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Total Phenolic and Flavonoid Contents Of *Pluchea indica* Less Leaves Extracts from Some Altitude Habitats

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Abstract: The diversity of secondary metabolites in plants, in general, can caused by genetic factor or environment which is interconnected. It can occur because of differences in population, life cycle, cultivation time, soil, climate, and geographic condition. The aim of the study was to quantitatively analyze the phenolic and flavonoid content of *Pluchea indica* leaves extract from different altitude habitats. *Pluchea indica* leaves were obtained from three different altitude habitat: lowland (Bangkalan; 28.3 - 31.72 m), middleland (Trawas; 727 – 937 m) and highland (Batu; 1303 – 1322 m). The simplicia of *P. indica* leaves were macerated and extracted with methanol, ethyl acetate, aquades and n-butanol. The total phenolic (gallic acid/GAE) and flavonoid (quercetin/QE) contents were determined using UV-VIS spectrophotometer. The results showed that the total phenolic contents of lowland (1,763 ±0,047 mg/mL) was found higher as compared to the middle-altitude land (1,455 ±0,295 mg/mL) and highland (1,212 ±0,608 mg/mL). The total flavonoid contents of *P. indica* were showed no mean difference of lowland (3,1 mg/mL), middle-altitude land (3.0 mg/mL) and highland (3.2 mg/mL). The highest phenolic and flavonoid content was found to be in ethyl acetate fraction of *P. indica.*
Key words: *Pluchea indica* Less, total phenolic content, total flavonoid, leaves extracts, altitude habitat.

Introduction

The phenolic compounds are secondary metabolite in plants and posses a wide structural role such as supplementary tissue, protection and defense strategy. The phenolic compounds plays a role in metabolic plasticity and its possible for the plants to adapt with environmental change both biotic and abiotic, and provide plant product in term of colour and flavor[1,2] .

Flavonoids are secondary metabolites that are toxic, often occur as glycosides, and is one of the largest natural phenols. Flavonoids are compounds reducing good, inhibits many oxidation reactions, both enzyme and non-enzyme[3]. Flavonoids are polar compounds because having a number of hydroxyl groups which have a sugar, which will dissolve in polar solvents such as ethanol, methanol, butanol, acetone, dimethylsulfoxide, dimethylformamide, and water[4,5]. One benefit of flavonoids is can be used as an active ingredient in making plant-based insecticide. Flavonoids can work as a powerful inhibitor of respiration, antimicrobial, and antiviral[6].

Pluchea indica plants are included in Asteraceae. Asteraceae family has a wide variety of phytochemicals, of which polyphenols, pyrethrum, triterpenoids, saponins, coumarin and flavonoids[7].

Asteraceae have benefits as a traditional medicinal plants and botanical pesticides (insecticides, fungicides, herbicides and nematicides).

Pluchea indica is a perennial shrub,that grows in subtropical climate. *Pluchea indica* is known to contain secondary metabolites released into the environment, either in the form of compounds evaporated from leaves or decomposed in soil[8]. *Pluchea indica* is known to contain secondary metabolites such as alkaloids, flavonoids, hydroquinone phenols, tannins, essential oils, benzenoid, phenylpropanoid, lignans, terpene, quercetin, and steroids that can be used as a herbicide or insecticide[9,10,11] . *Pluchea indica* with methanol extract has ~~the effect of~~ insecticide effect and the chloroform extract can be used as anti-nematode activity and antibacterial agent. Previous study resulted that the leaf extract of *Pluchea indica* have larvicidal activity on *Culex pipiens* mosquito[12]. Uchiyama *et. al.* isolated the terpenic glycoside compound from *Pluchea indica*[8], and Biswas *et. al*. isolated pure compounds from the extract of the roots of *Pluchea indica* and identified as antimicrobial compounds[13].

The secondary metabolites content in plants is formed as an attempt to defend themselves from its growing ecosystem. Hence the high and low contents of secondary metabolites in plants is influenced by the environment such as altitude, rain, and temperature[14]. Further said that the influence of environmental factors interact with genetic factors in the phenotypic expression of secondary metabolites, resulting in the production and excretion of secondary metabolites is influenced by temperature, light intensity, soil properties, microorganisms, and nutrient status[15].-Thus, if Pluchea indica is cultivated, it must adapted to its habitat in order to achieve maximum secondary metabolite content.

Based on above, this research aim to study the total phenolic compounds and total flavonoids in *Pluchea indica* found in a wide variety of habitats, namely in the lowland, middle-highland and highland. *Pluchea indica* has been selected because containing phenolic compounds, easy to grow in the lowland to the highland (altitude up to 1500 meters above sea level), easily cultivated, and can be developed as a biopesticide plants.

Materials and Methods

Plant material used

The leaves of *Pluchea indica* were obtained from three different altitude area: lowland <50 m asl, middle plain land 700-950 m asl and highland >1300 m asl. The sampling location are Bangkalan 28.3-31.72 m asl (E112.763368;S-7.021999) for lowland sampling, Trawas 727-937 m asl (E112.59425;S-7.681328) of middle-highland sampling and Bumiaji 1303-1322 m asl (E112.517002;S-7.804258). The plant material was dried in the shade in an airy place and the stored in paperbags and kept at room temperatur.

Chemicals and reagents

Petroleum eter (JT Baker), methanol (JT Baker), Ethyl acetate (JT Baker), sodium hydroxide/NaOH (Merck), sodium carbonate (Riedel-de Haen), Folin-Ciocalteu reagent (Sigma), sodium nitrite/$NaNO_2$ (Merck), aluminium cloride/$AlCl_3$ (Merck), aquades, n-butanol (Merck), quercetin, gallic acid (Merck).

Extraction and fractination method

The leaves of *Pluchea indica* were extracted by modifying Dorman and Hiltunen procedure (2004). The leaves powder of *Pluchea indica* (40 mesh) were macerated with petroleum ether (1:4 w/v) at room temperature for 2 hours. Then dried residue was extracted with methanol (1:15 w/v) using soxhlet extraction at a temperature of 65^{o}C for 3 hours. Methanol solvent was evaporated with rotary evaporator. The extract was fractionated with ethyl acetate and water solvents (1:1 v/v). Then water phase was fractionated again with n-butanol solvent (1:1 v/v). The ethyl acetate fraction, n-butanol fraction and water fraction were lyophilized by evaporating the solvent with a rotary evaporator. Each extract and fractions were stored at 4° C until subsequent analysis, respectively.

Extraction and Fractionation process of *P. indica* leaf using the procedure of Dorman and Hiltunen[16]. *P.indica* leaf powder (40 mesh size) in macerated with petroleum ether (1:4 b/v) at room temperature for 24 hours, then dried residue extracted with methanol (1: 15 b/v) using soxhlet extraction at a temperature of 65^{o}C for 3 hours. Methanol solvent was evaporated with a rotary evaporator. The extract obtained was fractionated with ethyl acetate and distilled water (1:1 v/v). Next, distilled water phase was fractionated with n-butanol

solvent (1:1 v/v). Solvents in fraction of ethyl acetate, n-butanol, and distilled water were evaporated with rotary evaporator, each extract and fractions were stored at 4°C until next analysis

Total Phenolic Contents

The total phenolic contents of *Pluchea indica* ekstract were determined using spectrophotometer according to Folin-Ciocalteu method, gallic acid was used as a standard (the concentration range 0.125 to 0.625 mg/mL)[17,18]. The reaction mixture was prepared by mixing 1 mL of the methanolic solution of the extract, 9 mL of distilled water, 1 mL of Folin-Ciocalteu reagent and 10 mL of 7% sodium carbonate.. After 90 min, the solution absorbance was measured in 765 nm wavelength against a blank consisting distilled water and Folin-Ciocalteau reagent. The total phenolic content was expressed as gallic acid equivalent (GAE) using calibration curve.

Total Flavonoid Contents

The total flavonoid content was determined according to the aluminium chloride colorimetric method, as described by Kumar *et.al*[19]. An aliquot of 1 mL of sample was mixed with 4 mL of distilled water and added with 0.3 mL of a 5% $NaNO_2$ solution (w/v). After 5 min added with 0.3 mL of 10% AlCl3 solution (w/v). After 6 min, the mixture was added with 2 mL of 1 mol/L NaOH and diluted to a total of 10 mL. The absorbance of the supernatant solution was measured at 352 nm against a methanol blank. The total flavonoid content was determined as quercetin equivalent (QE) using calibration curve.

Results and Discussion

Total phenolic contents

The measurement of total phenolic content of P. indica leaves extract with methanol solvent, ethyl acetate fraction, n-butanol fraction and water fraction at various altitudes habitats are shown in Table 1.

Table 1. Total phenolic contents of *P.indica* (mg/mL) with various solvent and height habitat

Location	Methanol extract	Ethyl acetate fraction	n-Butanol	Water fraction
Lowland	0.421±0.0026	0.475±0.0106	0.485±0.0321	0.382±0.0269
Middle altitude land	0.365±0.0101	0.400±0.3350	0.356±0.1026	0.335±0.0126
High land	0.298±0.0086	0.362±0.0242	0.219±0.0463	0.333±0.0164

The results analysis of variance (ANOVA) showed that there was a significant mean difference between total phenolic *P. indica* the methanol extract solvent, ethyl acetate fraction, n-butanol fraction and water fraction (14.230 F count > F table 3.008). Tukey test results showed that the fraction of water, butanol and methanol extract fraction are not significantly different, but the three significantly different from fraction of ethyl acetate. Ethyl acetate fraction have the highest phenolic content, while the lowest levels of phenolic contained in the water fraction.

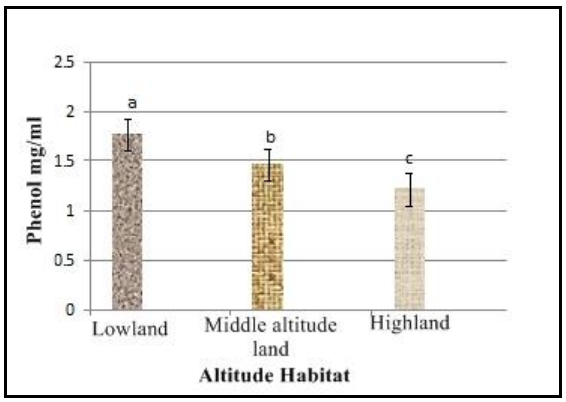

Figure 1. Total phenolic content of P. indica from various height locations

The total phenolic content of *P. indica* are significantly different (ANOVA) on leaf samples taken from three locations with different heights (106.374 F count> F table 2.508). Tukey test results showed that the highest phenol content was obtained from *P. indica* which lived in the lowlands (Bangkalan) of 1.763 ± 0.047 mg / ml, then the middle-highland (Trawas) of 1.455 ± 0.295 mg / ml, while the lowest phenolic content contained in plateau (Batu) amounted to 1.212 ± 0.608 mg / ml as shown in Figure 1.

Analysis of variance also indicate significant interaction between the sampling locations with variety of solvent extract (12.063 F count> F table 3.402). Interaction between height habitat and the solvent is shown in Figure 2. From the interaction plot (Figure 2) it is known, that the solvent methanol extract, n-butanol fraction, ethyl acetate fraction and water fraction *Pluchea indica* have the highest total phenolic content in leaf samples taken from the lowland (Bangkalan), while the lowest was obtained from leaf samples from the highland (Batu).

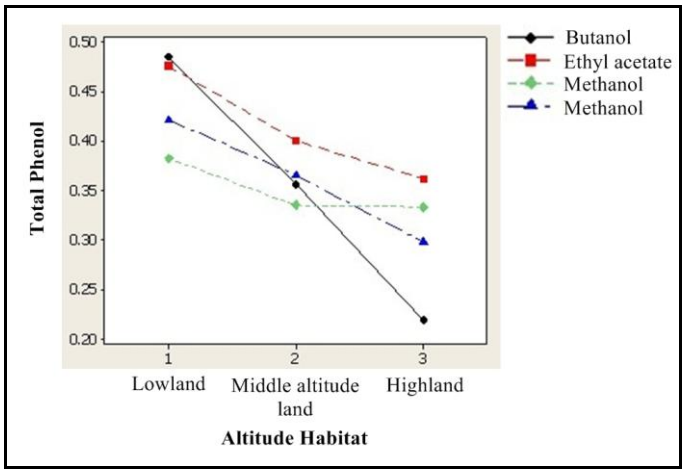

Figure 2. Interaction plot between sampling location with phenolic extraction solvent: Lowland (Bangkalan); Middle altitude land (Trawas); Highland (Batu)

Flavonoid contents

Results of measurement of total flavonoids content of *P. indica* leaves with methanol extract solvent, ethyl acetate fraction, n-butanol fraction and water fraction at various heights habitats are shown in Table 2.

Table 2. Total flavonoid content (mg/mL) of *P.indica* with various height locations

Location	Methanol extract	Ethyl acetate fraction	Butanol fraction	Water fraction
Lowland	0.97±0.057	0.97±0.057	0.64±0.057	0.57±0.057
Dataran menengah	0.77±0.057	0.90±0.000	0.60±0.010	0.73±0.057
HIghland	0.87±0.057	0.97±0.057	0.60±0.000	0.73±0.057

The results of ANOVA shows that there are differences in the total flavonoid content of *P. indica* significantly between methanol, butanol, ethyl acetate and water fraction (66.083 F count > F table 3.008). Tukey test results showed that butanol fraction and water fraction are not significantly different, but water and butanol fractions significantly different with ethyl acetate and methanol. Ethyl acetate fraction have the highest total flavonoid while the lowest was the total flavonoid fraction of butanol.

The total flavonoid contents of *P. indica* is not significantly different (ANOVA) on leaf samples taken from three locations with different heights (1.75 F count < F table 2.508). Total flavonoid of *Pluchea indica* taken on lowland of 3.1 ± 0.019 mg / mL, middle-highland of 3.0 ± 0.012 mg / mL, and highland of 3.2 ± 0.015 mg / mL.

Analysis of variance also showed significant interaction between sampling location with different solvents of leaf extract (5.750 F count> F table 3.402). Interaction between height habitat and solvent is shown in Figure 3.

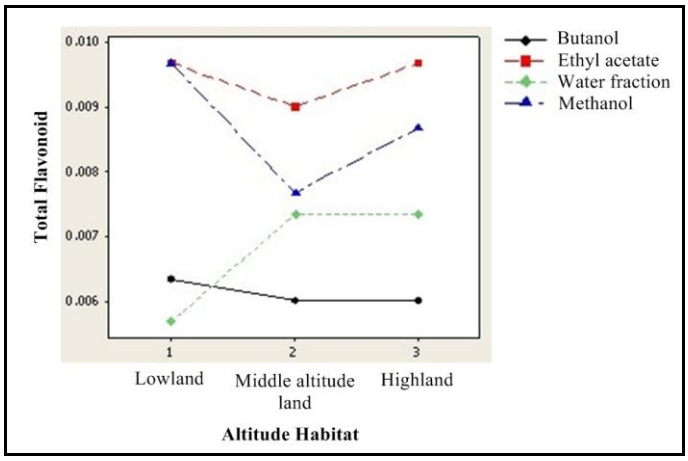

Figure 3. Plot interaction between the location of the leaves in various altitudes habitats and solvent extract of flavonoids: Lowland (Bangkalan); Middle altitude land (Trawas); Highland (Batu)

The interaction plot in Figure 3 shows that the methanol extract of *P. indica* has the highest total phenolic content in the lowland, while the lowest total flavonoid content was in middle-highland. N-butanol fractions of *P. indica* has a maximum content of flavonoids in the lowlands (Bangkalan), and the lowest levels of flavonoids contained in the highlands (Batu). Flavonoid fraction of water has a maximum in the middle-highland and highland, and the lowest is in lowland. Ethyl acetate fraction on *P. indica* has a maximum content of flavonoids in the lowlands and highlands, whereas the lowest is in middle-highland. Total phenolic and flavonoid compounds as a whole are shown in Figure 4.

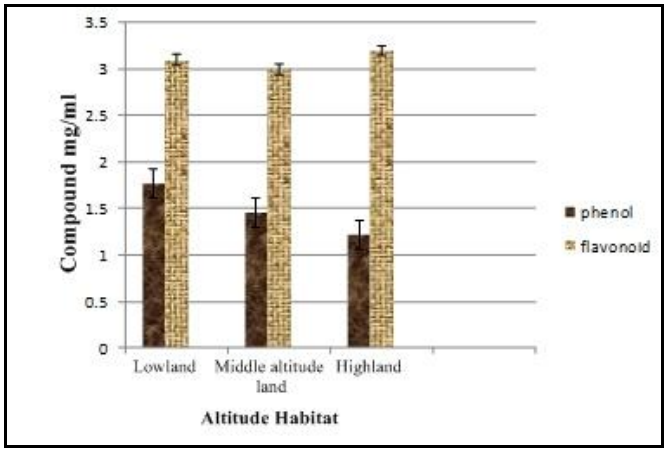

Figure 4. Total phenolic (Gallic acid) and flavonoid (Quercetin) content of *P. indica* in various locations

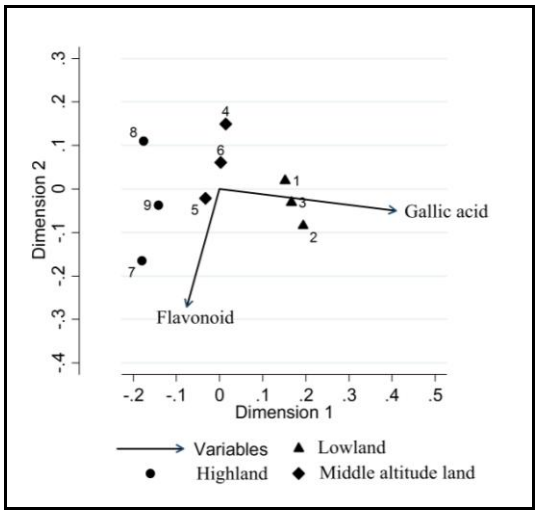

Figure 5. Relationship between the height habitat with total phenolic and flavonoid content: Lowland (Bangkalan); Middle altitude land (Trawas); Highland (Batu)

Total phenolic compounds in *P. indica* in the lowland amounted to 1.763 ± 0.047 mg / mL), middle-highland of 1.455 ± 0.295 mg / mL and highland of 1.212 ± 0.608 mg / mL. The total flavonoid compounds in *P. indica* showed the lowlands of 3.1 mg / mL, in the middle-highland (3.0 mg / mL), and total flavonoid on the highland of 3.2 mg / mL. Biplot among habitat with total phenolic and flavonoid contents can be seen in Figure 5.

Figure 5 showed that the highest total phenol *Pluchea indica* there are areas of lowland (Bangkalan), and the lowest was in the highland (Batu). The total flavonoid content of tend to be higher in highland and lowland. Thus, the higher a region, the smaller the total phenol produced. So the the cultivation of *Pluchea indica* should be done in the lowland.

Discussion

Pluchea indica plants more life in the lowlands and middle plains, while in the highlands rarely find plants of *P. indica*. This condition makes the growth of *P. indica* is strongly influenced by environmental factors. In this study, altitude habitat affects the levels of phenolic compounds (gallic acid and quercetin) on crop plants of *P. indica*, namely lowland (Bangkalan Madura) had the highest phenol content, followed by intermediate plateau (Trawas) and plateau (Batu).

The total phenolic and flavonoid contents are higher in lowland (Bangkalan, Madura) is supported by *P. indica* growth data which showed that *P. indica* leaf area in the lowlands amounted to 18.182 cm, in the middle area of 10.173 cm and the highlands of 3:57 cm. The broad leaf, has a high rate of photosynthesis ability, so it will supply more C elements required for synthesis of secondary metabolites compounds.

Altitude someplace affect climate (including the intensity of light, the quality of radiation, temperature, humidity, precipitation, CO2, O2 concentration, season, day length), and also affects the soil conditions (chemical, physical and biological soil). Thus the height of a plant habitat ultimately affect plant growth both primary and secondary metabolism metabolism. Many species of plants have adapted well to different environments, through different strategies. One of such strategy is to increase the production of secondary metabolites[4].

The diversity of secondary metabolites in plants caused no interaction between plants with ever-changing environment produces a variety of compounds to defend itself against environmental abiotic and biotic environment. The compound is the result of metabolic adaptation of plants to the environment. The secondary metabolites play several important functions in plants, including structural role in various supporting tissue and protective, and engagement in the defense strategy (herbivores and pathogens)[3,20].

Sharafzadeh and Kourosh[21], explained that the secondary metabolism was formed through a specific path of the primary metabolism, and its existence as a response to the stimulus, so that factors affecting primary metabolism influenced the presence of secondary metabolites. The potential of secondary metabolism (production, persistence and effectiveness) of a source organism and its effect on the target organism has a diversity that is generally caused by genetic or environmental factors. The influence of environmental factors need to be emphasized because of their interaction with genetic factors in the phenotypic expression of secondary metabolites[22].

Environmental factors affecting the availability of primary and secondary metabolites. The concentration of primary and secondary metabolites in a plant depends on variations in organ, tissue, period developments and the influence of environmental factors such as temperature, UV, light, nutrients, water availability and the concentration of CO_2 in the atmosphere[21]. Other factors that affect plant growth are organic matter and nutrients in the soil[2,23]. Soil organic matter and mineral soil has a primary function as a supplier of nutrients for plants and soil biota.

Effect of habitat on the content of secondary metabolites shown by research Pujiasmanto et.al.[24] which shows the content of androgapolid in *Andrographis paniculata* in middle-highland (2.27%) higher than in the lowland (1.73%) and highland (0.89%). Singh et al.,[25] reported that total phenolic compounds in *Pluchea lanceolata* that grows on cultivated land much more than the land that is not cultivated. Similarly, the quercetin was found on land treated but not found on land not cultivated. The higher the solar radiation on the highland have an impact on secondary metabolite profiles. For example the production of phenolic compounds increased in response to the increasing UV radiation[4]. Research on *Hypericum perforatum* L. growing under temperature and different light intensities showed that the accumulation of secondary metabolites is very depended on temperature, light intensity and phenological

cycle. The amount of phenolic compounds greatly changed during the development of the plant, and the highest level achieved in flowering phase with the intensity of light and higher temperatures[26]. The elevated ozone (mean 32.4 ppb) increased the total phenolic content of leaves and also has minor effects on the concentration of the individual compound[27].

Inderjit and Keating[28] reported the content of secondary metabolites (alelopati) are varied from one location to another and from one time to another time. It related to variations in climate conditions and soil such as air and soil temperature and soil moisture. Mazid et al.[29] previously described that environmental stresses caused by biotic and abiotic factors affecting the production of secondary metabolites, and in general tend to increase the production of secondary metabolites. So that when plants are stressed, an exchange occurs between carbon to biomass production or formation of defensive secondary compounds. A stress response is induced when plants recognizes stress at the cellular level. Secondary metabolites are involved in protective functions in response to both biotic and abiotic stress conditions[30].

The existence of the organic matter in the soil supporting also secondary metabolites in plants. The expression of secondary metabolites in the field is influenced by soil texture, nutrients, pH, organic C, soil processing techniques and cropping systems. Nitrogen and phosphate deficiency is directly affect the accumulation of phenylpropanoid. Potassium deficiency (K), sulfur (S), Magnesium (Mg) and iron (Fe) may also increase phenolic concentration and increase phenolic release in nature[22,30].

Phenol biosynthesis is catalyzed, one of them, by Phenylalanine ammonium lyase (PAL). PAL is the ramification between primary and secondary metabolism, so the catalysis reaction are an important step in the formation of many phenolic compounds. PAL activity can be increased due to several factors, such as low levels of nutrients, light (through its effect on phytochrome), and microbial infections. The control point is at the initiation of transcription. Invasion of fungi, for example, trigger the transcription of RNA messenger which encode PAL, thereby increasing the number of PAL in the plants, then stimulates the synthesis of phenol compounds. The PAL activity in plants is regulated by various genes encoding PAL in many species, and some expressed only in certain tissues or under particular environmental conditions[2,5]. Most enzymes such as PAL, induced by stress and play an important role in plant protection. PAL along with cinnamate 4-hydroxylase is an important group of enzymes in allocating large amounts of carbon from phenyl alanine into the biosynthesis of several important secondary metabolites[20]. The previous study showed that plants exposed to drought stress resulted in a large number of secondary products such as phenols, terpenes as well as substance N and S such as alkaloids, cyanogenic glucosides or glucosinolate. Generally when plants are stressed, secondary metabolite production may increase because growth is often inhibited more than photosynthesis, and the carbon fixed not allocated to growth is instead allocated to secondary metabolites[31,32].

Conclusion

The total phenolic content of *Pluchea indica* lowland (Bangkalan) higher (1.763 ± 0.047 mg/mL) compared with *Pluchea indica* which grows in the middle-highland (1.455 ± 0.295 mg/mL) and highland (1.212 ± 0.608 mg/mL). The total flavonoid contents in *Pluchea indica* showed no mean difference content flavonoids in the highlands (3.2 mg/mL), middle plateau (3.0 mg/mL) and lowland (3.1 mg/mL). Ethyl acetate fraction on *P. indica* has the highest total phenolic and flavonoid content.

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