Effects of Humic Acid and Planting Media on Antioxidant Production in the Medicinal Plant Valerian (*Valeriana officinalis* L.)

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Summary

Valerian (*Valeriana officinalis* L.) is a medicinal plant that contains active compounds such as flavonoids and alkaloids that are important as antioxidants. The aim of this research was to determine the effect of humic acid concentration in different planting media on the antioxidant content. The main factor of this experiment was the concentration of humic acid added to the planting media at concentrations of 0, 4, 8, and 12 g kg^{-1}. The second factor was the planting medium, of which there were three types: sand/soil (1 kg kg^{-1}), husk/soil (1 kg kg^{-1}), and zeolite/soil (0.5 kg kg^{-1}). We determined the biomass, antioxidant content (polyphenols, flavonoids, and alkaloids), and IC_{50} values of valerian plants grown in various media. Our results show that the combination of the humic acid and planting medium affected the antioxidant content and activity in valerian plants. In particular, the planting medium containing zeolite and 8 g kg^{-1} of humic acid increased the antioxidant content and activity. From the result it can be concluded that a combination of humic acid and zeolite medium can be applied for valerian's farmer.

Key words

alkaloids, flavonoids, humic acid, polyphenols, IC_{50}, valerian

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Introduction

Valerian (*Valeriana officinalis* L.) is a medicinal plant that grows from thick rhizomes and has dark green, lanceolate-shaped serrated leaves 5–8 cm long spreading from a central rosette and a 1–1.5 m tall flowering stem with clusters of flower buds (Pilerood and Prakash, 2013). The plant originated in Europe and Asia (Nandhini et al., 2018) and has been widely cultivated in Indonesia. Valerian contains several active compounds, including polyphenols and alkaloids, which are secondary metabolites that function as antioxidants (Shakeel et al., 2019) and serve as medicinal raw materials (Nandhini et al., 2018) Valerian has been used clinically as a sedative to treat anxiety and agitation, and as a mild sedative to improve sleep quality (Nandhini et al. 2018)). Previous studies have reported a wide variety of compounds in valerian, including essential oils, iridoids, flavones, alkaloids, amino acids, and lignans (Wiśniewski et al., 2016) Concentration of active constituents that impart a health benefit to consumers is the important determinant of quality in valerian raw material. However, the enhanced production of secondary metabolites per unit of inputs of medicinal plants is the prime requirement for quality raw drug production. Secondary metabolite production is regulated by nutrition, growth rate, enzyme activity and enzyme induction. Nutritional limitation and a consequent decrease in growth rate produce regulatory signals that cause changes in secondary metabolites and plant morphology (Tiago et al. 2017)).

According to (Li, 2020) effort should be conducted not only to indicate the best yielding, and content of active substances but also to determine the principles of growing, including identification of the planting medium and fertilizer, which determines the high quality of raw material. Planting media can also be modified to improve soil texture and aeration to increase nutrient availability. The cation exchange capacity of the planting medium can be increased by the addition of sand, husk, or zeolite to the soil. Sand and husk provide high porosity, while zeolite has a specific hollow crystal structure. The pore size of zeolite provides cavities that can be filled with water providing it with molecular filter, ion exchange and absorption properties (Man-hong et al., 2020) Rice husk (referred to as husk hereinafter) is added to planting media to increase soil porosity and is commonly used for potted plants. By adding husk, a planting medium becomes more easily penetrated by water due to an improved balance between microporosity and macroporosity. The addition of husk to planting media can also increase air circulation, allowing the soil to absorb more water

Humic acid affects soil permeability and water-binding capacity and confers a high exchange capacity that helps move nutrients from the soil to plants (Karami et al. 2020) to enhance plant growth (Rafeii and Pakkish, 2014),). Canellas and Olivares (2014) stated that humic substances increase plant growth, especially in the root system. Humic acid is an organic material that has a positive effect on plant physiology by improving soil structure and increasing soil fertility, thereby affecting nutrient uptake and root architecture (Mindari et al. 2014)). Recent research showed that the addition of humic acid increases antioxidant content in *Capsicum annuum* L. (Aminifard et al., 2012) *Thymus vulgaris* L. (Juárez et al. 2011), *Vicia alba* Medik. (Dawood et al. 2019), *Lactuca sativa* L. (Shahein et al. 2015),

In this study, we assessed the antioxidant content and activity of valerian plants grown in various planting media supplemented with various concentrations of humic acid. We also examined the potential of planting media to increase the antioxidant content and activity in valerian.

Materials and Methods

Materials

The materials used in this study were valerian seeds, soil, sand, rice husk, zeolite and humic acid. The seeds were obtained from local farmers and the humic acid used was AH-90 (Malang).

Methods

This study figwas conducted from February to October of 2019 in a greenhouse at the Plant Physiology Laboratory of the Faculty of Biology, Jenderal Soedirman Univerity, Purwokerto Central Java, Indonesia.

The study used a completely randomized design with a two-factor factorial pattern. The main factor was the concentration of humic acid, which was added to the planting media at concentrations of 0, 4, 8, and 12 g kg^{-1}, and the second factor was the planting medium of which there were three types: sand/soil (1 g kg^{-1}), husk/soil (1 g kg^{-1}), and zeolite/soil (0.5 g kg^{-1}). The parameters observed were plant biomass, antioxidant content (polyphenols, flavonoids, and alkaloids) and half-maximal inhibitory concentration (IC_{50}) values.

Sample Extracts

Samples were extracted by the maceration method. A dried plant sample (10 g) was placed in a maceration tube with 300 mL of ethanol solvent and allowed to soak for 24 h. The resulting extract was collected in a tube. The maceration process was performed three times. The collected extract was evaporated in a rotary vacuum evaporator until dry. The dried sample extract was used for analyses of antioxidant content and activity.

Polyphenol Content Determination

Polyphenol levels were determined by spectrophotometry according to the procedure of Malik and Achmad (2015) with some modifications, using gallic acid as a standard. Gallic acid standard solutions with concentrations of 10, 20, 30, 40, and 50 ppm were prepared. Folin-Ciocalteu reagent (0.4 mL) was added to each gallic acid standard solution, shaken and allowed to stand for 4–8 min followed by the addition of 4.0 mL of 7% Na_2CO_3 solution with shaking until mixed homogeneously. Aquadest was added up to 10 mL and allowed to stand for 2 h at room temperature. Absorbance was measured by UV-Vis spectrophotometry at a wavelength of 744 nm, and a calibration curve of gallic acid concentration (μg mL^{-1}) versus absorbance was made.

Extract solutions for polyphenol content determination were prepared by dissolving 10 mg of extract in 10 mL of pure methanol. Extract solutions (1 mL) were subjected to the same process as the standard solutions as described above and the absorption was measured at 744 nm. The phenol content was determined as mg equivalents of gallic acid/g of extract.

Flavonoid Content Determination

Flavonoid contents were determined by spectrophotometry using quercetin standards. Standard quercetin solutions were prepared by diluting 50 µg mL^{-1} of quercetin with 5% glacial acetic acid (in methanol) to produce concentrations of 3, 6, 12, 15, and 24 µg mL^{-1}. Absorption was measured at a wavelength of 370 nm to create a standard curve of quercetin concentration versus absorbance.

Extract solutions for flavonoid concentration determination were prepared by dissolving 15 mg of extract in 10 mL of ethanol to give a concentration of 1500 ppm. Aliquots (1 mL) of the extract solutions were then combined with 1 mL of 2% $AlCl_3$ solution and 1 mL of 120 mM potassium acetate. The samples were then incubated for 1 h at room temperature. Absorbance was determined at 435 nm. Three replicate samples were used for each analysis and average absorbance values were calculated (Stanković, 2011)

Alkaloid Content Determination

For each extract sample (100 µL), 50 µL of concentrated H_2SO_4 was added and vortexed followed by the addition of 100 µL of Dragendorff's reagent and vortexed again. Color changes were observed after 30 min producing light yellow, yellow and brown colors. Absorbance was measured with a spectrophotometer and concentration calculations were performed using the formula: concentration = absorbance of standard/absorbance of sample × 15.35%. The standard absorbance was 0.387 (Van Tan, 2018)

Determination of IC_{50} Values

Antioxidant activity was determined by the DPPH method. A DPPH stock solution (160 ppm), a stock solution of *n*-hexane extract (437.5 ppm), an ethyl acetate stock solution (20 ppm), 96% ethanol (10 ppm), and a vitamin C positive control (10 ppm) were prepared. DPPH was added to a series of extract and positive control concentrations and the absorbance were measured at a wavelength of 517 nm (Sebaugh 2011).

Statistical Analysis

To see the effect of treatment in general, the F test was conducted at $P \leq 0.05$ significance level using ANOVA and followed by the Least Significant Difference (LSD) Test at at $P \leq 0.05$ significance level.

Results and Discussion

The addition of HA showed that all planting media resulted in different biomass (F 2,8 $P < 0.05$). (Table 1). This finding is consistent with previous studies that indicated that HA affected the biomass as shown by Mindari et al., (2014) at corn, Kaled, and Fahwy (2011) at soil salinity, Ahmad et al (2013) at gladiolas.

Antioxidant activity improved with increasing HA levels, and the type of soil and the content of humic compounds in the soil can have a decisive effect: the higher the content of humic acid in the soil, the stronger antioxidant activity. (Aminifard et al. 2012) (Shahein MM et al., 2015) found that the addition of humic acid increased plant growth and chemical content of lettuce. The utilization of humic acid could positively change nutrient uptake, essential oil content and its major constituents in *T. vulgaris* (Dawood et al. 2019)

Table 1. Effects of humic acid concentration at different planting medium on biomass, polyphenol, and flavonoid content of valerian (*Valeriana officinalis* L.)

Humic acid	Media	Biomass (g)	Polifenol (ppm)	Flavonoid (ppm)	Alkaloid (ppm)
H0	Sand	6.41±0.48 a	28.45±0.22 a	29.15±4.56 a	6.41±0.48 a
	Rice ash	6.44±0.38 a	37.87±1.27 b	37.67±1.55 b	6.44±0.38 a
	Zeolite	7.22±0.67 b	49.13±1.98 d	45.92±3.04 c	7.22±0.67 b
H4	Sand	6.56±0.76 a	34.58±0.46 e	34.39±1.44 d	6.56±0.76 a
	Rice ash	6.41±0.21 a	41.98±2.58 f	40.58±0.90 e	6.41±0.21 a
	Zeolite	6.79±0.27 c	42.13±1.11 f	41.46 ± 2.56 e	6.79 ± 0.27 c
H8	Sand	6.61 ± 0.91 c	42.79 ± 1.84 f	38.67 ± 0.94 b	6.61 ± 0.91 c
	Rice ash	6.82 ± 0.34 d	44.88 ± 2.49 g	40.88 ± 0.48 e	6.82 ± 0.34 bc
	Zeolite	6.92 ± 0.23 e	52.71 ± 3.44 i	42.22 ± 2.77 e	6.92 ± 0.23 bc
H12	Sand	6.14 ± 0.76 f	41.49 ± 2.50 f	38.11 ± 0.89 b	6.14 ± 0.76 a
	Rice ash	6.69 ± 0.52 c	43.11 ± 2.20 f	37.62 ± 0.48 b	6.69 ± 0.52 c
	Zeolite	7.09 ± 0.16 e	46.62 ± 1.63 j	41.68 ± 0.83 e	7.09 ± 0.16 bc

Note: H0, H4, H8, H12 - planting media with humic acid concentration of 0, 4, 8, and 12 g kg^{-1}, respectively. Values marked by the same letter within the column are not statistically significant according to Least Significant Difference (LSD) Test at $P \leq 0.05$ significance level

The antioxidant content of valerian plants tended to be higher in soil containing zeolite, ($F = 4.12$ $P < 0.05$) which is because the primary usage of zeolites in agriculture is to absorb nitrogen, hold it and release it slowly. Zeolites, with their specific discrimination for ammonium (NH), can absorb this particular cation from manure, compost, or ammonium-containing fertilizer, thus reducing nitrogen losses to the environment (Karami et al., 2020). (Luis Moreno et al., 2017) suggested that the zeolite particles stimulated the biosynthesis of polyphenols and alkaloids; however, plant growth was not improved. A high antioxidant content does not always result in increased growth. It is thought that the biosynthesis of acetic acid from pyruvic acid does not always produce flavonoids and alkaloids but can be directed to produce other compounds instead. (Wiśniewski et al., 2016) assessed valerian growth in various planting media and found that under conditions for yielding good growth, lower amounts of valeric acid were produced.

The planting media that incorporated sand and husk into the soil supported plant growth as evidenced by higher biomass. This result may be because the sand composition of a planting medium can increase the porosity and aeration of the soil and optimize the nutrient absorption process (Badar and Qureshi 2014). The combination of humic acid and sand medium has a positive effect on growth parameters, photosynthetic pigments, seed yield and yield components as well as some biochemical constituents. This result is in accordance with Dawood et al. (2019) of the yielded faba bean seeds. The most promising treatment appeared due to the interaction between nicotinamide at 10 mg L^{-1} and humic acid. (Ezyan Badrul Hisham and Hanuni Ramli, 2019) report that the addition of husk charcoal to planting medium functions as a nutrient binder, which allows excess nutrients to be released slowly according to plant needs.

The planting medium containing zeolite with the addition of 8 g kg^{-1} of humic acid increased the antioxidant content, while the highest flavonoid content was obtained with the same medium without the addition of humic acid. We observed a correlation between polyphenol content and flavonoid content (Fig. 1).

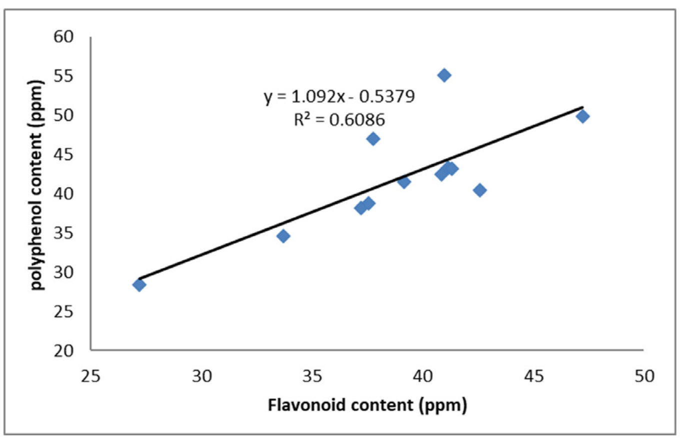

Figure 1. Correlation between flavonoid and polyphenol contents in valerian (*Valeriana officinalis* L.) plants

Because flavonoids are polyphenol compounds, the total flavonoid content is often associated with the antioxidant activity of plant extracts. Individual flavonoid compounds have different antioxidant potentials and do not always contribute to the total antioxidant activity of plant extracts.

Alkaloids are nitrogen-containing products of plant metabolism and are used as a reserve for protein synthesis ((Ekin, 2019)); therefore, alkaloid content is related to plant biomass.

Plants grown in the planting media containing husk with various concentrations of humic acid exhibited high biomass, as well as a high alkaloid content. Planting media containing zeolite can increase nutrient availability because zeolite has a high cation exchange capacity (Luis Moreno et al. 2017), which improves the availability of nutrients, including nitrogen. Antioxidants can be produced from primary metabolites in the form of monosaccharides and pyruvic acid which contribute to the production of flavonoids through the malonic acid pathway and alkaloids through the aliphatic amino acid and aromatic amino acid pathways (Pilerood and Prakash, 2013).

Our analysis of antioxidant activity based on IC_{50} values generated by the DPPM method showed that the smaller the IC_{50} value, the higher the antioxidant activity. The IC_{50} values of valerian extracts made from plants grown in the various planting media are shown in Table 2. IC_{50} values of < 50, 50–100, 101–150, and 151–200 correspond to very strong, strong, moderate and weak antioxidant activities, respectively, of a compound (Moharram and Yossef, 2014). Since the IC_{50} value is the effective concentration of an extract needed to reduce 50% of the total DPPH in the assay, the value of 50 is substituted for the value of y in the equations shown in Table 2, and solution/result for the value of x gives the IC_{50} value.

Table 2. IC_{50} values of valerian (*Valeriana officinalis* L.) extracts from plants grown in various planting media

Treatment	Line equation	y value	IC_{50}
Vitamin C positive control	$y = 1.6611x - 0.6$	50	30.46
Soil/sand without humic acid	$y = 0.0313x + 0.01$	50	159.7
Soil/sand + humic acid (4 g kg^{-1})	$y = 0.0408x + 0.18$	50	122.1
Soil/sand + humic acid (8 g kg^{-1})	$y = 0.3362 + 28.775$	50	63.13
Soil/sand + humic acid (12 g kg^{-1})	$y = 0.3076x + 38.9$	50	36.09
Soil/husk without humic acid	$y = 0.3501x - 4.63$	50	156.04
Soil/husk + humic acid (4 g kg^{-1})	$y = 0.4162 + 22.105$	50	67.02
Soil/husk + humic acid (8 g kg^{-1})	$y = 0.8238x + 6.425$	50	52.90
Soil/husk + humic acid (12 g kg^{-1})	$y = 0.3534x + 34.805$	50	43.00
Soil/zeolite without humic acid	$y = 0.248x + 42.40$	50	30.67
Soil/zeolite + humic acid (4 g kg^{-1})	$y = 0.740x + 0.14$	50	67.38
Soil/zeolite + humic acid (8 g kg^{-1})	$y = 2.0063x + 17.86$	50	16.02
Soil/zeolite + humic acid (12 g kg^{-1})	$y = 0.5709 - 1.98$	50	91.05

As shown in Table 2, IC_{50} values < 50 were obtained for extracts from plants grown in the planting media containing sand/soil with 12 g kg^{-1} of humic acid added and zeolite/soil without humic acid, with the highest antioxidant activity obtained from plants grown on zeolite/soil with 8 g kg^{-1} of humic acid. High antioxidant levels corresponded to high antioxidant activity, indicating that polyphenols, flavonoids, and alkaloids contributed to antioxidant activity. According to (Tiago et al., 2017), a high alkaloid content reflects high antioxidant activity.

Conclusions

The addition of humic acid to the planting media significantly affected the antioxidant content and antioxidant activity in valerian. In particular, the planting medium consisting of a mixture of zeolite and soil with the addition of 8 g kg^{-1} of humic acid effectively increased the antioxidant content and activity in valerian plants.

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