The Effect Of NaCl Salinity Stress To Phenolic Compound, Total Flavonoid And Antioxidant Activity Of Pegagan (Centella asiatica (L.) Urban) Leaves

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Abstract. Pegagan (Centella asiatica (L.) Urban) is a herbal plant that contain secondary metabolite compounds like phenol and flavonoid. NaCl salinity is one of abiotic stress that enhanced synthesis of some secondary metabolites in plants. This study was investigated the effect of NaCl salinity stress to phenolic compound, total flavonoid and antioxidant activity of pegagan leaves. Pegagan were treated with five different NaCl concentrations, 0 mM (1), 50 mM (2), 100 mM (3), 150 mM (4) and 200 mM (5) for a week. Morphological leaves were observed for the present of necrotic symptom. Phenolic compound and total flavonoid content were measured using spectrophotometer at wavelength 765 nm and 415 nm. Antioxidant activity was measured based on DPPH method. The result showed that increasing NaCl concentration cause increasing necrotic spot in leaves. Phenolic compound, total flavonoid and antioxidant activity are increased by increasing NaCl concentration. The result indicated that phenol and flavonoid have important role in plant defense mechanism against NaCl toxicity effects.

1 Introduction

Pegagan (*Centella asiatica* (L.) Urban) is a herbal plant that have large biological activities [8]. Those plant contain secondary metabolites like terpenoid, phenol and flavonoid [24]. Secondary metabolites in pegagan can be found in root, petiole, and leaves while leaves contain highest phenolic compound and antioxidant activity than other part [13]. The present of secondary metabolites make pegagan have important role in medicinal field and industry. Jahan et al., [17] said that in China, Afrika and India pegagan has been used as traditional medicine for more than 3,5 century e.g. wound healing, headache, fever, constipation, dysentry, stomach disorder, etc. Furthermore, pegagan has been processed into several products for modern medicine and cosmetic. Those products are Mandukaparni, Mentat, Gertiforte (Geri Care/Stress Care), Abana (Heart Care), Menosan, Nourishing Skin Care, SNP Control Cream, Eye Treatment Serum, Diamond Shiny Pearl BB, Organic Baby Skin Cream, Mandarin O2 Foaming Cleanser, Weight Loss Tea, and Anxocare [22].

Environmental factors influence the production and accumulation of secondary metabolite in plants. There are a lot of kind environmental factors sec as salinity, temperature, soil fertility, light etc [10]. Salinity is one of abiotic stress that caused by salt such as NaCl, Na_2SO_4, $CaSO_4$, $MgSO_4$, KCl, $MgCl_2$ and Na_2CO_3 which NaCl is dominan salt in the soil [5]. Soil are classified saline when have electrical conductivity (EC) minimum 40 dS/m or equivalent with 40 mM NaCl [20]. Salinity stress interfere biochemical and physiological processes.

Ashraf [11] said that salinity stress lead osmotic stress, decrease turgor cell, accumulation of amino acids and soluble sugar, decrease net photosynthetic rate, etc. On the other hand, several study reported that NaCl salinity treatment increased phenolic compound and flavonoid content in plant leaves [3, 15, 16, 19, 26].

Salt stress lead increasing ROS (Reactive Oxygen Species) production and caused oxidative damage in plant [12]. Phenol and flavonoid have important role in plant defense mechanism to against oxidative stress. Plant adaptation are affected by the balanced between phytochemical compound (phenol and flavonoids) and ROS [18].

Phenolic compound exhibit antioxidant activity by giving hydrogen atom and make free radical inactive [4]. Flavonoid also can be act as antioxidant to scavenge ROS and protect plant from oxidative damage [6]. Thus, increasing phenol and flavonoid content also increased antioxidant activity in plant.

The existence of both compounds make pegagan can be act as medicinal plant. Medicinal plant is source of natural antioxidant [27]. Researchers reported that antioxidant in leaves were increased by increasing NaCl salinity treatment [2]. However, no report has been studied about correlation between phenolic compound, flavonoid and antioxidant activity in pegagan leaves under NaCl salinity stress. So, the focus of this study was to investigated the relationship between phenolic

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compound, total flavonoid and antioxidant activity in pegagan leaves under NaCl salinity stress.

2 Materials and Methods

2.1 Plants material

Pegagan were collected from Magelang, Central Java, Indonesia. Pegagan seedlings with 4 leaves were planted in greenhouse for two month. Pegagan were treated with NaCl with 5 different concentration 0, 50, 100, 150 and 200 ppm after 7 week planting. NaCl solution watered to the soil medium for a week. Leaves were collected and dried in oven (50 °C). Leaves extract were obtained on watherbath. Phenolic compound, total flavonoid and antioxidant activity were recorded in methanol on spectrophotometer UV-Visible (Genesys 20).

2.2 Plant Extraction

The leaves of pegagan was dried and ground into powder. The powder (200 mg) was extracted by maseration with 20 ml 80% methanol (phenol and flavonoid) and pure methanol (antioxidant assay). The mixture was filtered after 24 h extraction with filter paper no 8, then the extract were concentrated by using water bath for about 20 menit.

2.3 Chlorophyll Content

100 mg fresh leaves was crushed using mortar and added 10 ml of aceton 80%. The solution filtered using filter paper no 8. 2/3 volume of kuvet are taken and measured the absorbance by spectrofotometer at wavelength 645 nm and 663 nm. Aceton 80% was used as blanko. Chlorophyll content calculated using this following equation [14].

$$\text{Chlorophyll a (mg/g)} = \frac{[(12.7 \times A663) - (2{,}69 \times A645)] \times V}{(1000 \times W)}$$

$$\text{Chlorophyll b (mg/g)} = \frac{[(22.9 \times A645) - (4{,}68 \times A663)] \times V}{(1000 \times W)}$$

$$\text{Chlorophyll total (mg/g)} = \frac{[(20.2 \times A645) + (8{,}02 \times A663)] \times V}{(1000 \times W}$$

2.4 Total Phenol and Flavonoid

Total phenol was measured based Folin-Ciocalteu method [9]. Leaves extract (100 µl) were added with water (5 ml) and Folin-Ciocalteu reagent (500 µl). After 8 min, 20% Na_2CO_3 (1.5 ml) was added and stand for 30 min in room temperature. The absorbance was measured at wavelength 765 nm. Gallic acid was used as standar solution (40 – 120 ppm) and prepared as above.

Total flavonoid was measured according to Sakthidevi and Mohan [7]. leaves extract (0.5 ml) was mixed with 0,1 ml of 5% $NaNO_2$. After 5 min added with 0.1 ml of 10% $AlCl_3$. After 5 min added with 0,1 ml 1M NaOH. Then, 80% metanol was added to make 5 ml. The absorbance was measured at wavelength 415 nm. Qurcetin was used as standard solution (20 – 100 ppm) and prepared as above.

2.5 Antioxidant Activity

Antioxidant activity was measured based on DPPH (2,2-diphenylpicrylhydrazyl) method [23] with modified. 1 ml of sample in methanol at four different concentration (40, 80, 120 and 140 ppm) was added into 2 ml of freshly prepared 0,1 mM DPPH solution and stand for 30 min in dark room. The absorbance was measured at wavelength 517 nm. Percentage of inhibition calculated using this following equation:

$$\% \text{ inhibition} = [(Abs_{control} - Abs_{sample}) / Abs_{control}] \times 100,$$

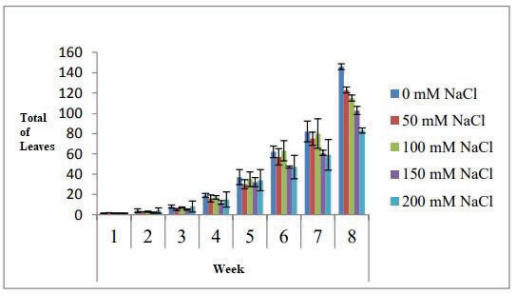
$Abs_{control}$ is absorbance of DPPH + methanol and Abs_{sample} is absorbance of DPPH + sample. Vitamin C (0.5 – 5 ppm) was used as positive control [25]. Antioxidant activity index (AAI) calculated using the following equation:

AAI = concentration of DPPH (µg/ml) / IC50 (µg/ml) [21].

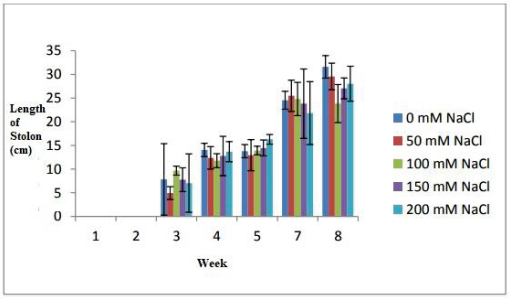
3 Result and Discussion

3.1 Plant Growth

NaCl salinity stress decrease plant growth. The result show that total leaves decrease after NaCl treatment (Figure 1.a). It is because ion Na^+ and Cl^- in cytoplasm increased by increasing NaCl concentration. Those ion inhibit metabolism process so many leaves fall. Except that, ion Na^+ and Cl^- also inhibit water absorption and reduce stolon length (Figure 1b). Water have important role in cell division and expansion.



(a)



(b)

Fig. 1. Total number of leaves (a) and length of stolon (b) in response to NaCl treatment

Decrease of plant growth followed by increasing necrosis spot in leaves (Figure 2). However, increasing total phenol and flavonoid followed by increasing necrotic spot in leaves. Necrosis due to the degradation of chlorophyll (Table 1) and change leaves colour become brown. In the other hand, necrosis also reduced leaves area for photosynthesis, thus decrease plant biomass (Table 2).

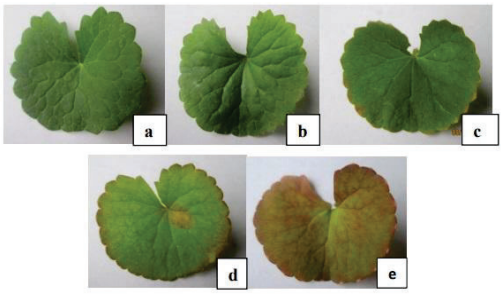


Fig. 2. Leaves necrosis in respons to NaCl treatment

NaCl treatment was given at five different concentration 0 mM (a), 50 mM (b), 100 mM (c), 150 mM (d) and 200 mM (e).

Table 1. Chlorophyll a, Chlorophyl b and Chlorophyll Total of Pegagan Leaves

NaCl Concentration (mM)	Chlorophyll Content **(mg/g) ± SD**		
	Chl a	**Chl b**	**Chl Total**
0	0.00168^{a} ± 0.00018	0.00051^{a} ± 0.00014	0.00219^{a} ± 0.00022
50	0.00133^{ab} ± 0.00024	0.00042^{ab} ± 0.00008	0.00175^{ab} ± 0.00033
100	0.00106^{bc} ± 0.00033	0.00035^{ab} ± 0.00009	0.00141^{bc} ± 0.00042
150	0.00101^{bc} ± 0.00026	0.00033^{ab} ± 0.00010	0.00133^{bc} ± 0.00035
200	0.0008^{c} ± 0.00022	0.00026^{b} ± 0.00006	0.00106^{c} ± 0.00027

Table 2. Biomass of Pegagan Leaves

NaCl Concentration (mM)	Wet Weight (gram) ± SD	Dry Weight (gram) ± SD
0	90.39^{a} ± 3.12	20.36^{a} ± 7.039
50	37.12^{b} ± 3.63	16.78^{ab} ± 5.26
100	46.50^{b} ± 6.13	9.35^{ab} ± 5.32
150	42.69^{b} ± 3.09	9.25^{b} ± 2.03
200	33.10^{c} ± 2.08	8.12^{b} ± 0.83

3.2 Total Phenol and Flavonoid

NaCl salinity stress arising oxidative stress and increased total phenol and flavonoid in plant. The result showed that total phenol and flavonoid content of pegagan leaves were increased by increasing NaCl concentration, although total phenol its not significantly different (Fig. 3 and Fig. 4). Salt stress also increase phenol total and flavonoid total in *Ae. cylindrical* leaves [17].

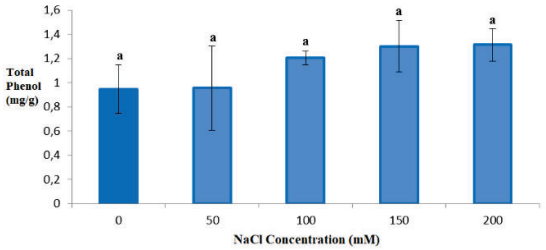


Fig. 3. Total Phenol Contents of Pegagan Leaves in Response to NaCl Treatment

Total phenol (mg/g DW) of pegagan leaves in response to NaCl treatment at five different concentration 0, 50, 100, 150 and 200 mM. Means (of 3 replicates) followed by bars with same letter are not significantly different (α = 0.05). Level as determined by analysis of variance (ANOVA).

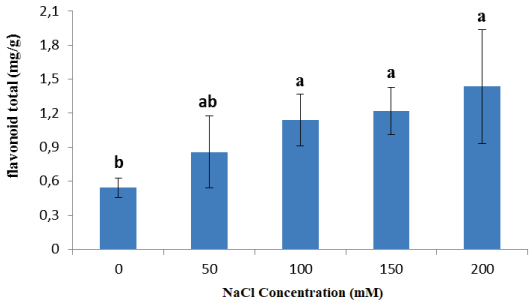


Fig. 4. Total Flavonoid Contents of Pegagan Leaves in Response to NaCl Treatment

Total flavonoid contents (mg/g DW) of pegagan leaves in response to NaCl treatment at five different concentration 0, 50, 100, 150 and 200 mM. Means (of 3 replicates) followed by bars with different letter are significantly different (α = 0.05). Level as determined by analysis of variance (ANOVA).

Phenol content in pegagan leaves (0.945 – 1.314 mg/g) higher than phenol content in *Anethum graveolens* leaves (0.23 – 1.06 mg/g) [25] and lower than *Rosmarinus officinalis* leaves (0.23 – 1.06 mg/g) [13]. However, flavonoid content in pegagan leaves (0.543 mg – 1.434 mg/g) lower than total flavonoid in *Colubrina asiatica* leaves (8.1 – 14.5 mg/g) [3] and *Origanum majorana* leaves (1.94 – 3.19 mg/g) [16].

Increasing total phenol and flavonoid indicated that those compound have important role in pegagan adaptation to salinity stress. Increasing those compound was related their function as antioxidant to prevent cellular damage as result of increasing ROS formation. Plant also increased synthesis of osmoprotectant and compatible solute to protect cellular component and osmotic potential in cytoplasm [11].

3.3 Antioxidant Activity

Antioxidant activities of pegagan leaves with five different concentration of NaCl treatment were studied by DPPH method. Free radical of DPPH are neutralized by interaction of antioxidants and DPPH through electron transfer [1]. The result showed that increasing NaCl salinity level increased antioxidant activity in pegagan leaves (Fig. 6).

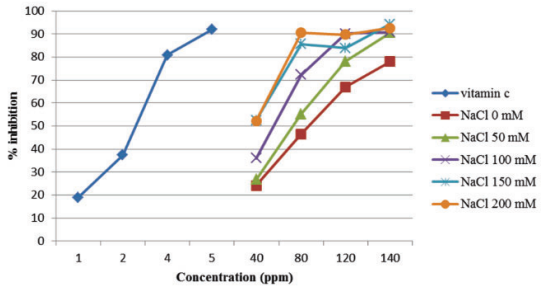


Fig. 5. % Inhibition of Pegagan Leaves in Response to NaCl Treatment

% inhibition of pegagan leaves in response to NaCl treatment at five different concentration 0, 50, 100, 150 and 200 mM. Vitamin C was used as control in this experiment. Each assay was performed in triplicate.

IC_{50} value exhibit the concentration of sample required for scavenging 50% of free radical (Figure 6). Increasing NaCl treatment showed smaller IC_{50}. The result showed strong antioxidant activity by increasing salinity treatment. Pegagan without NaCl treatment showed weak antioxidant activity (AAI 0.45) with IC50 87.59 ppm. NaCl treatment 50 mM and 100 mM showed moderate antioxidant activity (AAI 0.53 and 0.72) with IC50 74.89 and 54.79 ppm, respectively. NaCl treatment 150 mM and 200 mM showed very strong antioxidant activity (AAI 2.49 and 10.46) with IC50 15.86 and 10.46 ppm. The classification of AAI according to Vasic et al. [21]: (a) poor antioxidant activity (AAI < 0.5), (b) moderate antioxidant activity (AAI > 0.5-1), (c) strong antioxidant activity (AAI > 1-2) and (d) very strong antioxidant activity (AAI > 2).

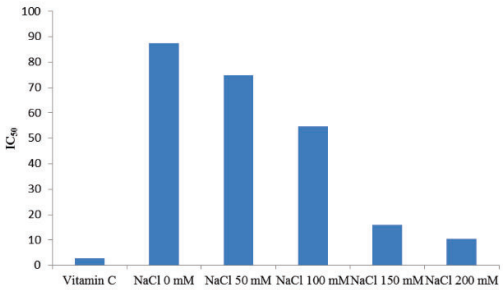


Fig. 6. IC_{50} of Pegagan Leaves in Response to NaCl Treatment

IC_{50} of pegagan leaves in response to NaCl treatment at five different concentration 0, 50, 100, 150 and 200 mM. Vitamin C was used as control in this experiment. IC_{50} show the concentration of samples required to inhibit 50% DPPH free radicals.

Total flavonoid and phenol content is linier with antioxidant activity in pegagan leaves. Increasing total flavonoid and phenol in pegagan leaves under salinity stress showed that pegagan leaves have high antioxidant activity. So, this plant recommended as a medicinal plant.

4. CONCLUSION

The result showed that increasing NaCl concentration cause increasing necrotic spot in leaves. Phenolic compound, total flavonoid and antioxidant activity are increased by increasing NaCl concentration. The result indicated that phenol and flavonoid have important role in plant defense mechanism against NaCl toxicity effects. Except that, the antioxidant activity of pegagan leaves indicate those plant can be used as medicine.

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