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INVESTIGATION OF TOTAL FLAVONOID, PHENOLIC AND ANTIOXIDANT ACTIVITY OF FERMENTATION BROTH OF FERMENTED CLIMBING SWAMP FERN (*Sthenochlaena palustris*)

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ABSTRACT

Fermented vegetable has become more popular as part of daily diet due to their increased shelf life and, more importantly, their beneficial effect for health. Climbing swamp fern (*Sthenochlaena palustris*) is an edible plant that known has medicinal properties. Fermentation can improve functionality of food, and therefore in the present study we prepare fermented fern and examine antioxidant activity, total phenolic and flavonoid content of the fermentation broth. The fronds of fern were collected from local farmer and fermented with different salt concentration (2, 4, 6% w/w), addition of exogenous lactic acid bacteria (none vs *Lactobacillus plantarum*) and time of fermentation (72 vs 144 hours). The experiment was performed using completely randomized design with factorial design approach. Broth formed during fermentation was collected and examined for their total flavonoid content (TFC), total phenolic content (TPC) and antioxidant activity, measured as percentage of DPPH inhibition. The result indicated that salt has significant effect on TFC, while all the three factors have significant effect TPC and DPPH inhibition. The highest TFC was observed when lowest salt was used, and the highest TPC was observed when the lowest salt concentration was used with the addition of *L. plantarum* and the lowest fermentation time. While the highest antioxidant activity was observed when the highest concentration of salt used with the addition of *L. plantarum* and the lowest fermentation time. TPC has significant and moderate correlation with TFC and DPPH inhibition, while TPC and TFC indicate no significant and no correlation. Further investigation is required to examine antioxidant compounds other than polyphenol and flavonoid in the fermentation broth. Furthermore, the solid fraction of the fermented fern fronds needs to be examined for its antioxidant property.

1. Introduction

Climbing swamp fern (*Stenichaena palustris*) is classified as a wild plant that can thrive on peatlands without special treatment (Jaelani *et al*., 2019) and easily found in Borneo Island, Indonesia. The Dayak tribes in Central Kalimantan use this plant to prevent anemia, increase breast milk production and apply it as antiaging (Zannah *et al*. 2015). The young leaf of the plant are greenish red and when it getting older the color become greener and become rich of antioxidant and nutritional compounds.

Some phytochemical that are found in the fern leaf include phenolic and flavonoid

compounds (Chai *et al*., 2012; Chear *et al*., 2016). Mature fronds tend to have higher antioxidant activity and can be used as exogenous antioxidant (Chai *et al*., 2012). Fronds of the fern have some important nutritional component including protein, iron, copper, vitamin C, β-carotene, and folic acid (Irawan *et al*., 2006). Iron in the fern fronds considered plays important roles to overcome anemia (Cahya *et al*., 2016). In addition, it also contains fatty acid, phytosterol, and kaempferol glycoside (Chaer *et al*., 2016). Like other vegetables, fern has low shelf life, due to high water and nutritional content which can promote food deterioration, therefore processing method to improve shelf life and, if possible, improve nutritional value and functional properties is required.

Fermentation of vegetables can increase shelf life and become source of probiotic microorganism (Torres *et al*., 2020), increase the quality of fermented product by increasing secondary metabolite that can promote antioxidant properties (Wu *et al*., 2015; Septembre-Malaterre *et al*., 2018). Fermentation process can increase folic acid and vitamin B12 content (Masuda *et al*., 2012) and remove anti nutritional component in food (Marco *et al*., 2017). The fermentation can be performed as a spontaneous process without or with addition of exogenous microorganism (Yang *et al*., 2020; Pejcz *et al*., 2021). Lactic acid bacteria (LAB) is the most widely used microorganism in vegetable fermentation (Gerardi *et al*., 2019; Lee *et al*., 2016; Wang *et al*., 2021).

Some important factors for optimal fermentation process include the microbes used, substrate and conducive environment for the microbial growth (Septembre-Malaterre *et al*., 2018). Different conditions applied during fermentation may affect the product quality and properties such as profile of volatile compounds and other chemical in the fermented products (Wang *et al*., 2021; Liang *et al*., 2020), which in turn may affect functionality of the food such as safety and stability, flavour and taste, nutrition, bioactivity and health, and texture and rheology (Terefe & Augustin, 2020). During vegetable fermentation, including climbing fern, liquid portion is usually accumulated known as fermentation broth (Gerardi *et al*., 2019; Kim *et al*., 2003). The fermentation broth is of our interest in the present study.

İn the present study we investigate whether salt concentration, exogenous LAB and fermentation time affecting total flavonoid content (TFC), total phenolic content (TPC), and antioxidant activity of fermentation broth of fermented climbing fern.

2. Materials and Methods

2.1. Materials

Fronds of climbing fern were purchased from farmers in Rasay Jata Umum III Village, Kubu Raya District, West Kalimantan Province. Salt (NaCl) used in the present study is purchased in traditional market in Kemuning market, Pontianak, West Kalimantan. Folin-Ciocalteu solution, sodium carbonate, gallic acid, aluminium chloride, potassium acetate, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were provided by the Agriculture Laboratory, Universitas Tanjung Pura, Pontianak with analytical grade. While ethanol used in the present experiment were technical grade.

2.2. Preparation of Climbing Fern Fronds

The fronds were collected early in the morning by picking the second and third fronds which aged 12-14 days. The age of the fronds was determined based on the appearance of leaf shoot until the age that usually consumed by local people according to initial survey. The collected fronds were then taken to the laboratory, sorted and washed with running water. It was cut to give around 4-5 cm fronds.

2.3. Preparation of *Lactobacillus plantarum* Culture

The culture was grown on MRSB broth and shaken at room temperature for 48 hours. The cell density was determined using haemocytometer by direct counting using light microscope with 400 × magnification.

2.4 Fermentation of Fern Fronds

Fermentation was performed with 3 independent variables, i.e. salt concentration (3 levels), exogenous LAB (2 levels), and time of fermentation (2 levels). The experiment was conducted as a completely randomized design. The independent variables and their levels are presented in Table 1. The responses (dependent variable) in the present study were TFC, TPC and antioxidant activity measured as percentage of DPPH inhibition. The experiments were run in triplicates.

The fermentation was performed in a plastic container. As much as 100 g of the fronds was put into the container and salt was added according to the concentration presented in Table 2. For exogenous microbe, 5 mL of the starter culture was added with the density of the cell was 10^7 cell/mL. The lid of the container was then closed and the fermentation was performed at room temperature for the designated time as indicated in Table 2.

After the fermentation concluded, the broth was separated from the solids, by filtering through a cheese cloth and the broth was collected and keeps in a dark vial. The sample was deposited in a freezer until analysis was conducted.

Table 1. Independent variables and their levels used in this experiment

Independent variables	Levels		
Salt concentration (%w/w)	2	4	6
Exogenous microbial	none	*L. plantarum*	-
Fermentation time (hours)	72	144	-

Table 2. Experiments run in the present study

Salt concentration (%w/w)	Exogenous LAB	Fermentation time (hours)
2	None	72
4	None	72
6	None	72
2	*L. Plantarum*	72
4	*L. Plantarum*	72
6	*L. Plantarum*	72
2	None	144
4	None	144
6	None	144
2	*L. Plantarum*	144
4	*L. Plantarum*	144
6	*L. Plantarum*	144

Note: "None" in exogenous microbial column indicate that the fermentation was spontaneous. The experiments were run in triplicate

2.5. Determination of Flavonoid Content

Flavonoid content was determined using method described by Dewi *et al*. (2020). Briefly, 500 µL of sample was mixed with 0.1 mL 10% w/v $AlCl_3$ solution, 1.5 mL methanol, 2.8 mL distilled water and 0.1 mL 1 M potassium acetate in a test tube. The mixture was then homogenized and incubated for 30 minutes for in dark room at room temperature. The absorbance of the mixture at 415 was then recorded. The total flavonoid content was indicated as percentage of quercetin equivalent per 1 g sample (mg QE/g).

2.6. Determination of Total Phenolic Compound

Total phenolic compound was determined using Folin-Ciocalteu reagent as described by

Dewi *et al*. (2020). As much as 200 µL samples was mixed with 1 mL of 10% v/v Folin-Ciocalteu reagent and 3 mL of 2% sodium carbonate solution. The mixture was then homogenized and leave stand for 30 minutes in dark room at room temperature. The absorbance at 765 nm was then recorded. The total phenolic compound was indicated as percentage of gallic acid equivalent per gram sample (mg GAE/g).

2.7. Determination of Antioxidant Activity

The antioxidant activity was determined using DPPH method as described by Safari *et al*. (2019). As much as 4 mL of sample was mixed with 1 mL 0,2 mM DPPH. The mixture was homogenized and incubated for 30 minutes at room temperature in dark room. The absorbance at 517 was recorded, and the inhibition of DPPH was calculated against control using equation (1), where control was prepared by substituting sample with ethanol.

$$\text{Percentage inhibition (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\% \quad \ldots (1)$$

2.8. Statistical Analysis

All collected data was tabulated to Minitab 17 statistical software and analyzed to give Anova table and factors considered to have significant effect when p-value < α, where in this study α = 0.05.

3. Results and Discussions

3.1. Experimental Data

The experiment was performed based on factorial design with completely randomized design run in triplicate. The data obtained is presented in Table 3.

Table 3. Experimental data of experiments run in the present study

Salt concentration (%w/w)	Exogenous LAB	Fermentation time (hours)	TFC (mg QE/g)			TPC (mg GAE/g)			DPPH inhibition (%)		
			Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
2	None	72	0.69	0.76	0.87	4.47	4.16	4.43	2.7	3.5	2.2
4	None	72	0.69	0.53	0.54	5.36	5.40	5.33	10.1	9.6	11.1
6	None	72	0.60	0.41	0.71	4.26	4.57	4.50	5.4	5.3	4.4
2	*L. Plantarum*	72	1.00	1.01	1.12	8.57	8.67	8.53	14.5	14.2	14.3
4	*L. Plantarum*	72	0.52	0.52	0.69	6.50	6.40	6.22	12.3	11.7	12.7
6	*L. Plantarum*	72	0.47	0.52	0.54	7.24	7.24	7.05	18.0	17.7	18.4
2	None	144	0.63	0.51	0.69	4.60	4.67	4.33	3.8	4.5	3.1
4	None	144	0.94	1.01	1.12	5.36	5.64	5.33	2.0	1.1	2.6
6	None	144	0.60	0.69	0.43	4.57	4.64	4.57	6.0	7.2	5.1
2	*L. Plantarum*	144	0.81	0.85	0.65	8.26	8.43	8.57	12.3	12.5	12.2
4	*L. Plantarum*	144	0.54	0.56	0.51	1.43	1.40	1.33	13.0	12.6	11.6
6	*L. Plantarum*	144	0.80	0.61	0.72	6.67	6.53	6.78	10.6	10.4	11.2

3.2. Total Flavonoid Content of Fermentation Broth

Fermentation may change chemical composition of the product, including flavonoid (Nazarni *et al*., 2016; Lee *at al*., 2016) due to the presence of enzymatic activity releasing free flavonoid (Lee *et al*., 2016). The result of the present study indicate that salt concentration has significant effect on TFC of fermentation broth ($p<0.001$) as shown in Anova table (Table 4). As can be seen on Figure 1, higher salt concentration led to lower TFC. This observation is in agreement with Lee *et al*. (2016) who found that higher salt concentration may inhibit β-glucosidase and therefore lowering the number of flavonoid aglycones, which eventually reduce the TFC. No significant effect detected when exogenous LAB incorporated into the fermentation process ($p=0.937$) and different fermentation time ($p=0.403$).

Table 4 also showed that there is significant effect of interaction of salt concentration vs exogenous LAB ($p<0.001$), salt concentration vs fermentation time ($p<0.001$) and exogenous LAB vs fermentation time ($p=0.049$). Table 4

also show that interaction of the three factors significantly affect TFC (p= 0.001). This result indicates that all factors contribute to the change of TFC when interact each other even though exogenous LAB and fermentation time do not individually affect TFC.

Table 4. Anova of total flavonoid content (TFC, mg QE/g) versus salt concentration (%w/w), exogenous LAB, and time (hours)

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	11	1.09893	0.099902	11.22	0.000
Linear	4	0.26652	0.066631	7.48	0.000
Salt Conc. (%w/w)	2	0.26002	0.130008	14.60	0.000
Exo. LAB	1	0.00006	0.000057	0.01	0.937
Time (hours)	1	0.00645	0.006451	0.72	0.403
2-Way Interactions	5	0.65758	0.131516	14.77	0.000
Salt Conc. (%w/w)*Exo. LAB	2	0.33481	0.167405	18.80	0.000
Salt Conc. (%w/w)*Time (hours)	2	0.28457	0.142284	15.98	0.000
Exo. LAB*Time (hours)	1	0.03820	0.038201	4.29	0.049
3-Way Interactions	2	0.17482	0.087412	9.82	0.001
Salt Conc. (%w/w)*Exo. LAB*Time (hours)	2	0.17482	0.087412	9.82	0.001
Error	24	0.21367	0.008903		
Total	35	1.31260			

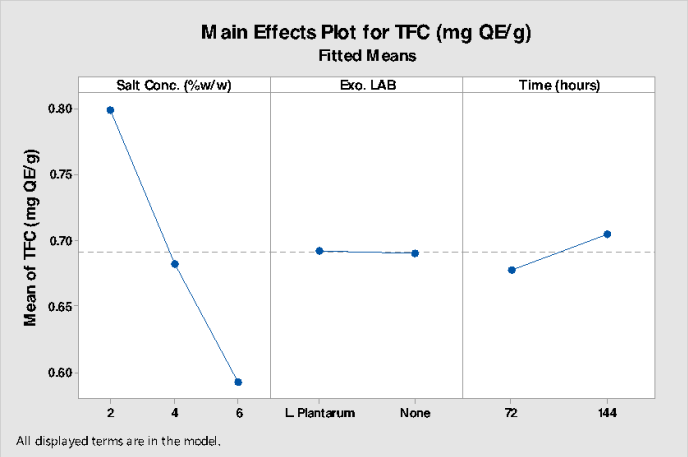

Figure 1. Main effect plot of salt concentration, exogenous LAB and fermentation time for total flavonoid content (TFC)

3.3. Total Phenolic Content of the Fermentation Broth

Some published results indicate that fermentation can change the TPC of fermented product, including the fermentation broth (Kim *et al*., 2003). The result of the present study indicates that all of the factors investigated in this study have significant effect on TPC. Anova table as shown in Table 5 indicate that all factors, either individual, interaction between 2 factors and interaction between 3 factors have p-value < 0.05. Study reported by Kim *et al*. (2003) indicate that TPC data tend to show inconsistencies trend either between different fermentation time for different type of vegetable. Some vegetable showed increased TPC up to 3-4 months of fermentation and decrease after 6 months, and even lower than the unfermented sample. However, in general increase in TPC was detected in all treatment even the increase appeared at different time point (Kim *et al*., 2003). Plot of main factors (Figure 2) showed that fermentation with the lowest salt concentration, addition of *L. plantarum* and shorter fermentation time (72 hours) give higher TPC. Therefore our present result is in agreement with the result reported by Kim *et al*. (2003) where in general fermentation increases the TPC of the fermentation product.

Table 5. Anova of total phenolic content (TPC, mg GAE/g) versus salt concentration (%w/w), exogenous LAB, and time (hours)

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	11	132.059	12.0054	723.90	0.000
Linear	4	51.745	12.9363	780.03	0.000
Salt Conc. (%w/w)	2	20.375	10.1873	614.27	0.000
Exo. LAB	1	24.438	24.4375	1473.53	0.000
Time (hours)	1	6.933	6.9332	418.06	0.000
2-Way Interactions	5	69.896	13.9793	842.92	0.000
Salt Conc. (%w/w)*Exo. LAB	2	49.377	24.6884	1488.66	0.000
Salt Conc. (%w/w)*Time (hours)	2	11.236	5.6181	338.76	0.000
Exo. LAB*Time (hours)	1	9.283	9.2834	559.77	0.000
3-Way Interactions	2	10.418	5.2088	314.08	0.000
Salt Conc. (%w/w)*Exo. LAB*Time (hours)	2	10.418	5.2088	314.08	0.000
Error	24	0.398	0.0166		
Total	35	132.457			

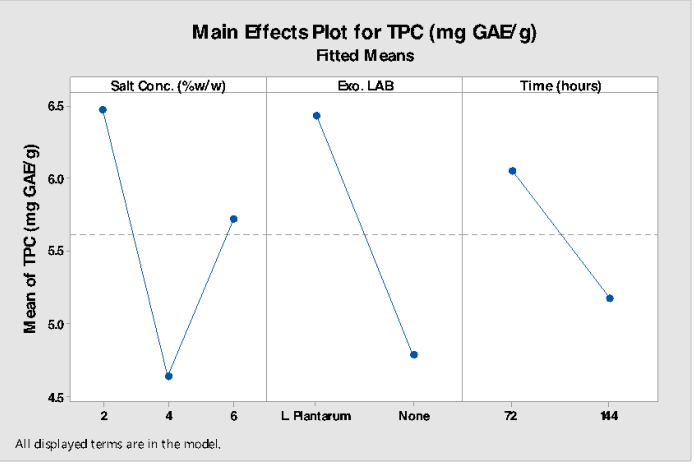

Figure 2. Main effect plot of salt concentration, exogenous LAB and fermentation time for total phenolic content (TPC)

3.4. Antioxidant Activity of the Fermentation Broth

Antioxidant activity is very important properties of food and one of factors that make functional properties of food. Fermentation is reported to increase antioxidant properties of fermented vegetables (Sayin & Alkan, 2015; Lee *et al*., 2016; Wang *et al*., 2021). Antioxidant activity was found either in the fermentation broth (Kim *et al*., 2003) or solid part (Wang *et al*., 2021; Lee *et al*., 2016) of the fermentation product. In the present report we focused on fermentation broth formed during the fermentation process.

Anova of DPPH inhibition is presented on Table 6. The results indicate that all factors affect the DPPH inhibition significantly. All of the individual factors and interaction of the factors has p-value < 0.05 as shown in Table 6. The highest antioxidant activity was found at the highest salt concentration, with addition of *L. plantarum* and fermented for 72 hours (Figure 3.). This result is in agreement with finding by Lee *et al*. (2016) who also found that fermentation and, even more, addition of LAB to the fermentation process increase antioxidant activity of the fermentation product. Kim *et al*. (2003) also found some increase in antioxidant activity of fermentation broth of some of the vegetable they used in their experiment.

The results of the present study indicate that most likely the antioxidant activity of the fermentation broth is predominantly determined by the TPC. However, according to Kaur &

Kapoor (2001) antioxidant activity not only given by the presence of phenolic and flavonoid compound but also the presence of other compounds such as vitamin E, coenzyme Q10, lycopene, β-carotene, α-carotene and vitamin C. Therefore, the presences of other antioxidant compounds in fermentation broth of fermented fern need to be elucidated.

3.5. Correlation Analysis between Factors

Correlation between factors can explain whether one factors correlate with the other factors in the experiment. The correlation analysis results are presented on Table 7. TPC has significant ($p<0.05$) and moderate association with TFC ($r=0.405$) and DPPH inhibition ($r = 0.452$). While TFC is not significant ($p=0.295$) and negative very week or no association ($r= -0.179$) (Fowler *et al*. 1998).

Table 6. Anova of DPPH inhibition (%) versus salt concentration (%w/w), exogenous LAB, and time (hours)

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	11	860.028	78.184	205.36	0.000
Linear	4	703.221	175.805	461.78	0.000
Salt Conc. (%w/w)	2	16.235	8.117	21.32	0.000
Exo. LAB	1	627.611	627.611	1648.51	0.000
Time (hours)	1	59.375	59.375	155.96	0.000
2-Way Interactions	5	45.292	9.058	23.79	0.000
Salt Conc. (%w/w)*Exo. LAB	2	22.544	11.272	29.61	0.000
Salt Conc. (%w/w)*Time (hours)	2	20.681	10.340	27.16	0.000
Exo. LAB*Time (hours)	1	2.067	2.067	5.43	0.029
3-Way Interactions	2	111.515	55.758	146.46	0.000
Salt Conc. (%w/w)*Exo. LAB*Time (hours)	2	111.515	55.758	146.46	0.000
Error	24	9.137	0.381		
Total	35	869.165			

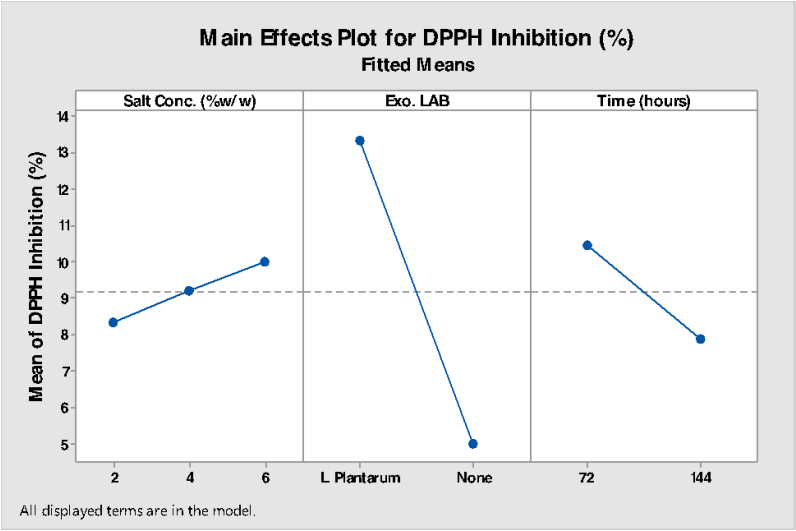

Figure 3. Main effect plot of salt concentration, exogenous LAB and fermentation time for DPPH inhibition

This result indicate that antioxidant activity of fermentation broth of fermented fern is most likely dictated by TPC which is in agreement with statement by Kaur & Kapoor (2001) who

stated that polyphenol compound account for the majority of antioxidant activity of a sample.

Table 7. Correlation between TFC, TPC and antioxidant activity

	TFC (mg QE/g)	TPC (mg GAE/g)
TPC (mg GAE/g)	r = 0.405 p = 0.014	
DPPH Inhibition (%)	r = -0.179 p = 0.295	r = 0.452 p = 0.006

4. Conclusions

As far as we are aware, our present work is the first to report fermentation of fern fronds. We investigated the effect of salt concentration, LAB and fermentation time on TFC, TPC and antioxidant activity of the fermentation broth formed during fermentation process of fern fronds. We found that only salt concentration has significant effect on TFC. While all factors investigated in the present study has significant effect on TPC and antioxidant activity. Correlation analysis showed that there is a moderate correlation between TPC vs TFC and DPPH inhibition vs TPC while no correlation for DPPH inhibition vs TFC. Further investigation of the antioxidant compounds in fermentation broth of fermented fern need to be performed.

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