones, flavanonols and khalcones (Marby *et al*, 1970; Susilowati and Estingingrum, 2016). Flavonoids may act as natural antioxidants (Shahidi and Naczk, 1995) due to the presence of phenolic hydroxyl groups in their molecular structure. The total flavonoids in lemongrass, stem and leaves in this study is as presented in Figure 3.

The highest total flavonoids obtained in the ethanolic extract of lemongrass stem (3.31 mg GAE/g); followed by ethanolic extract of lemongrass leaves (2.53 mg GAE / g; and semi-polar solvent stem (2.38 mg GAE / g). The high content of lemongrass stem flavonoids is suggested to be caused by the presence of parenchyma cells which contain high number of bioactive components, including flavonoids.

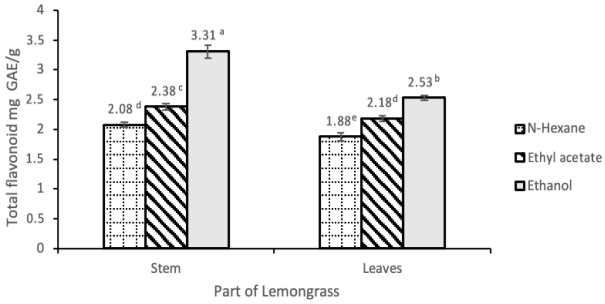

Figure 3. Flavonoid content of lemongrass stem and leaves

Flavonoid have different levels of polarity depending on their structure in plants, but it is known that flavonoid compounds are generally semi polar to polar. The structure of flavonoids, which are more dominant in polar, as seen in lemongrass leaves and stems, which is thought to affect the resulting flavonoids. The higher total flavonoid content in the polar solvent influenced by the multi-step extraction process, in which the extraction can extract specific bioactive components according to their solubility level. N-hexane is a non-polar solvent, that will extract non-polar compounds, suitable to extract lemongrass, because it is insoluble in water, and capable to dissolve nonpolar compounds at low boiling points (Gomarjoyo *et al.*, 2015). Likewise, ethyl acetate as semi-polar solvent extracts semi-polar compounds. Ethanol 70 % can easily extract polar compounds without any interference from being extracted from other group compounds, resulted highest yield of flavonoid content in both lemongrass stem and leaves.

Arbaayah and Kalsom (2013) reported the higher yield of flavonoid content in extracts using ethanol or distilled water as solvent. Solvent penetrates the plant cell wall, entering cell cavity containing

flavonoid. Efficiency of the extraction may differ due to flavonoid content, plant pat, nature/type of flavonoids especially its polarity and therefore its affinity to polar or less polar solvents. Similarity of flavonoid characters with polar and semi-polar solvents enhance the extraction of flavonoid content in lemongrass.

Flavonoids are phenolic compounds that are widely isolated from plants because of their antioxidant, anti-microbial and anticancer benefits. Flavonoids contribute to its antioxidant activity in vitro by binding (chelating) metal ions such as Fe and Cu. Metal ions such as Cu and Fe, can catalyze reactions that eventually produce free radicals (Mira *et al.*, 2002; Muchtadi, 2012). As antioxidants, flavonoid not only can prevent oxidation but also possess an anticancer activity. Several prenylated flavonoids that have been isolated from plants show cytotoxicity activity against several cancer cells, such as artelastin, artelastochromene, artelasticin in MCF-7 cells (breast cancer), TK-10 (kidney cancer), UACC-62 (melanoma cancer); artelastoxanthone and artonol A in A549 (lung cancer), Hep3B (liver cancer), HT-29 (colon cancer), MCF-7 (breast cancer) (HH *et al.*, 2005). According to previous studies by Itoigawa *et al.*, 2002, prenylated flavonoids may induce cytotoxicity in some cancer cells.

The first mechanism of flavonoids as anticancer, is through activation of the apoptotic pathway of cancer cells correlation to the DNA fragmentation. This fragmentation begins with the release of the proximal DNA chain by reactive oxygen compounds such as hydroxyl radicals. This compound formed from the redox reaction of Cu (II). These copper compounds are mobilized by flavonoids; both from extra cells and intra-cell, especially from chromatin. Secondly, flavonoids as inhibitors of tumor / cancer proliferation, one of which by inhibiting protein kinase activity, so it blocks the signal transduction pathway from the cell membrane to the cell nucleus. Thirdly, by inhibiting the activity of tyrosine kinase receptor, to increase the activity of tyrosine kinase receptors in the growth of malignancy.

Antioxidant activity (DPPH)

DPPH Method to determine the ability of antioxidants to inhibit free radicals by donating hydrogen atoms. Compounds that are active as antioxidants reduce DPPH free radicals (2,2-diphenyl-1-picrylhydrazine) to diphenyl picryl hydrazine resulting in a color change in the DPPH solution in methanol from dark purple to yellow (Novianti, 2012; Romadanu *et al*, 2014) The antioxidant activity of lemongrass is expressed in the inhibition percentage. The results of the analysis of various treatments had a significant effect of DPPH radical scavenging. The value of DPPH radical scavenging capacity of lemongrass at 2000 ppm inhibition can be seen in Figure 4.

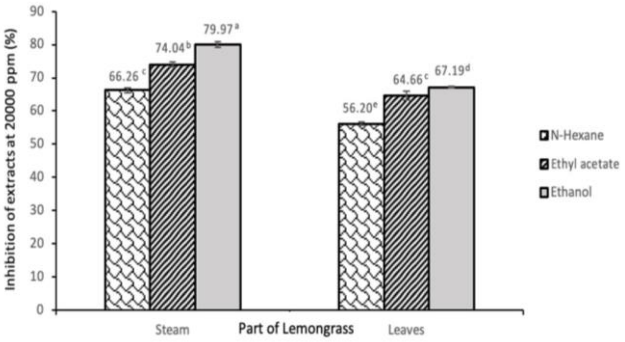

Figure 4. DPPH radical scavenging capacity of lemongrass stem and leaves extracts at 20000 ppm

As shown in Figure 4, the highest antioxidant activity found in the lemongrass stem using ethanol (79.967%). This result higher compare to the previous research by Aliyudin (2019) with the same concentration and solvent, which of (73.1%).

In conjunction to study of Suratmo (2009), the lower antioxidant activity of lemongrass stems and leaves using n-hexane solvent is suggested to be the impact of non-polar component with relatively lower strength of antioxidant activity. Suratmo (2009) observed that the hexane extract fraction, containing only nonpolar compounds, for example essential oils, fats and oils, did not show antioxidant activity. Multi-step extraction is therefore suggested to have more specific

performance in extraction according to the polarity degree of the compound. The multi-step extraction is effective in obtaining bioactive compound as the extraction was initiated using non-polar solvents to extract non-polar compounds such as fats and oils, and then followed by ethyl acetate and ethanol to extract semi-polar and polar compounds such as flavonoids, phenolic tannins.

Differences in the distribution of compounds in lemongrass stems, compared to leaves, affect the capacity for free radical scavenging. The content of bioactive components in lemongrass is different for each part, the stem has higher of type flavonoid are geranial, neral, and myrcene compounds than the lemongrass leaves. There were α-citral (geranial) and β-citral (neral) components which act as antioxidants and free radical scavengers (Mirghani *et al.,* 2012). The more hydroxyl groups, the greater is it antioxidant activity of a material because it will donate more hydrogen to radicals (Gazali *et al.*, 2019).

Research conducted by Mongkolsilp *et al.* (2004) about five medicinal plants from Thailand indicated that higher total phenolic content is associated with higher free radical scavenging activity; this is consistent with this study. One of the antioxidants found in lemongrass is phenolic. According to Winarsi (2007) phenolic compounds in the form of tannins, flavonoids are antioxidants that can quickly donate one hydrogen atom to DPPH radicals and produce derivatives in the form of antioxidant radicals; which are more stable because of the resonance in the benzene ring.

Beside flavonoids, phenolic components such as tannins are known to have antioxidant activity (Amarowicz, 2007; Saxena, *et al.,* 2013). Al Jaber *et al.* (2011) stated that the phenolic components (flavonoids and tannins), alkaloids, terpenoids, and organic sulfur components may act as natural antioxidants. Shah *et al.* (2011), identified several compound in *Cymbopogon citratus* including terpenes, alcohols, ketones, aldehyde and esters. Asaolu *et al.* (2009), analysed the phytochemical constituents of lemongrass leaves. The major phytoconstituents were essential oils (that contain α-citral, β-citral, nerol, geraniol, citronellal, terpinolene, geranyl acetate, myrecene and terpinol methylheptenone), flavonoids and phenolic compounds, which consist of luteolin, isoorientin 2'-O-rhamnoside, quercetin, kaempferol and apiginin.

Lemongrass high antioxidant activity not only inhibit oxidation reactions in food but also suggest potential health benefits. A study conducted by Oboh *et al.* (2010) in Nigeria showed that *C. citratus* contribution to the its health promotion potential as antioxidant actity. In that study, *C. citratus* that was extracted by hot water had significantly higher DPPH radical scavenging ability, Fe^{2+} chelating ability and OH* scavenging ability than those with cold water. Other research on animal studies was conducted by Gayathri *et al.* (2010), in which lemongrass decreased the toxic of lipid perroxidation (TBARS) on groups IV in both serum and heart tissue compatible with Vitamin E tested on male wistar albino into five different groups is group I and II rats were treated with vehicle. Groups III and IV rats treated with 100 and 200 mg/kg body weight. Group V with 100 mg/kg body weight of vitamin E. on 58 could replace it partially. Another study of lemongrass in human subject by Ray. (2010) using supplemented 250 ml of lemongrass decoction to 31 hypertensive individuals for the period of 16 weeks, showed that lemongrass decoction with a dose 2 times a day gave significant impact on the improvement of arterial pressure.

CONCLUSION

The multi-step extraction combined with the Microwave-Assisted Extraction (MAE) method starts with the use of a nonpolar solvent (n-hexane), following with a semi-polar solvent (ethyl acetate) and a polar solvent (ethanol) to produce specific bioactive components including total flavonoids and total phenolic; with high antioxidant activity. This method is more effective applied than the single extraction. The bioactive components in the specific part of the lemongrass *(Cymbopogon citratus)* stem are polar; extracted with polar solvents (ethanol); total phenolic content of 19.31 mg GAE/g, flavonoids of 3.31 mg GAE/g and antioxidant activity of 79.96%; which are higher than that of lemongrass leaves, and also higher than that of other solvents (ethyl acetate and n-hexane).

The high content of antioxidants proves that lemongrass has the potential to be used as a natural food antioxidant agent, especially in food products that contain high unsaturated fats.

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Code of Ethics

For Authors

Main Concern:

- Originality and plagiarism
- Authorship of the paper
- Data access and retention
- One journal submission
- Conflict of interest
- Timeliness

Plagiarism

When an author deliberately uses another's work without acknowledgment, credit, or permission. Plagiarism has many different forms, from literal copying to paraphrasing someone else's work or your work and can include:

- Data
- Words and Phrases
- Ideas and Concepts

Authorship

Authorship should be limited to those who have made a significant contribution to the conception, design, execution, or interpretation of the reported study. You must obtain their agreement beforehand.

Data Access and Retention

Authors may be asked to provide the raw data in connection with a paper for editorial review, and should be prepared to provide public access to such data. Authors are responsible for their data and the analysis.

One Journal Submission

Submitting your manuscript to one journal only at a time. Avoid to submit the same manuscript to various journals.

Conflict of Interest

All submissions must include disclosure of all relationships that could be viewed as presenting a potential conflict of interest.

Timeliness

Probably, there will be several revisions in order to meet our journal standard. Be prompt to deal with it. Contact the editorial team if you require more time.

How to Avoid Plagiarism

1. *Use your own ideas*

Write your own work with your own idea.

2. *Cite the sources*

Always acknowledge the sources. That is why you need a **good citation** and **reference system**.

3. *Rewrite someone ideas in your own words*

Effective **paraphrasing** can help you prevent plagiarism! **Remember to CITE!**

4. *Use notes*

Record all details about the source and **distinguish** carefully between any idea from your reading and your own ideas.

Journal of Functional Food and Nutraceutical

User Account Registration Guideline

REGISTRATION

To make a submission to Journal of Functional Food and Nutraceuticals, you need to **register** a user account and log in. After log in, click the **submission** tab and **make a new submission**, then you will be directed to your Dashboard.

SUBMISSION

1. Click **New Submission** on the left side of the screen.

In the **Section** area, choose which **type of papers** you want to submit → Read and agree the **Submission Requirement** by clicking all the checkboxes → In addition, you can write an email to the editor by filling the **Comments for Editor** or leave it blank → Click **Save and Continue**

2. You will be directed to the **Upload Submission File** section.

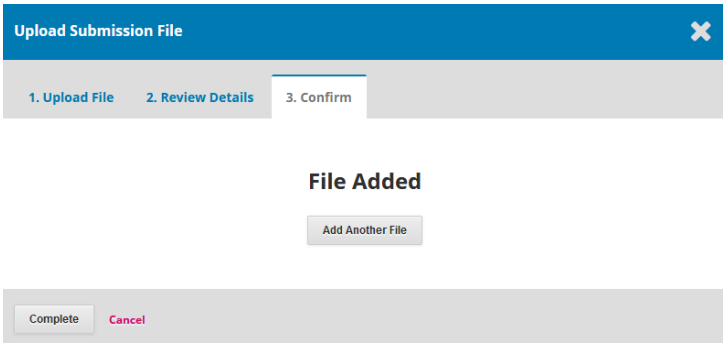
A. Upload your file in the **Upload Submission File** section.

Select **Article Text** in the **Article Component** → **Upload** your **file**. The type of file that accepted is in **.doc** or **.docx** → Click **Continue** to go to the next process

B. You need to **reconfirm** that you have uploaded the correct file in the **Review Details**.

You can edit the file name by clicking the **Edit** button → Click **Continue** to go to the next step

C. On the **Confirm** section, you can add another file.



You may be able to **add another file** for your submission, otherwise leave it blank → Click **Complete** button

Note:
1. You will be able to see your uploaded file in the **Submission Files** list.
2. You will be able to upload another file by clicking the **Upload File** button on the right side of the **Upload Submission** section.
3. You will be able to modify your file by clicking ▶button next to your uploaded file.
4. To continue, click the **Save and Continue** button.

3. You have to enter the **Metadata** of your article.

Fill the **Title** with capital letters → Fill the **Abstract** of your article → You can add more **contributors** if you wrote the article with another author(s) → Fill the **keywords** by pressing enter button at the end of each → Click **Save and Continue**

4. For the last step, you will be asked to **Confirm** your submission.

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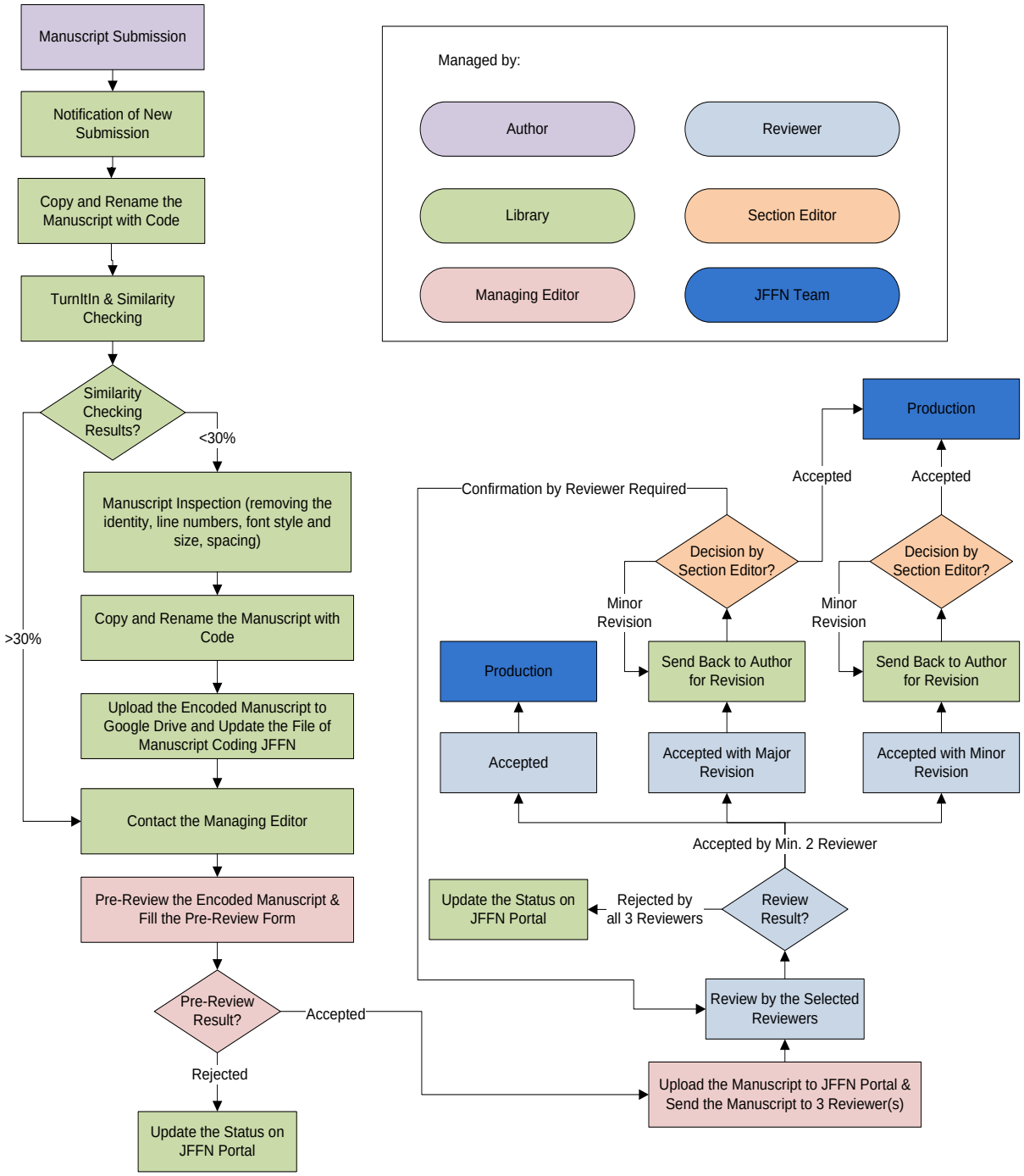
Flow of Manuscript Acceptance Process in JFFN



Guideline for Authors

NOTE: Please read the instructions carefully and strictly follow them to ensure smooth submission process. Papers that do not adhere to the guidelines will be rejected, hence full cooperation from the authors is highly appreciated.

- **Type of Papers**

There are three types of paper which are accepted by Journal of Functional Food and Nutraceutical: Research Papers, Review Articles, and Short Communication and Notes. Please note that the papers have not been and will not be published elsewhere, the Chief Editor reserves the right to change the paper into Short Note. The Author(s) shall retain all copyright rights held by the Author in the Manuscript.

a. **Research Papers**
   Original full-length research papers that have not been published previously, except in a preliminary form, and should not excess 7,500 words from introduction to conclusion (not including references). Research paper should not contain more than 40 references.

b. **Review Articles**
   By invitation only.

c. **Short Communications and Notes**
   Short communications of up to 3000 words from introduction to conclusion (optional), not including references, describing work that may be of a preliminary nature but merits publication. These papers should not contain more than 40 references.

- **Manuscript Preparation**

Manuscripts are accepted either in English or Indonesian language. For content written by Indonesian author/s, the author should provide title and abstract in both Indonesian and English.
Author is asked to provide manuscripts as electronic files and should be prepare using common word processor software (e.g. Microsoft-Word®) in following formats: DOC, DOCX, or RTF.
For *Research Papers* and *Short Communications and Notes* please refer to the **Template** to prepare the manuscript accordingly. Please note that the paper should be uploaded in a **SINGLE** file where title page information, manuscript, figure(s) and/or table (s) are included.

- **Originality**

The manuscript that submitted must be an original work. Authors should refer to the Code of Ethics to ensure its originality. Ensure the manuscript has not been previously published, nor is it before another journal for consideration (including published in different language).

It is recommended to check the manuscript for any possible plagiarism by using any program such as TurnitIn or any other software before submitting the manuscripts. Authors are responsible for the integrity of the work as a whole (including method, analysis, calculation, or other details), from inception to published article.

- **Publication Fee**

Submission Fee
Journal of Functional Food and Nutraceutical **will be charged IDR 500.000 per article** at the time of manuscript submission. Submission fee exemption can be applied with term and condition.

Membership Fee
For P3FNI and Swiss German University members will receive 50% discount for the submission fee.

- **Submission Preparation Checklist**

As part of the submission process, authors are required to check off their submission's compliance with all of the following items, and submissions may be returned to authors that do not adhere to these guidelines.

- The originality of the manuscript and ensure the manuscript has not been published previously – Please see "Originality"
- Manuscript text is prepared in accordance with the author guidelines and given template (for *Research Papers* and *Short Communications and Notes*).
- Ensure that some of the provisions are checked and present – **Please see file Check List:** http://bit.ly/checklistjffn.

- **Article Submission**

Online submission via *Open Journal System* (https://journal.sgu.ac.id/jffn). Please refer to the User Account Registration Guideline for submission process. After the submission, author who submit the manuscript will get a confirmation email and able to track their submission status by logging in to the system. The submission tracking includes a status of manuscript review and editorial process. If authors have any problems with the online submission, please contact JFFN admin at the following email: jffn@sgu.ac.id

Reviewer Guideline

Interested to become a reviewer?

The JFFN Editorial Team will send the manuscripts to the relevant reviewers according to the expertise of respective reviewers. If you are interested in becoming a reviewer of JFFN, please fill out the reviewer application form: http://bit.ly/revregform along with a brief summary of your expertise and your CV. Send all the documents to jffn@sgu.ac.id. The reviewers who pass the selection will obtain many benefits. All review process will be processed through JFFN online system.

1. **Confirmation (Accept or Decline)**

   The reviewers will receive an email invitation that will be sent by the JFFN system. Use the links to accept or reject the invitation. If you decide to accept the invitation as the reviewer, you will be responsible to input the review result/s to the JFFN journal editor as the requirement whether the manuscript is appropriate to be published in JFFN.

2. **Submitting the review**

   Reviews must be entered in the JFFN submission system. Drop us an email if you encounter trouble accessing the manuscript or entering your comments to jffn@sgu.ac.id.

3. **Timing**

   The deadline for completing the manuscript review process is 14 days. If you are unable to complete or need additional time for the review process, please notify us immediately so that we can keep the authors informed and assign alternative solution if necessary.

Confidentiality

All reviewers are required to maintain the confidentiality of the manuscript and never share information to the other parties without the editor's consent. The involvement of third parties in the review process, must be declared during the review process. Correspondence as part of the review process is also to be treated confidentially by all parties.

All reviewers are strongly required to keep the confidentiality of process reviews, maintain material confidentiality of manuscripts, and will not take advantage during the review process.

Anonymity

Reviewers are anonymous by default. Reviewers' identities are not revealed to authors or to other reviewers unless reviewers specifically request to be identified by signing their names at the end of their comments.

All reviewer's identity will be kept confidential. The reviewer's identity will not be revealed to anyone unless reviewers specifically request to notify the identity by writing the name on the review form or comments.

Writing the Review

Here are the generic questions to the reviewer. Please evaluate the submission based on the general scientific journal guideline. Please download the review form from: http://bit.ly/revformjffn.

The form covers:

1. Is the manuscript technically sound and do the data support the conclusion?
2. Has the statistical analysis been performed appropriately and rigorously?
3. Is the manuscript presented in an intelligible fashion and written in standard English/Indonesian?
4. Review comments to the author? Please state the positive suggestion that might support the authors to improve the manuscript.
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Revisions

When an author revises a manuscript, the Academic Editor will often ask the original reviewer(s) to evaluate the authors' revision. We expect the reviewers to be available to provide these additional comments. You will be requested to suggest the acceptance of the manuscript.

In the revision process, the editorial team frequently will ask reviewers to evaluate the author's revision. The editorial team expects that all reviewers will be available to conduct evaluation and provide valuable suggestions to improve the manuscript quality. In the end, the reviewer will be asked to decide the appropriateness of the manuscript according to several categories:

- Accept without revision
- Accept with minor revision
- Accept with major revision
- Decline

Thank you to our reviewers

Peer-review is an important step to maintain the high quality of a journal. Reviewers provide scientific critiques based on their expertise that assist editors to make acceptance decision professionally. Therefore, the Editors would like to acknowledge our reviewers listed below who have contributed their valuable time for maintaining the quality of Journal of Functional Food and Nutraceutical.

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Registrasi anggota P3FNI

Perhimpunan Penggiat Pangan Fungsional dan Nutrasetikal Indonesia (P3FNI) juga mengembangkan kontribusinya di kancah internasional bersama dengan International Society for Nutraceutical and Functional Food (ISNFF). Secara internasional terdapat klaster ISNFF seperti di Korea dan China. Untuk kepentingan percaturan internasional P3FNI menggunakan nama Indonesian Society for Functional Food and Nutraceutical (ISFFN).

Keterlibatan P3FNI atau ISFFN ini memberi manfaat anggotanya untuk memberikan kontribusi ilmu pengetahuan dan teknologi serta mengikuti pemutakhiran pangan fungsional dan nutrasetikal. Bersosialisasi dan berkesempatan dalam pertemuan ilmiah bersama penggiat pangan fungsional dan nutrasetikal dari berbagai negara untuk terus-menerus mengarah pada optimalisasi penggunaan pangan untuk kesejahteraan manusia secara bijaksana dan menjaga kelestariannya.

Siapa Yang Perlu Menjadi Anggota?

1. Akademisi dan peneliti yang terus-menerus mengembangkan dan memajukan ide alternatif dan kreatif untuk menuju kemajuan dan kesejahteraan manusia dengan menggunakan data basis ilmiah.
2. Praktisi kesehatan maupun industri yang menerapkan pangan fungsional dan nutrasetikal.
3. Mahasiswa sebagai penerus masa depan untuk melestarikan praktik-praktik pengadaan dan penggunaan pangan fungsional dan nutrasetikal secara bijaksana dan berkelanjutan.
4. Memberi advokasi dalam kasus-kasus pangan dan kesehatan.
5. Individu yang memiliki perhatian dalam pangan fungsional dan nutrasetikal.

Fasilitas Anggota P3FNI

1. Link Internasional untuk kegiatan atau program riset dan pembelajaran pangan fungsional dan nutrasetikal meliputi direktori laboratorium dan professor, lembaga kesehatan, LSM/NGO, skim hibah, dan internship/magang di industri.
2. Biaya partisipasi dalam pertemuan ilmiah yang diselenggarakan P3FNI.
3. Informasi tentang pangan fungsional dan nutrasetikal Indonesia dan isu internasional terbaru (international current issues).

Iuran Keanggotaan P3FNI

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Akademisi, Peneliti non-komersial	Rp 400.000
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