

The Combination of *Phyllanthus niruri*, *Euphorbia hirta*, and *Loranthus* sp. as a Source of Antioxidant Agents

Elizabeth Betty Elok Kristiani, Sri Kasmiyati*

Faculty of Biology, Universitas Kristen Satya Wacana, Indonesia

*Corresponding Author: kas@uksw.edu

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Abstract. Meniran (*Phyllanthus niruri*), patikan kebo (*Euphorbia hirta*), and benalu (*Loranthus* sp.) have often been used by people as medicinal plants. This research aimed to measure the levels of flavonoids, phenolics, and ascorbic acid compounds, as well as the Free Radical Scavenging (FRS) activity of ethyl acetate extract from the mixture of *P. niruri*, *E. hirta*, and *Loranthus* sp. The FRS activity was measured with the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. The level of compounds was measured by using the spectrophotometry method with specific reagents. The result of the FRS activity in ethyl acetate extract from the mixture of *P. niruri*, *E. hirta*, and *Loranthus* sp. varied depending on its dose. The measure of FRS in the *P. niruri*: *E. hirta*: *Loranthus* sp 0:0:1 (K-OOL) composition showed a strong result with a value of $IC_{50} 97.2 \pm 2.1$ ppm, while in the 0:0.5:0.5 (K-OEL) composition it was moderate with a value of $IC_{50} 147.6 \pm 6.5$ ppm. The other compositions showed weak and inactive results. The K-OOL composition had the highest flavonoid and phenolic content that were 298.8 ± 0.00 mg QE/g extract and 141.5 ± 2.85 mg GAE/g extract respectively. The composition with the highest ascorbic acid content (298.8 ± 0.00 mg/g extract) was K-OEO composition. So far, research on medicinal plants is still limited to one type of plant. The combination of several types of plants in several formulations allows obtaining a composition that can produce maximum antioxidant capacity. Therefore, this research is expected to produce a combination formulation of various types of medicinal plants that have the K-OOL composition very strong antioxidant activity and can be used as herbal medicines.

Key words: antioxidant; secondary metabolites; *Phyllanthus niruri*; *Euphorbia hirta*; *Loranthus* sp.

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INTRODUCTION

Medicinal plants play an important role in treating human's health disorders (Sofowora et al., 2013). The history has shown that ever since the ancient times, humans have been utilizing natural ingredients to cure diseases and preserve health. Commonly, they turn various types of herbal plants into medicinal potions to use whether as a treatment or prevention. In Indonesia, medicinal potions are known as *Jamu*. The traditional use of herbal medicine is rooted in the cultural practices and customs that was passed from generation to generation (Mahmood et al., 2011). At present days, the role of medicinal plants is rising because of their ability to cure various diseases. These abilities are related to the presence of secondary metabolites in plants (Petrovska, 2012). Plants are excellent sources of secondary metabolites such as phenolics, flavonoids, alkaloids, lignans, and terpenoids (Karakaya et al., 2019). The various capabilities of secondary metabolites in plants including as sedative (Bahmani et al., 2019), antibacterial (Ibrahim et al., 2013; Kasmiyati et al., 2021) antispasmodic (Martinez-Perez et al., 2018), antidepressant (Eloziia et al., 2017; Rahman et al., 2017), anticancer (Kristiani et al., 2016; Middleton et al., 2000; Kristiani et al., 2021), anti-inflammatory,

analgesic, antipyretic, hepatoprotective, and nephritic (Yusufoglu, 2014; Haidara et al., 2020), analgesic, immunomodulatory, and/or antioxidant properties (Majid et al., 2015; Safriani et al., 2021). Currently, many studies have been carried out to scientifically prove the efficacy of medicinal plants for health.

Antioxidants are compounds that slow down or prevent oxidation so as to prolong the life of oxidizable materials. The majority of human's diseases or health disorders are mainly related to oxidation, specifically due to a free radical activity (Kumar & Pandey, 2015). Exogenous intake of antioxidant sources can help repair the damage caused by oxidative stress by slowing down the initiation or propagation of oxidative chain reactions, free radical of scavenging, an absorber singlet oxygen or other the agents of reduction (Baiano & Nobile, 2015). Exogenous antioxidants can derive from food such as fruits, flowers, vegetables, and cereals, or from traditional herbs which are generally a mixture of spices (Deng et al., 2012).

There are various scientific studies of the antioxidant activity of some plants that have been used by people for their health benefits. Meniran (*Phyllanthus niruri*), patikan kebo (*Euphorbia hirta*), and benalu (*Loranthus* sp.) have been widely used by

people as medicinal plants. Based on the the results of those studies by several researchers, the differences of solvent affect the phytopharmaceutical ability of plant extracts because of the different bioactive compounds extracted. Ethyl acetate extracts, either direct or by fractionation, were reported to show strong antioxidant activity, such as on the roots of *Phyllanthus amarus* (Maity et al., 2013), *Euphorbia dracunculoides* (Majid et al., 2015), as well as on the bark, leaves, and seeds of *Loranthus pulverulentus* (Raza et al., 2013).

Several secondary metabolites have been found to exhibit free radical scavenging activity, such as polyphenols (phenolic acids, flavonoids, anthocyanins, lignins, and stibenes), carotenoids (xanthophylls and carotenes), and vitamins (vitamins C and E) (Xu et al., 2017). Wafaa et al. (2016) also reported an antioxidant activity from flavonoid and tannin compounds. Meanwhile, phenolic compounds from the methanol extract of the leafy twigs of *Loranthus micranthus* showed the most potent free radical scavenging ability among other compounds (Zainol et al., 2003).

In daily practice, the herbal preparation of medicine was consisting of more than one type of material. Each material will have different phytopharmaceutical active ingredients so that enables interactions in their activities. So far, the study of medicinal plants has been limited to a single plant species. The combination of several types of plants in several formulations allows the acquirement of a composition that can produce maximum antioxidant capacity. Therefore, this research is expected to produce a combination formulation of various types of medicinal plants that have very strong antioxidant activity and can be used as herbal medicines. This study aimed to measure the levels of flavonoid, phenolic, and ascorbic acid compounds, as well as the antioxidant activity of the combination of ethyl acetate extract of *Phyllanthus niruri*, *Euphorbia hirta*, and *Loranthus* sp.

METHODS

This research was carried out in October to December 2020, at the Laboratory of Biochemistry and Molecular Biology, Faculty of Biology, Satya Wacana Christian University, Salatiga. The main materials of *P. niruri*, *E. hirta*, and *Loranthus* sp. weeds (Figure 1) were taken at the Satya Wacana Christian University campus in Salatiga. Each sample composition was extracted by maceration using ethyl acetate as solvent. Parameters measured including the total bioactive compounds content that were flavonoid, phenolics, and ascorbic acid, as well as Free Radical Scavenging (FRS) activity. Before the

mixing was conducted, each plant was dried first (Figure 1) and then made into dry simplicia powder. The mixing of materials is presented in Table 1.



Figure 1. Plants used in the study. (A-C) Fresh plants were taken from the sampling location; (D-F) Plant dry simplicia; (A and D) Meniran (*Phyllanthus niruri*); (B and E) Patikan kebo (*Euphorbia hirta*); and (C and F) Benalu (*Loranthus* sp.).

Table 1. The combinations of *Phyllanthus niruri*, *Euphorbia hirta*, and *Loranthus* sp. used as research samples.

Combination	Ratio of combination		
	<i>Phyllanthus niruri</i>	<i>Euphorbia hirta</i>	<i>Loranthus</i> sp.
K-POO	1	0	0
K-OEO	0	1	0
K-OOL	0	0	1
K-PEO	0.5	0.5	0
K-POL	0.5	0	0.5
K-OEL	0	0.5	0.5
K-PEL	0.33	0.33	0.33

Preparation of extracts

Ethyl acetate was added to the dry powder of each combination until it completely submerged. The mixture was macerated for three hours while being shaken using a magnetic stirrer. The maceration result was filtered using a filter paper and the filtrate was set aside. The maceration process was repeated until the immersion filtrate appeared clear. All filtrate was combined and then concentrated using a rotary evaporator (Rotavapor R-114 Buchi) with the help of a vacuum pump (Eyela A-1000S). The concentrated extract was ready for further analysis.

Total flavonoid content assay

The total flavonoid content was measured by the AlCl₃ method (John et al., 2014). Quercetin was used as a standard for flavonoid compounds in the concentration range of 0-100 ppm. A total of 1.0 ml of sample was added with 0.3 ml of 10% AlCl₃ and was incubated for 5 minutes. After that, 0.3 ml of 5%

NaNO₂ was added and was incubated for another 5 minutes. In the final step, 2.0 ml of 1M NaOH was added. The absorption of the mixture was measured using an ultraviolet-visible spectrophotometer (Shimadzu Mini 1240 UV spectrophotometer) at a wavelength of 510 nm. The measurement results of the absorption of the quercetin solution were represented in a linear regression equation. The flavonoid level in the sample was determined based on the quercetin standard curve equation.

Total phenolic content assay

The total phenol content was determined using the Folin Ciocalteu method (Almey et al., 2010) with gallic acid as the standard for the phenolic compounds. A total of 1.0 ml of sample was added with 1.0 ml of Folin Calteau reagent and incubated for 5 minutes. After that, 10 ml of 7% Na₂CO₃ was added next and incubated for 90 minutes. The absorption of the mixture was measured using an ultraviolet-visible spectrophotometer (Shimadzu Mini 1240 UV spectrophotometer) at a wavelength of 550 nm. The measurement results of the absorption of the gallic acid solution were represented in a linear regression equation. The total of the flavonoid was calculated based on the standard curve equation of gallic acid.

Ascorbic acid content assay

The ascorbic acid content testing was based on the research done by Balogh and Szarka (2016). The ascorbic acid level was measured with the sulfosalicylic acid method. An amount of 1.0 ml sample was added with 3.0 ml of sulfosalicylic acid, 2 ml of Na-molybdate, 2 ml of 0.15 N H₂SO₄, and 1 ml of 1.5 mM Na₂HPO₄. The absorption of the mixture was measured using an ultraviolet-visible spectrophotometer (Shimadzu Mini 1240 UV spectrophotometer) at a wavelength of 550 nm. The measurement results of the absorption of the ascorbic acid standard solution were represented in a linear regression equation. The total level of the ascorbic acid was calculated based on the standard curve equation of ascorbic acid.

Free Radical Scavenging (FRS) activity assay

The FRS activity was measured by using the DPPH method (Chan et al., 2007). Ascorbic acid was used as a standard free radical scavenging agent. The series of sample concentrations were in the range of 0.001 - 0.1 g/ml. A total of 1 ml of the sample was added with 2 ml of DPPH (1.1-diphenyl-2-picrylhydrazyl) 50 ppm in methanol. The mixture was incubated in the dark for 30 minutes and its absorbance was measured using an ultraviolet-visible spectrophotometer (Shimadzu Mini 1240 UV

spectrophotometer) at a wavelength of 517 nm. The value of the FRS activity was calculated using the equation = $1 - (\text{sample absorption} / \text{standard uptake}) \times 100\%$. The absorption data for each test concentration was represented in a linear regression equation, and the IC₅₀ value was then determined.

Data analysis

The differences in IC₅₀ values and test compound content between extracts were analyzed statistically through analysis of variance (ANOVA) using SAS ver. 9.1.3. If there was a significant effect between the treatment and the control, the test was then continued with the Tukey's test at the 5% test level. All experiments were carried out in five replicates.

RESULTS AND DISCUSSION

Free Radical Scavenging (FRS) activity

Antioxidants are molecules that are able to inhibit the oxidation of other molecules by donating its electrons so as to break the chain of free radical reactions (Dontha, 2016). In general, *in vitro* antioxidant tests using free radical traps are relatively easy to do. In this study, the antioxidant ability of the extract was tested *in vitro* using 2,2-Diphenyl-1-picrylhydrazyl (DPPH), a stable free radical. The DPPH method is one of the most widely used free radical scavenging method because the steps are simple, fast by using an ultraviolet-visible spectrophotometer, and low in cost (Dontha, 2016; Burda & Oleszek, 2001). In this test, DPPH as a stable radical (purple color) will be reduced by the presence of an antioxidant compound into a radical form of DPPH-H which gives a yellow color. The more color removal there is, the greater the ability to reduce free radical scavenging of antioxidant compounds (Bandoniene & Murkovic, 2002). The antioxidant ability test using the DPPH method is expressed through the ability to inhibit free radicals (PRB). Ascorbic acid or commonly known as vitamin C is used as a standard antioxidant compound. Vitamin C is a non-enzymatic antioxidant group which the mechanism interferes the free radical chain reactions (Dontha, 2016).

The results have shown that the FRS activity of the mixed extract of the three weeds was dose dependent, in which the higher the concentration of the extract was, the greater the FRS activity (Figure 2). Figure 2 shows that a single extract composition of *Loranthus* sp. or the composition of *P. niruri*: *E. hirta*: *Loranthus* sp. 0:0:1 (K-OOL) and the composition of *P. niruri*: *E. hirta*: *Loranthus* sp 0:0.5:0.5 (K-OEL) have a greater antioxidant activity than other compositions.

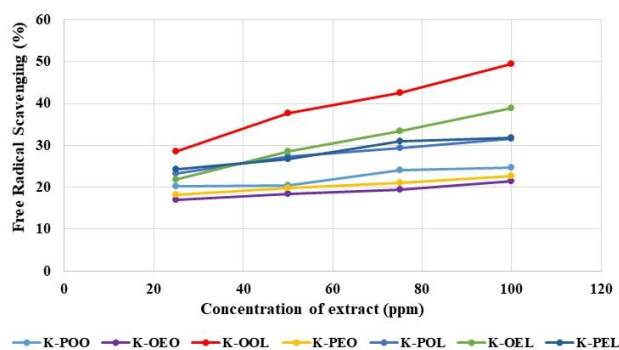


Figure 2. Graph of the dose-dependent relationship between extract concentration and FRS activity of the mixed extract of *Phyllanthus niruri*, *Euphorbia hirta*, and *Loranthus* sp. combination. The ratio of *P. niruri*: *E. hirta*: *Loranthus* sp. in K-POO = 1:0:0, K-OEO = 0:1:0, K-OOL= 0:0:1, K-PEO = 0.5:0.5:0, K-POL= 0.5:0:0.5, K-OEL= 0:0.5:0.5, and K-PEL= 0.33:0.33:0.33.

The antioxidant activity of a material is expressed through how much the value of antioxidant concentration required to inhibit 50% of free radical compounds, or commonly referred to as the IC_{50} value. The category of the antioxidant strength of a material can be determined based on the IC_{50} value (Jun et al., 2003). The IC_{50} value and antioxidant strength of each mixture of *P. niruri*, *E. hirta*, and *Loranthus* sp. are presented in Table 2.

Table 2. The antioxidant activity and strength of the mixed extract of *Phyllanthus niruri*, *Euphorbia hirta*, and *Loranthus* sp.

Plants combination	IC_{50} value* ($\mu\text{g/ml}$)	The antioxidant power
K-POO	508.5 ± 16.9^b	Not active
K-OEO	599.7 ± 17.7^a	Not active
K-OOL	97.2 ± 2.1^d	Strong
K-PEO	562.4 ± 19.5^{ab}	Not active
K-POL	272.1 ± 32.4^c	Weak
K-OEL	147.6 ± 6.5^d	Medium
K-PEL	283.3 ± 81.1^c	Weak
Ascorbic acid	21.4 ± 0.1^e	Very strong

* a-f letters indicate significant differences at 5 % significance level. The classification of antioxidant power based on IC_{50} value (ppm): < 50: Very strong; 50-100: Strong; >100-250: Medium; >250-500: Weak; >500: Not active (Jun et al., 2003). The ratio of *P. niruri*: *E. hirta*: *Loranthus* sp. in K-POO = 1:0:0, K-OEO = 0:1:0, K-OOL= 0:0:1, K-PEO =

0.5:0.5:0, K-POL= 0.5:0:0.5, K-OEL= 0:0.5:0.5, and K-PEL= 0.33:0.33:0.33.

In the condition of *P. niruri* and *E. hirta* as a single extract (K-POO and K-OEO respectively), the PRB activity of the extract was inactive ($IC_{50} > 500$ ppm), while the single extract of *Loranthus* sp. (K-OOL) showed a strong antioxidant activity ($IC_{50} < 100$ ppm). Da'i et al. (2016) found that the ethanol extract of *P. niruri* from an Indian local market showed a potential of antioxidant activity, both in *in vitro* and *in vivo* model tests. In that study, the IC_{50} value *in vitro* test of the extract was 14.21 ± 0.73 mg/ml. In line with this result, the ethanol extract of *P. niruri* leaves grown in the Conservation and Cultivation Unit of the Bogor Indonesia Biopharmaceutical Research Center showed showed higher than water and methanol extracts, with the IC_{50} value of 14.5 g/ml (Nurcholis et al., 2012). Research using aqueous extracts of *P. niruri* also showed that the ability was still low (IC_{50} value 90.86 g/mL) compared to ascorbic acid (IC_{50} value 25.31 g/mL) (Giribabu et al., 2014).

In all compositions, the FRS ability was still lower than that of ascorbic acid, which expressed a higher IC_{50} value of the mixture than ascorbic acid. The weak FRS ability of the mixed extracts compared to ascorbic acid was probably due to the extract being a crude extract produced by a maceration, while ascorbic acid was a pure compound. Further fractionation towards the extract may extract more antioxidant-specific compounds to increase its ability to scavenge free radicals. Abbasi et al. (2013) performed a stratified fractionation of a methanol extract of *Euphorbia heterophylla*, and it was found that the ethyl acetate fraction had a very strong antioxidant activity (the IC_{50} value using the DPPH method was 10.33 g/ml). The fractionation process can increase the antioxidant ability as seen in the comparison between the research results of Caroline et al. (2018) and Masruro and Tukiran (2017). The IC_{50} value of PRB with the DPPH method of *E. hirta* methanol extract was 72.20 g/ml (Caroline et al. 2018), while the methanol fraction of graded maceration using n-hexane, chloroform, and methanol showed 30.02 g/ml (Masruro & Tukiran, 2017).

In the mixed composition, the presence of *P. niruri* and *E. hirta* that were K-PEO, K-POL, and K-PEL (Figure 3A), K-PEO, K-OEL, and K-PEL (Figure 3B), and K-POL, K-OEL, and K-PEL (Figure 3C) significantly decreased the FRS ability of the mixture. The effect of *P. niruri* on the mixture containing *Loranthus* sp. decreased by 5 – 29%, while the effect of *E. hirta* decreased by 8 -23%. On the other hand, the presence of *Loranthus* sp. in the mixture was able to increase the FRS activity of the

mixture by 25-60%. The composition of K-POL, K-OEL, and K-PEL became more active in the presence of *Loranthus* sp. (IC_{50} values are 272.1; 147.6; and 283.3 ppm, respectively). There might be compounds, both antioxidant and non-antioxidant, in *P. niruri* that were antagonistic to compounds in *E. hirta* and *Loranthus* sp., while the compounds between *E. hirta* and *Loranthus* sp. were synergistic and thus the PRB activity increased. Khanum et al. (2011) reported that there was a synergistic effect on the addition of *Oreganum vulgare* in increasing the PRB activity ($\pm 5\%$) of *Trachyspermum ammi* and *Plectranthus amboinicus* in all test concentrations, but they did not mention which compounds were synergistic. Some researchers have found that the combination of polyherbs that have antioxidant activity can be synergistic, additive, or antagonistic. The synergistic effect was seen in the combination of green tea with *Phyllanthus emblica* L., *Punicagranatum*, *Cinnamomum cassia*, *Ginkgo biloba* L., and *Camellia sinensis* Linn. (Jain et al., 2011), the combination of *Pterospartum tridentatum* and *Cymbopogon citratus*, and the combination of *Gomphrena globosa* and *C. citratus* (Bag & Chattopadhyay, 2015).

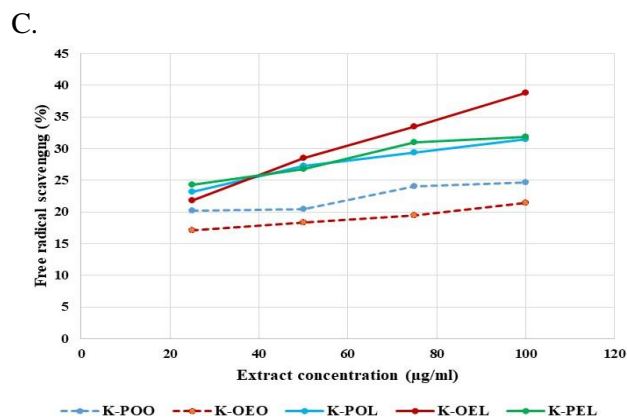
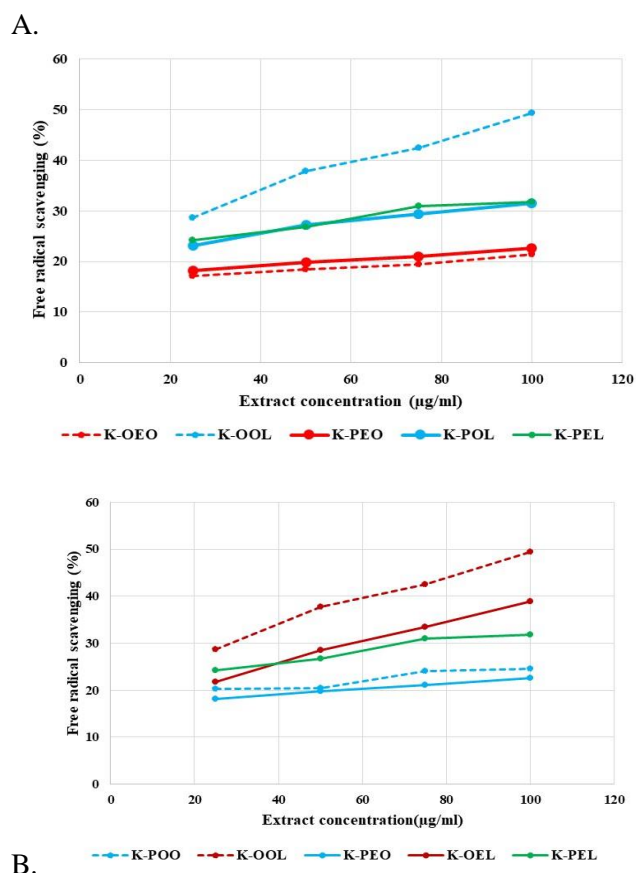


Figure 3. Patterns of the influence of one plant on another to the PRB activity in mixed extracts of *P. niruri*, *E. hirta*, and *Loranthus* sp. (A) The influence of the presence of *P. niruri*; (B) The influence of the presence of *E. hirta*. (C) The influence of the presence of *Loranthus* sp. The ratio of combination *P. niruri*: *E. hirta*: *Loranthus* sp. in K-POO = 1:0:0, K-OEO = 0:1:0, K-OOL = 0:0:1, K-PEO = 0.5:0.5:0, K-POL = 0.5:0:0.5, K-OEL = 0:0.5:0.5, and K-PEL = 0.33:0.33:0.33.

Wang et al. (2015) conducted a study on essential oils from several herbs and spices including bay leaf, black pepper, coriander (seeds and leaves), cumin, garlic, ginger, mustard greens, shallots, and turmeric. The result showed that only the combination of coriander oil with cumin was synergistic, while the other combinations were additive to the antioxidant activity (Wang et al., 2015). The study towards the combination of *Strobilanthes crispus*, *Phyllanthus niruri*, *Orthosiphon aristatus*, and *Stevia rebusiana* (23.96:0.62:75.42:0) also showed the highest antioxidant activity (Rahim et al., 2018). The study of the combination between green tea with some abundant of phenolics and flavonoids herbs included *Vitis vinifera*, *Phyllanthus emblica* L., *Punica granatum*, *Cinnamomum cassia*, *Ginkgo biloba* L., and *Camellia sinensis* Linn. showed the synergism relationship, that were the requirement of doses to scavenge the free radical compounds on the combination form lower than a single form (Jain et al., 2011).

The content of flavonoid, phenolics, and ascorbic acid

The pharmacological ability of herbal medicines is influenced by the presence of bio-active compounds, which usually are secondary metabolites (Haddad-Kashani et al., 2012; Prakash & Gupta, 2013). The production of secondary metabolites in plants is determined by intrinsic factors in the form of the genes of the plant itself and extrinsic factors in the form of environmental conditions where they grow

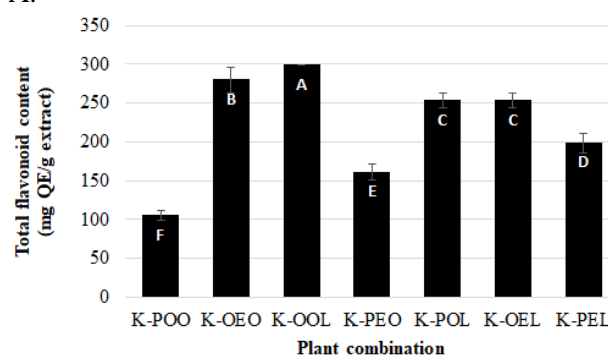
(climate and soil) (Figueirode et al., 2008). The quality, quantity, and biological activity of bio-active compounds in plants are related to the development stage of plants, plant organs, and the solvents used for extraction and isolation (Senguttuvan et al., 2014; Chekroun-Bechlaghem et al., 2019). The total of the secondary metabolite compounds and their antioxidant abilities are influenced by the solvent used, the extraction method used, and the plant part extracted (Rafińska et al., 2019). This research studied the antioxidant ability of three weeds that have been known to cure various diseases namely meniran (*P. niruri*), patikan kebo (*E. hirta*), and benalu (*Loranthus* sp.).

Natural antioxidant compounds from plants are diverse, such as polyphenols (phenolic acids, flavonoids, anthocyanins, lignans and stilbenes), carotenoids (xanthophylls and carotenes), or vitamins (vitamins E and C) (Baiano & Nobile, 2015; Manach et al. 2014). This study measured the content of flavonoids, phenolics, and vitamin C (ascorbic acid). In this study, the flavonoid levels were determined using quercetin as a standard compound. This compound is the most abundant polyphenol found in fruits and vegetables (Dabeek & Marra, 2019). Quercetin is one of the flavonoid members that have the most efficient antioxidant ability. The structural nature of its strong antioxidant capacity is caused by several factors, including ortho-dihydroxy or catechol group in the B-ring, 2,3 double bonds, and hydroxyl substitution at position 3 and 5 (Ozgen et al., 2016). Flavonoids with $AlCl_3$ forms a stable complex with C4 keto cluster and C3 or C5 hydroxyl cluster in flavones and flavonols (Petry et al., 2001). This complex gave a color that will be detected by a visible spectrophotometer. In the measurement of the total phenolic compounds, pure gallic acid was used as a standard to calculate the levels of phenolic compounds in the extract. Gallic acid has a hydroxy cluster and a conjugated double bond in the aromatic ring and can form a complex compound with the Folin-Ciocalteu reagent giving a deep blue color (Gulçin, 2012). Phenolic compounds can be released from the cell wall. Samples in the form of dry powder helped facilitate the release of a large number of bound phytochemical compounds into the medium during the extraction process, including phenolic compounds from the matrix (Ngamsuk et al., 2019).

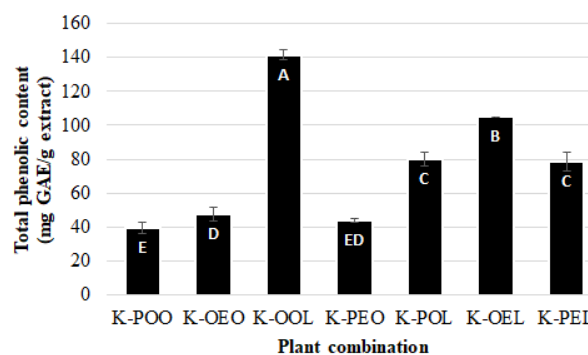
This study used ethyl acetate as a solvent in the maceration process. Ethyl acetate is a solvent with a medium polarity (0.228). This chemical nature allows the extraction process to attract various non-polar and polar compounds at a moderate level, making it possible to attract antioxidant parameter compounds such as flavonoids, phenolics, and vitamin C. Based on its structure, vitamin C has a carboxylate cluster is

a polar compound, while for phenolic and flavonoids compounds which are the compound groups, some tend to be polar and some are non-polar, so they will be carried away during the extraction process. Phenolic compounds and flavonoids are secondary metabolites with various groups and are often found in vascular plants (Haminiuk et al., 2014). Therefore, flavonoid, phenolic, and ascorbic acid compounds will also be present in the three tested plants, and the test results are shown in Figure 3. Giribabu et al. (2014) conducted a phytochemical test on the aqueous extract of *P. niruri* dried leaves and it showed a positive presence of alkaloids, flavonoids, saponins, tannins, lignin, terpenoids, and curcumin.

A.



B.



C.

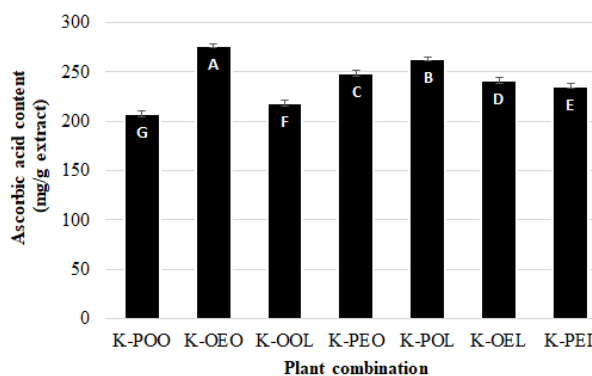


Figure 4. The level of total flavonoids (A) total phenolics; (B) and ascorbic acid; (C) contents in the extract of the *P. niruri*, *E. hirta*, dan *Loranthus* sp. combination. The ratio of combination *P. niruri* : *E.*

hirta: *Loranthus* sp. in K-POO = 1:0:0, K-OEO = 0:1:0, K-OOL= 0:0:1, K-PEO = 0.5:0.5:0, K-POL= 0.5:0:0.5, K-OEL= 0:0.5:0.5, and K-PEL= 0.33:0.33:0.33.

The synthesis and accumulation of non-enzymatic antioxidant compounds in plants might be caused by their natural tendency to respond to conditions of stress from their surroundings and/or their genetic structure that does exist to perform normal physiological functions to protect itself from microbial pathogens and animal herbivores (Kasote et al., 2015). Based on the environment where they grow, these three tested plants which are considered as weeds may produce antioxidant compounds for both of the reasons mentioned. In this research, the composition of K-POO, K-OEO, and K-OOL represented the single extract conditions of *P. niruri*, *E. hirta*, and *Loranthus* sp. In the single composition of *P. niruri* and *E. hirta*, the ascorbic acid level was the highest (207.4 ± 2.89 and 276.1 ± 1.67 mg/g extract), followed by flavonoid (105.0 ± 6.25 and 280.0 ± 16.54 mg quercetin/g equivalent extract), and phenolic as the lowest content (39.6 ± 3.23 and 47.6 ± 3.88 mg gallic acid/g equivalent extract) with an inactive free radical scavenging activity (IC_{50} value of 508.5 ± 16.9 B and 599.7 ± 17.7 g/ml). The single composition of *Loranthus* sp. had the highest flavonoid and phenolic content (298.8 ± 0.00 mg quercetin/g equivalent extract and 141.5 ± 2.85 mg gallic acid/g equivalent extract) among all other compositions, but had the lowest ascorbic acid content (218.7 ± 3.01 mg/g extract), while the PRB activity was strong (IC_{50} value of 97.2 ± 2.1 g/ml). When it is associated with the ability of antioxidants, it can be seen that the presence of phenolics has most prominent positive correlation with it. The higher the level of phenolic compounds, the stronger the antioxidant ability. The *Loranthus* sp. extract (KOOL) with the highest phenolic acid showed the highest antioxidant activity.

In the single extract of *P. niruri* and *E. hirta* (K-POO and K-OEO) with low phenolic content, the antioxidant activity was also low. In the combination of *P. niruri* and *E. hirta* (K-PEO), the activity remained low. The antioxidant activity turned higher when *Loranthus* sp. was put into the mixture. Phenolic compounds generally have one or more aromatic rings with one or more hydroxyl clusters. The ability of phenolic compounds to act as antioxidants depends on the redox characteristics of the phenolic hydroxyl clusters and the potential of electron delocalization in their chemical structure (Gulçin, 2012). *Loranthus* sp. might be synthesizing

large amounts of phenolic compounds which contain many hydroxyl clusters because it is generally assumed that the antioxidant capacity of phenolics will increase with the amount of free hydroxyl and side-chain conjugation to an aromatic ring. Some researchers stated that phenolic compounds, which consist of phenolic acids, flavonoids, lignans, stilbenes, and tannins are the most important because in both *in vitro* and *in vivo* test results, this type of compound shows a strong antioxidant activity (Myburgh, 2014; Blokhina et al., 2003; Duthi et al., 2000).

The phenolic content in the ethyl acetate extract of stem bark of *Loranthus pulverulentus*, which partitioned from methanol : water extract (90:10) extract was 151 ± 2.1 to 396 ± 1.6 GAE/g extract while the leaf was 137 ± 0.9 to 430 ± 2.2 mg GAE/g extract (Raza et al., 2013). The difference of results within this study may be influenced by the extraction method and the *Loranthus* sp. species. The ethanolic extract of *P. niruri* contained a total of 81.59 ± 2.85 mg phenolic equivalent to gallic acid/g extract (Da'i et al., 2016). The study of *E. hirta* leaf extract showed that the ethanolic extract contained a higher level of phenolic compounds (291.74 ± 2.46 mg gallic acid equivalent/g extract) and flavonoids (40.32 ± 1.67 mg quercetin equivalent/g extract) compared to methanol and water extracts (Asha et al., 2016).

The same thing was found in the antioxidant activity as the results of the DPPH test, the hydroxyl radical scavenging test, and the superoxide radical scavenging test showed that the antioxidant activity of ethanol extract was stronger than the other two extracts, namely methanol dan aqueous extract (Asha et al., 2016). This phenomenon that antioxidant activity of ethanol extract stronger than aqueous extract was also seen in a study by Venkatachalam et al. (2018). This indicated that a higher content of bioactive compounds will result in a higher antioxidant activity as well. Ethanol extract, which had a higher flavonoid content than an aqueous extract, also exhibited stronger antioxidant abilities based on the DPPH free radical scavenging test, ferric reducing power (FRAP) determination, and Lipid peroxidation inhibition (Maya et al. 2018). The phytochemical test of ethanol and aqueous extract of *E. hirta* resulted in positive for tannins, flavonoids, coumarins, reducing sugars, steroids, and triterpenoids. The FRS activity using the DPPH test for the ethanol extract was stronger (IC_{50} value of 4.93 ± 0.40 g/mL) than aqueous extract (IC_{50} value of 46.33 ± 3.21 g/mL (Maya et al., 2018). The level of phenolic compounds in the aqueous extract of *E. hirta* was 52.92 ± 5.62 mg equivalent to gallic

acid/g extract with an FRS activity IC_{50} value of 175 ± 0.098 mg/ml (Sharma et al., 2007). Do provide more sentences regarding the role of solvent used in extraction related to this study to summarize the information mentioned above to provide a clear and good paragraph. Haddad-Kashani et al., 2012 stated that the pharmacological properties of natural product depend on the content of secondary metabolites. Generally, the polarity of secondary metabolite compounds is different so that the use of solvent for extraction affected the type of compound to be extracted (Visht & Chaturvedi, 2012). Extraction using short-chain alcohol as solvents could extract compounds such as terpenoid, saponin, phenolic compounds, and/or flavonoids (Wu et al., 2011; Lalee et al., 2012). In this research, all of the extract contained both flavonoid and phenolic acid, although we used ethyl acetate as solvent extraction. This is may be cause of ethyl acetate is a semipolar solvent. Ethyl acetate is could attract both of lipophilic and hydrophilic compounds (Hardiana et al., 2012). Flavonoid are an intermediates compound which could act as lipophilic and hydrophilic (Middleton et al., 2000). The results of research by Sulmartiwi et al. (2018) showed that phenolic compounds can be dissolved using non-polar, semi-polar, and polar solvents.

The research on the antioxidant activity of benalu (*Loranthus* sp), meniran (*P. niruri*) and patikan kebo (*E. hirta*) has been carried out and reported by many researchers, but research is still limited to testing the antioxidant activity of each plant, and no studies have reported the antioxidant activity of the combination of the three plants. Based on the results of this study, it was found that the combination of the three herbal plants (*Loranthus* sp, *P. niruri* and *E. hirta*) had higher antioxidant activity than single plants. The presence of *Loranthus* sp in each plant mixture composition tested significantly increased antioxidant activity. The results of this study are expected to provide new information that can be used in the development of herbal medicines to increase endurance and traditional medicine. Generally, in traditional medicine using herbal medicine, the ingredients used are a mixture of various species of medicinal plants, therefore the information obtained from this study is expected to be additional useful information for the development of herbal medicine.

CONCLUSION

Free radical scavenging activity (FRS) in mixed extracts of *Phyllanthus niruri*, *Euphorbia hirta*, and *Loranthus* sp. is dose dependent. The higher doses of

the extract, the higher ability of Free Radical Scavenging. The strongest of FRS ability was K-OOL composition (ratio of combination *P. niruri*: *E. hirta*: *Loranthus* sp.= 0:0:1) with IC_{50} value of 97.2 ± 2.1 ppm. In the composition of the mixture tested, the presence of *Loranthus* sp. significantly increased antioxidant ability whereas *P. niruri* attenuated. Phenolic compounds were more correlated with the ability of antioxidants than flavonoid or ascorbic acid. The ratio of combination *P. niruri*: *E. hirta*: *Loranthus* sp.= 0:0:1 (K-OOL) had the highest of flavonoids and phenolics content, which were 298.8 ± 0.00 mg quercetin equivalent/g extract and 141.5 ± 2.85 mg gallic acid equivalent/g extract, respectively. The highest of ascorbic acid content showed by K-OEO composition (ratio of *P. niruri*: *E. hirta*: *Loranthus* sp.= 0:1:0), which was 276.1 ± 1.67 mg/g extract.

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