Enhancement of Flavonoid and Anthocyanin Levels of Herbal Beverage Containing Butterfly Pea Flower and Lemon Fruit to Improve Anti-oxidant Activity

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Abstract: Butterfly pea flower (*Clitoria ternatea L.*) and lemon (*Citrus limon*) have bioactive compounds such as flavonoids and anthocyanin that were known the potential to be processed as functional beverage for health. The combination of them was never been done before. This experiment aimed to analyze the levels of total flavonoids, anthocyanin, and antioxidant activity in butterfly pea flower and lemon beverage. This experimental research was experimental method while dried butterfly pea flower was brewed at temperature of 60 degrees Celsius for 15 minutes combined with lemon fruits with formulation TL (100:0%) F1 (75:25%), F2 (50:50%), F3 (25:75%), and JL (0:100%). The formulation beverages were analyzed for total flavonoid by spectrophotometric method, anthocyanin by pH differential method, and antioxidant activity IC50 by DPPH method. The formulation beverage of butterfly pea flower and lemon F3 (25:75%) was the best formula based on the results for total flavonoids and antioxidant activity. Besides, formula F1 (75:25%) was the best in anthocyanin levels. More composition butterfly pea flower in the beverage, contained more the flavonoid and anthocyanin. Adding more lemon fruits into beverage made greater antioxidant activity IC50. Further research is needed to examine other phytochemical compounds and the effect of its administration on health.

1 INTRODUCTION

Butterfly pea flower and lemon are quite commonly found in Indonesia. The butterfly pea flower is a flower from the Fabaceae family with the international name blue pea flower because it is blue which indicates the presence of anthocyanin (Lestario, 2017). In addition, lemon is one of the fruits from the genus Rutaceae which has the highest hesperidin content compared to other citrus types (Chaturvedi et al., 2016; Klimek-szczykutowicz et al., 2020). No one has ever studied the total content of flavonoids and anthocyanin related to the combination of them.

Anthocyanin are natural pigments from fruits, vegetables, and edible flowers. Anthocyanin are glycosides (3-O-β-glucosidase) that release anthocyanidin aglycones through glycosidases with 6 types of aglycones, including; cyanidin, delfinidine, pelargonidine, malvidin, peonidin, and petudinine. In addition, anthocyanin have the effect of inhibiting DNA fragmentation by H^2O^2, inhibiting LDL-induced Cu^{2+} oxidation and improving mitochondrial function (Sunarti, 2021)

Delfinidine are known to reduce triglyceride accumulation, adipogenesis and lipogenesis by activating Adenosine Monophosphate-Activated Protein Kinase (AMPK) and decreasing the expression of Peroxisome Proliferator Activator Gamma-Receptor (PPAR-γ). AMPK activation inhibits the Acetyl Co-A Carboxylase (ACC) enzyme in fatty acid synthesis and the HMG-CoA reductase enzyme in cholesterol synthesis, and increases adipose triglyceride lipase (ATGL) in fatty acid catabolism resulting in lipolysis (Miey Park, 2019; Parra-Vargas et al., 2018). This causes the butterfly pea flower to potentially lower lipid profile levels and reduce fat accumulation.

Furthermore, flavonoids of the flavanone group such as hesperidin and its aglycone namely hesperetin have several biological activities, including; as an

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antioxidant, anti-inflammatory, and anti-carcinogenic vitamin. Hesperidin upregulates the expression of Nuclear Factor Erythroid-2 related factor 2 (Nrf2) and Extracellular Signal Regulated Kinases 1 and 2 (ERK 1/2) genes which stimulate upregulation of Heme Oxygenase-1 (HO-1) expression, resulting in a decrease in intracellular prooxidants. In addition to HO-1 expression, the Nrf2 gene also increased levels of antioxidant enzymes such as glutathione peroxidase, superoxide dismutase, and catalase. Hesperidin also inhibits the formation of Advanced Glycation End Products (AGEs) which triggers the accumulation of extracellular protein damage so as to prevent premature aging and degenerative diseases (Sunarti, 2021).

Hesperidin is also known to suppress appetite by stimulating the release of the appetite-regulating hormone cholecystokinin into enteroendocrine cells, decreasing the expression of LDL receptor coding genes, increasing AMPK expression which plays a role in increasing the oxidation of glucose and fat to become energy, and inhibiting lipid metabolism pathways on expression by PPAR-γ (Xiong et al., 2019). This causes lemons to have the potential to reduce lipid profile levels, body weight, and premature aging.

Butterfly pea flower and lemon are available in Indonesia in sufficient quantities to be developed. This experiment aimed to analyze the levels of total flavonoids, anthocyanin, and antioxidant activity in combination beverage of butterfly pea flower and lemon. The beverage is expected to be developed into a functional beverage for the prevention and treatment of diseases such as obesity, dyslipidaemia, and premature aging.

2 MATERIALS AND METHODS

This experimental study was carried out at the Central Laboratory of Food and Nutrition Studies, Gadjah Mada University Yogyakarta and Food Technology Laboratory, Faculty of Agriculture, Universitas Sebelas Maret, Surakarta from January to February 2022. Dried butterfly pea flowers were obtained from a restaurant garden, Pringsewu, Lampung Province, Indonesia. Lemon fruits were obtained from a local lemon plantation at Batu City, East Java Province, Indonesia.

2.1 The Preparation of Butterfly Pea Flower and Lemon Beverage

The preparation of the beverage referred to a previous study Purwaniati *et al.,* (2020) with slightly modification. In brief, fresh butterfly pea flowers were picked up from a plantation and then were dried under sunlight during the day for 4-5 days. Dried butterfly pea flowers were brewed using boiled mineral water (60^{o}C) 1:100 in ratio for 15 minutes and lemon fruits were freshly harvested in 1-2 weeks before the research began. The lemon fruits were squeezed and which directly mixed with dried butterfly pea flower brew. A combination beverage of butterfly pea flower and lemon fruits was categorized into three groups: F1 (75:25%), F2 (50:50%), F3 (25:75%) respectively (Table 1).

Table 1: The formulation of butterfly pea flower and lemon beverages.

Materials	Formulation				
	TL	F1	F2	F3	JL
Butterfly pea flower (ml)	100	75	50	25	0
Lemon fruits (ml)	0	25	50	75	100
Total (ml)	100	100	100	100	100

2.2 Total Flavonoid Measurement

Determination of total flavonoid levels using UV-Vis spectrophotometry method and was carried out by weighing 1-2 g of the beverage sample and then dissolving it in 10 ml of 96% ethanol. The solution was then taken 1 ml of the solution and added 5 ml of $FeCl_3$ solution to form a red colour. The solution was added with 96% ethanol so that the final volume became 10 ml. The absorbance was read with a spectrophotometer at a wavelength of 520 nm (Martini et al., 2020).

2.3 Anthocyanin Measurement

Determination of total anthocyanin levels using pH differential method and was carried out by diluting a combination beverage of butterfly pea flower and lemon with KCl buffer pH 1 until an absorbance was found to be less than 1.2 with a wavelength of 510 nm. After that, two types of samples were prepared, namely the first sample using KCl buffer at pH 1 and the second sample using Na-acetate buffer with pH 4.5. Both samples were read at a wavelength of 510–750 nm (Pasaribu et al., 2021).

2.4 Antioxidant Activity Measurement

Determination of an antioxidant activity using Diphenyl Picryl Hydrazyl (DPPH) method and was carried out by weighing the sample 1 - 2 g, dissolving with methanol at a certain concentration. After that, 1 ml of base liquor was taken and put into a test tube and added 1 ml of 1,200 micro molar DPPH solution and incubated in a dark room for 30 minutes. After that, making solution contained 1 ml of DPPH solution and 4 ml of ethanol. The solution was calibrated at a wavelength of 515 nm and the total antioxidant capacity was obtained.

$$\text{Antioxidant Capacity.} = \frac{\text{OD blank} - \text{OD sample}}{\text{OD blank}} \times 100\%$$

After obtaining the percentage of antioxidant capacity, then a graph is made between the concentrations of with the average % antioxidant activity so that the value of y = bx + a is obtained, then calculated IC50 value (Antarti & Lisnasari, 2018).

2.5 Statistical Analytic

The data were tested using One Way Anova Post Hoc Tukey HSD to analyze the difference result and the greatest of total flavonoids, anthocyanin, and antioxidant activity IC50 in the beverages.

3 RESULTS

3.1 Total Flavonoids Levels

From Table 2, it indicated that total flavonoids levels increased in combination of butterfly pea flower and lemon beverages regarding to increased percentage of lemon fruits. The highest total flavonoids was found in the F3 (1,391.5±72.83 ppm), significantly different from other formulation (p<0.001). The F2 and F3 formulas had significantly higher of total flavonoids levels than the TL formula (Table 3).

Table 2: Total Flavonoids of Combination between Butterfly Pea Flower and Lemon Beverages.

Formulation	Total Flavonoid (ppm)
TL (100%)	881.00±24.04
JL (100%)	756.50±12.02
F1 (75:25%)	993.50±6.36
F2 (50:50%)	1,146.5±81.31
F3 (25:75%)	1,391.5±72.83
P value	0,001*

Note: *significant

In contrast, significant higher of total flavonoids levels were observed in the F1-F3 formulas, compared to the JL formula (p=0.003; 0.027; <0,001, respectively). Besides, F3 formula was significant higher of total flavonoids levels compared to the F1 and F2 formulas (p=0.003; 0.023, respectively).

Table 3: Mean Differences of Total Flavonoids in Combination Between Butterfly Pea Flower and Lemon Beverages.

Formulation Differences		**Mean Difference (ppm)**	**P Value**
TL (100%)	F1 (75:25%)	-112.50	0.300
	F2 (50:50%)	-265.50	0.017*
	F3 (25:75%)	-510.50	0.001*
JL (100%)	F1 (75:25%)	-237.00	0.027*
	F2 (50:50%)	-390.00	0.003*
	F3 (25:75%)	-635.00	<0.001*
TL (100%)	JL (100%)	124.50	0.234
F1 (75:25%)	F2 (50:50%)	-153.00	0.130
	F3 (25:75%)	-398.00	0.003*
F2 (50:50%)	F3 (25:75%)	-245.00	0.023*

Note: *significant

3.2 Anthocyanin Levels

Table 4: Anthocyanin Levels of Combination between Butterfly Pea Flower and Lemon Beverages.

Formulation	Anthocyanin (ppm)
TL (100%)	592.50±0.45
F1 (75:25%)	424.41±25.75
F2 (50:50%)	205.42±15.58
F3 (25:75%)	68.44±1.56
P value	0,001*

Note: *significant.

Anthocyanin levels in the beverage formulas reduced in conjuction of decreased percentage of butterfly pea flower concentration (Table 4). Table 5 showed that the highest anthocyanin level was found in the TL formula (592.50±0.45 ppm), which significantly differed from the F1 (424.41±25.75 ppm, p=<0.001), F2 (205.42±15.58 ppm, p=<0.001) and F3 (68.44±1.56 ppm, p=<0.001). F1 formula had the

highest anthocyanin levels compared to F2 and F3 formulas with very significantly different.

Table 5: Mean Difference of Anthocyanin Levels in Combination Between Butterfly Pea Flower and Lemon Beverages.

Formulation Differences		Mean Difference (ppm)	P Value
TL (100%)	F1 (75:25%)	168.09	<0.001*
	F2 (50:50%)	387.08	<0.001*
	F3 (25:75%)	524.06	<0.001*
F1 (75:25%)	F2 (50:50%)	218.99	<0.001*
	F3 (25:75%)	355.97	<0.001*

Note: *significant

3.3 Antioxidant Activity

Table 6: Antioxidant Activity of Combination Between Butterfly Pea Flower and Lemon Beverages.

Formulation	Antioxidant Activity IC50 (ppm)
F1 (75:25%)	11,287.49±123.70
F2 (50:50%)	8,267.80±130.63
F3 (25:75%)	7,594.55±172.80
P value	0,001*

Note: *significant

Table 7: Mean Differences in Combination Between Butterfly Pea Flower and Lemon Beverages.

Formulation Differences		Mean Difference(ppm)	P Value
F1 (75:25%)	F2 (50:50%)	3,019.69	<0,001*
	F3 (25:75%)	3,698.44	<0,001*
F2 (50:50%)	F3 (25:75%)	678.74	0,036*

Note: *significant

It can be seen from Table 6 that the lower percentage of butterfly pea flower concentration had the higher antioxidant activity than the higher percentage of butterfly pea flower concentration. However, the lower percentage of lemon fruits concentration had lower antioxidant activity or vice versa. From Table 7, the antioxidant activity of the F3 formula was 7,594.55±172.80 ppm, significantly higher than the F2 formula (8,267.80±130.63 ppm, p=<0.001) and F1 (11,287.49±123.70 ppm, p=<0.001).

4 DISCUSSION

Herein, we demonstrated that combination of butterfly pea flower and lemon beverages had different total flavonoids and anthocyanin levels and antioxidant activity from butterfly pea flower or lemon fruits alone. The total flavonoids levels and antioxidant activity in the F3 formula were higher than the F1 and F2 formulas. While, the anthocyanin levels in the F1 formula were higher than the F2 and F3 formulas.

Our findings indicated that the F1 formula contained 75% butterfly pea flower and 25% lemon fruits to have low total flavonoids levels and antioxidant activity or vice versa in the F3 formula. The posible cause of low total flavonoids levels and antioxidant activity is come from the solvent that we used mineral water to dissolve dried butterfly pea flowers. In general, flavonoids compounds are difficult to dissolve in water and have to use organic solvents like ethanol or methanol. According to Styawan et al., (2020) stated that there was 4.65×10^4 ppm total flavonoid contained in methanol extract of butterfly pea flower. Rahayu et al., (2021) also stated that there was 5.94×10^4 ppm total flavonoids level contained in etanol extract of butterfly pea flower (Rahayu et al., 2021). Same as Andriani and Murtisiwi (2020), antioxidant activity IC50 in ethanol extract of butterfly pea flower was very strong, namely 41.36 ±1.191 ppm (Andriani & Murtisiwi, 2020).

Flavonoids were the major phytochemicals in lemon juice. Hesperidin and hesperetin levels in lemon juice were 85.77±0.03 ppm and 26.94±0.30 ppm, respectively (Liu et al., 2016). The lemon juice also contains the flavanone group, including: hesperidin, neohesperidin, naringin and eriocitrin (Klimek-szczykutowicz et al., 2020). Therefore, the total flavonoids levels and antioxidant activity in the F1-F2 beverages come from the present of flavonoids and flavanone compounds in lemon juice. It suggests that a sinergistic effect of both butterfly pea flower and lemon fruits does not occur in our research study.

In contrast to the total flavonoids and antioxidant activity, the F1 formula has higher anthocyanin levels (424.41±25.75 ppm). The lower in anthocyanin occurred along with the lower in the composition of dried butterfly pea flower brew in the beverage. It was because the anthocyanin contents were known to be only found in butterfly pea flowers and not in lemons. According to Andriani and Murtisiwi (2021), the extract of butterfly pea flowers had a very strong antioxidant activity is probably due to contained the phenolic contents. The antioxidant mechanism of

phenolic compounds is based on an oxidation-reduction reaction and act as reducing agents (Andriani & Murtisiwi, 2020). According to Utari et al., 2017, the phenolic compounds contained in the ethanol extract of rosela flower also had strong antioxidant activity (Utari et al., 2017). Phenolic compounds are semi-polar so they are not easily soluble in water (Rondonuwu et al., 2017).

Anthocyanins are natural pigments contains glycosides (3-O-β-glucosidase) that known have strong antioxidant activity. Its antioxidant ability is 2x greater than other antioxidants such as catechins and vitamin E (Sunarti, 2021). Butterfly pea flower is known to contain delfinidine, alkaloids, saponins, and phenols (Khaerani, 2013; Nuraini, 2014). Butterfly pea flower extract alone were known as strong antioxidant activity, namely IC50 95.30-132.50 ppm (Kumar, 2019). In contrast to this experimental results that the best antioxidant activity of the combination butterfly pea flower and lemon beverages were still classified as very weak. In contrast to the results that the higher anthocyanins in the beverage, more weak antioxidant activity (11,287.49±123.70 ppm). This could be due to the fact butterfly pea flower although high in anthocyanins, had weak antioxidant activity because that other phytochemical compounds that can increase antioxidant activity in butterfly pea flower do not dissolve optimally in the brewing process.

Water is a good solvent for dissolving anthocyanin because it has a relatively similar level of polarity (Lestario, 2017). This experiment used drinking water as a medium for brewing dried butterfly pea flowers at 1g/100 ml (1:100) to dissolve 881.00±24.04 ppm flavonoid compounds and anthocyanin levels 592.50±0.45 ppm. The use of water as a solvent in extraction was safer and applicable for use as a formulation of food or beverage products, although the amount of phytochemical compounds extracted were lower when compared to using other solvents.

Anthocyanin compounds are also easily degraded by heating factors (Priska et al., 2018; Surianti et al., 2019). Differences in extraction methods can also affect the total anthocyanin levels produced. According to Purwaniati et al., (2020) which stated that dried butterfly pea flowers brewed at a temperature of 50-80 degrees Celsius without knowing the length of boiling time produced anthocyanin levels 2-3 times higher, ranging from 1,365±0.77 ppm to 1,487±0.62 ppm but total flavonoids were not examined (Purwaniati et al., 2020).

Dried butterfly pea flower brew was richer in anthocyanin levels than fresh one. It was proven by another experiment stated 10 petals of fresh butterfly pea flower that was brewed by water in 250 ml with a temperature 100^{o} Celsius for 30 minutes had anthocyanin levels were about 510 ppm (Handito et al., 2022). Same as Pramitasari et. Al (2022), 2 g fresh butterfly pea flower was brewed with a temperature 30^{o} Celsius had lower anthocyanin levels, namely 486±0.95 ppm (Pramitasari et al., 2022). They were lower than this experimental result although it had been assisted with tartaric acid in the extraction process. There was 592,50±0,45 ppm from dried butterfly pea flowers which was brewed in 100 ml with lower temperature and shorter time.

Although the extraction of anthocyanin was better by boiling at a higher temperature, but the butterfly pea flower which also contains high flavonoids can potentially be damaged during the process so that the extraction method by brewing was the best alternative to maintain the flavonoid content of butterfly pea flower. Anthocyanin in butterfly pea flower are known to be more extractable at temperatures ranging from 50-80 degrees Celsius (Purwaniati et al., 2020). Boiling dried butterfly pea flower with lower temperature may be needed to extract more phytochemical compounds.

However not synergistic, combination of butterfly pea flower and lemon contained more total flavonoids than butterfly pea flower or lemon alone (Table 2). The type of flavonoids between butterfly pea flower and lemon were different so that it enriched the beverages. Each type of flavonoids have different functions so that they will be expected to optimize health and aim for many diseases. Futher research about other phytochemical compounds in the beverage is needed.

The limitation of our study is we did not investigate the phytochemical contents in all formula beverages, which influent the antioxidant activity. Formulas F3 (25:75%) has better potency than other formulas as functional beverages because they contain more odd flavonoids and antioxidant activities, but lowest in anthocyanin levels compared to other formulas. The formulas have the potential to be investigated further regarding another phytochemical compounds and the effect of its administration on health.

5 CONCLUSION

Combination of butterfly pea flower and lemon beverage in the F3 formula (25:75%) has higher total

flavonoids levels (1.391.5±72,83 ppm) and antioxidant activity (7.594,55±172,80 ppm) than in the F1 and F2 formulas. Meanwhile, the anthocyanin levels in the F3 formula is lower than F1 and F2 formulas. Futher research is needed to examine phytochemical compounds for this beverage and other treatment of dried butterfly pea flowers like boiling with mineral water.

STATEMENT

There are no conflicts of interest in this research.

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