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Effects of zeaxanthin extract of red paprika (*Capsicum annum.* L.) to the red skin color in kohaku koi fish (*Cyprinus carpio*)

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Abstract

Free zeaxanthin crystal extraction of red paprika using the solvent hexane and acetone (65: 35). Separation of the carotenoid zeaxanthin with others using column chromatography with acetone and petroleum ether (2: 8). The purpose of this research was to determine the increase in red color on the skin after kohaku koi fish (± 11.2 to 13 cm) were fed enriched with extracts of red pepper with different doses (0, 4, 8, 12 mg / kg of feed) for 60 day. The measurement of the red color skin using a color meter every 10 days at the beginning and end of maintenance. *Chromatophore* cell count at the start of the maintenance is ± K: 385 - 440, A: 378 - 458, B: 392-483 and C: 403-483 and at the end of the maintenance is ± K: 540 - 590, B: 1428 - 1640 and C: 2430-2888. The addition of zeaxanthin was highly significant (P <0.01) in improving the red color on kohaku koi fish skin with the optimal dose is 12 mg / kg.

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Introduction

One source of zeaxanthin can be obtained from the fruit of red paprika (*Capsicum annum*. L) that much containing carotenoid pigments. Zeaxanthin, capsorubin and capsanthin is the main constituent of the carotenoid fraction that was exclusively found in red paprika (Minguez, 1997).

Koi (*Cyprinus carpio*) much in demand because of the appeal of the color. Its color related due to the number and location of pigment cells (*chromatophore*) in the dermis (Sari *et al*., 2012). But fish can not synthesize their dye pigmen itself by *de novo*, so that pigments like melanin, carotenoids, pteridine and purine need to be included as part of the ornamental fish feed formulations to intensify its color (Katayama *et al*., 1973).

Colors on the skin of a fish caused by integument cells that containing a color substance (*chromatophore*) that provides true color in fishes that located in the lining of dermis. According to the color of the pigments which they contain, chromatophore on fish in generally classified as *melanophores* (brown or black), *erythrophores* (red), *xanthophores* (yellow), *leucophores* (white) and *iridophores* (sheen), (Hoar, 1969).

To intensify the color in freshwater fish, should be done by providing the carotenoids from zeaxanthin group, lutein or astaxanthin in fish feed (Tanaka, 1978).

The purpose of this research are to know the content of zeaxanthin compounds in red paprika, to find the increase in red color in the skin of kohaku koi fish after fed that enriched with zeaxanthin extracts from red paprika and to know the optimal dose of red paprika zeaxanthin extracts toward increase of red color in the skin of kohaku koi fish.

Material and method

Zeaxanthin Extraction of Red Paprika

The extraction of free crystalline from red pepper (0.5 ounces) using the solvent hexane and acetone (65: 35). The filtrate obtained dried using evaporator at a temperature of 40-45 °C in order to obtain a concentrated extract. Then the extract spotted on the Thin Layer Chromatography (TLC) plate (5 x 1 cm) with a value of *Rf* zeaxanthin is 0.87. Zeaxanthin isolation done with column chromatography with stationary phase silica gel G_{60} and the mobile phase acetone and petroleum ether (2: 8) ± 100 ml. Zeaxanthin isolate concentrad in nitrogen evaporator at a temperature of 40-45 °C so that obtain dry zeaxanthin extract.

Zeaxanthin Identification

Identification of zeaxanthin from red paprika was carried out using the UV-Vis T70. Red paprika (0.5 ounces) dissolved with 50 ml acetone then homogenized using magnetic strirrer for 1 minute. The filtrate was filtered through a Buchner funnel and Whatman 42, then inputed into spectrophotometer and scanned at a wavelength of 400 nm to 550 nm, within absorbancy used is 460 nm. The amount of zeaxanthin concentrations calculated using the equation of Delia, (2001) with $E^{1\%}_{1cm}$ = 2340, the specific absorbance coefficient zeaxanthin in acetone.

$$\text{Zeaxanthin } \mu g = \frac{A \times Y\,(ml) \times 10^6}{E^{1\%}_{1cm} \times 100}$$

$$\text{Zeaxanthin } \mu g/gr = \frac{\text{Zeaxanthin } \mu g}{G}$$

Annotation :

A = Absorbansy obtained

Y = Solution volume (ml)

G = Sample weight (gr)

$E^{1\%}_{1cm}$ = 2340

Analisys of Feed Total Carotenoid

Total carotenoid analysis of the feed using acetone and methanol (1: 1) for 30 minutes. This procedure is repeated until the extract is colorless. The extract was collected and mixed with 20 ml of petroleum ether. Aquadest 50 ml were added until phase separation occured. The upperpart of the phase dried in nitrogen evaporator, at a temperature of 40 °C. Residue taken and mixed in 5 ml of petroleum ether and the absorbancy measured in 450 nm using a

spectrophotometer (Lee, 2010). The total number of carotenoids calculated using equation Delia, (2001) with $E^{1\%}_{1cm}$ = 2500, the specific absorbancy coefficient of carotenoids in petroleum ether.

Zeaxanthin Aplication in Feed

Zeaxanthin extract (K: 0 mg, A: 4 mg, B: 8 mg, and C: 12 mg) each mixed with tapioca flour (5 g) using a magnetic stirrer. Zeaxanthin extract with different doses and then mixed with 1 kg of commercial feed and aerated at room temperature for night and then stored in a refrigerator with the temperature of -25 °C until use.

Kohaku koi fish (size ± 11.2 to 13 cm) is maintained in the same pond and given the net bulkhead (1 x 0.5 x0,8 m) which the density of 10 fish/container. Before receiving treatment, the addition of zeaxanthi the fish not fed for 3 days. Water quality management use aeration and circulary systems (± 10 liters / minutes). Feed given three times a day on at 8 am 12 pm and 4 pm at a dose of 3% biomass and provided in a *ad libitum*.

Histology of Chromatophore

The calculation of *chromatophore* cells in the lining of dermis in the fish body that colored red by applying histologicy technique use hematoxylin and eosin staining. The histology chromatophore cell done in the beginning and end of the research.

Color Analysis

The measurement of red color in skin of kohaku koi fishes using Samsung SM-C111 color meter every 10 days at the beginning and end of maintenance.

Statistic Analysis

The design of experiment carried out using *Completely Randomized Design* (*CRD*) with 4 treatments and 4 remedial. Which the parameters is increase of red color in the skin of kohaku koi fish, increase in number of *chromatophore* cell and dose of best treatment on each treatment. Statistics data processing carried out using using *Analysis of Variance* (*ANOVA*). Then, to know the most effective (optimum) treatment, should be conducted by *Least Significant Difference* (*LSD*) test.

Result and discussion

To know the concentration zeaxanthin in red paprika, identification was done using Uv-Vis T70 spectrophotometer. To the 0.5 ounces of fresh paprika using acetone 50 ml. The amount of zeaxanthin concentration of red paprika calculated by equation of Delia, (2001) and can be seen on Table 1 below :

Table 1. Total concentrations of zeaxanthin and other carotenoids in red paprika based on Uv-Vis spectrofotometer.

Faction	Type of carotenoids	Absorbance (nm)	Volume (ml)	$E^{1\%}_{1cm}$	Amount µg/gr	Rendemen (%)
1	Capsanthin	3.827	3	2100	10.93	21.87
2	Zeaxanthin	1.502	3	2340	3.85	7.70
3	β- Karoten	1.824	3	2500	4.38	8.76
4	Cucurbitaxanthin-A	1.428	3	2350	3.65	7.29

Based on Table 1 above can be seen that the concentrations of red paprika zeaxanthin ± 3.85 mg / g with a rendemen of ± 7.70%. While other carotenoids that capsanthin ± 10.93 mg/g with a rendemen of 21.87%, ± β-carotene ± 4.38 mg / g with a rendemen of 8.76%, cucurbitaxanthin-A ± 3.65 mg/g with a rendemen ± 7.29%. According Deli (2001), the total carotenoid content of red paprika is about 1.3 g/100 g, capsanthin ($C_{40}H_{56}O_3$) is 37%, zeaxanthin ($C_{40}H_{56}O_2$) is 8%, cucurbitaxanthin A ($C_{40}H_{56}O_3$) is 7%, capsorubin ($C_{40}H_{56}O_4$) is 3.2%, and β-carotene ($C_{40}H_{56}$) accounted for 9%. According Purseglove *et al.,* (1981), the number and ratio of carotenoid component in peppers varies, depending

on maturity, varieties, agrotechnic, and climatology.

Before zeaxanthin mixed with commercial feed, the feed analyzed to prove that the commercial feed does not contain other carotenoids that can cause a significant influence on fish studied. The total amount of carotenoids can be seen in Table 2 below:

Table 2. Nutrition and total carotenoids in commercial feed.

The content of substances	Amount (%)
Dry matter	90,42
Ash	10,94
Crude protein	37,01
Crude fiber	2,58
Crude fat	1,91
Total carotenoid	0,13

Based on Table 2 above can be seen that the commercial value of the nutrients in the feed given that dry matter 90.42%, ash 10.94%, 37.01% crude protein, crude fiber 2.58%, crude fat 1.91% and total carotenoids in commercial feed ranges ± 0.13%. According to Takeuchi (1988), the need for macro-nutrients: proteins koi 30-35 g.

Table 3. *Analysis of variance* (*ANOVA*) influence of the use of zeaxanthin toward the red color in kohaku koi fish skin on the 60th day maintenance.

Treatment	Degrees of freedom	Sum of spuares	Mean spuare	F ratio	F table	
					5%	1%
Zeaxanthin	3	4893 .69	1631 .23	860 .42**	3 .49	5 .95
Error (residual)	12	22 .75	1 .90			
Total	15	4893 .69				

Annotation: ** = really significant different (P<0,01).

100 g^{-1}, fatty 5-15 g 100 g^{-1}, energy 13-15 MJ kg (310-360 Kcal) and carbohydrates 30-40 g 100 g^{-1}. According Yuangsoi (2009), that the amount of carotenoids that can be given to the fish is the range of 6-8 mg / kg of feed will give different colors on the fish skin with the help of color comparator scoring tool.

Table 4. The results of *Least Significant Difference* (*LSD*).

Zeaxanthin (mg/kg feed)		Red color value (color meter)	Different with		
			Ho	H1	H2
0	K	205 .25	-		
4	A	232	26. 75**	-	
8	B	241 .5	36. 25**	9.5**	-
12	C	252 .5	47 .25**	20.5**	11**
LSD 0 .05 =2. 12 LSD 0.01 = 2.97					

Annotation :* = significant different (P<0,05)

**= really significant different (P<0,01).

Based on Figure 1 above can be known that the value of the red color on kohaku koi fish at the beginning of maintenance based on the color meter, is an average of 198. Until the 60th day, the increase in coloroccured with an average value that is K: 205, A: 232 ., B: 242 and C: 253. The most dominat value of red color on the 60th day is on treatment C (12 mg zeaxanthin / kg feed).

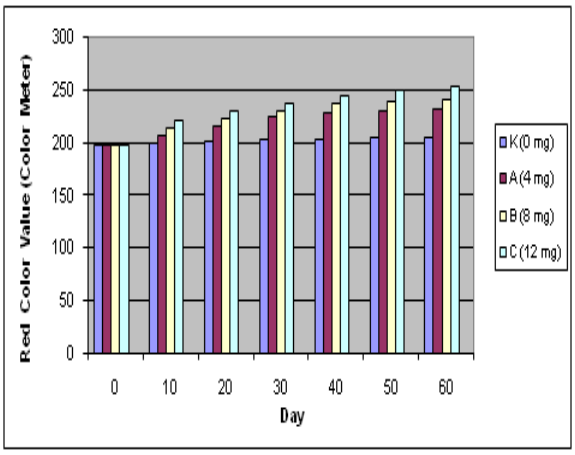

Fig. 1. The value of the color in the red skin of kohaku koi fish based on color meters for 60 days maintenance.

At treatment K (without zeaxanthin addition) did not show a significant increase in the color kohaku koi fish. Allegedly because in the feed was not given the addition of pigment so *chromatophore* not thrive and not suffered pigmentation increased in color. While on the kohaku koi that was given additional of zeaxanthin treatment in its feedstock, the intensity of color from the 10th to 60th day. Allegedly zeaxanthin contained in the feed absorbed in fish physiological and transferred into *erythrophore* so that more will accumulate in *erythrophore* and will cause to an increase in color on the skin of fish.

Evans (2002), argues that carotenoids gleaned from feed will be distributed in fatty tissues of fish body. Physiologically, fish will change a pigment obtained from the feed to produce a variation of color. Physiological changes in color is a discoloration caused by the movement activity of the pigment granules or chromatophore. The movement of pigment granules accumulate or dispersed in the color pigment cells, resulting from different stimulation, as temperature, light, etc.

Fig. 2. Increased of Red Color Intensity Kohaku Koi Fish For 60 Days Maintenance.

The number of *chromatophore* cells in the kohaku koi fish skin at beginning of research ranging from ± K: 385 - 440, A: 378 - 458, B: 392-483 and C: 403 - 483. While in the end of research ranged from ± K: 540 - 590., A: 731 - 974., B: 1428-1640 and C: 2430-2888. Based on the calculation of the intensity of the color using the color meter and chromatophore cell counts showed that the more the number of cells in the epidermis koi *chromatophore* will increase the intensity of the color.

According to Sari *et al*., (2012) that the more number of *chromatophore* cells in the epidermis layer, koi fish will improved color intensity. There is a positive correlation between the intensity of color and *chromatophore* cells. That means that the relationship between the number of *chromatophore* cell and color intensity is very closely. The increase of color intensity occurred because of change on *chromatophore* cells. While Hoar (1969), argues that grouped pigment will be transferred to *xanthophore*

or *erythrophore*, in which large quantities can be stored in it. Initially *xanthophore* or *erythrophore* not contain carotenoids, then the longer this carotenoid pigments began to accumulate in the *xanthopore* or *erythrophore* cell, so pigmentation occurred that causes fish looks yellow colored or red in visible.

To know the best zeaxanthin treatment toward red color in kohaku koi fish to each of the different treatment was then done the *Least Significant Difference* (*LSD*) can be seen in Table 3.

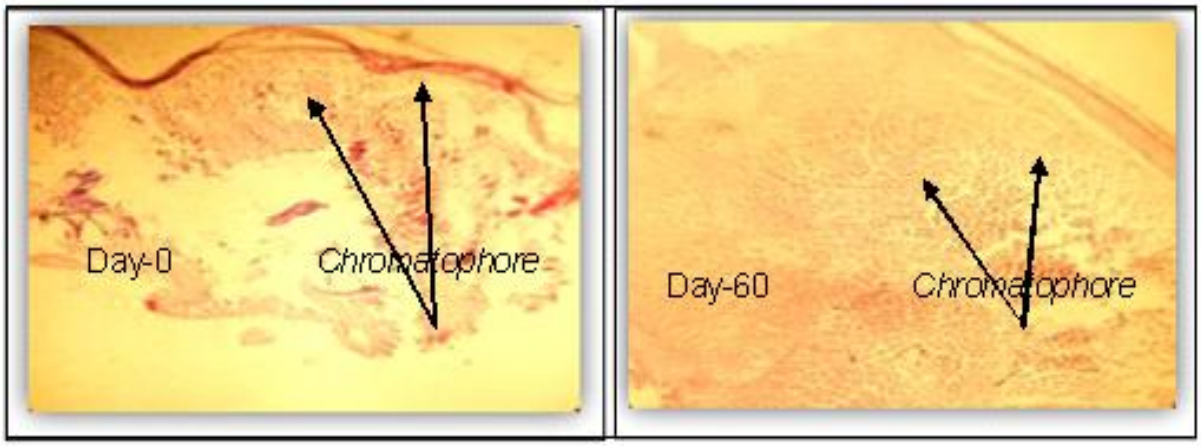

Fig. 3. Histology of *Chromatophore* in koi fish skin (H&E, 100 x).

Based on Table 4 it can be concluded that the best treatment of zeaxanthin addition toward red color in skin of kohaku koi fishes is on the concentration of 12 mg / kg of artificial feed (P <0.01). Lee (2010), reported that in a research about *Zacco platypus* a kind of koi fish fed enriched with red paprika powder containing zeaxanthin 8 mg/kg of pellets give significant pigmentation color (p<0.05) during 8 weeks of maintenance.

Conclusion

The concentrations of red paprika zeaxanthin ± 3.85 mg/g with a rendemen of ± 7.70%. Red color value Kohaku koi fish at the beginning of maintenance based on the color meter, the average is 198. On the 10th day began to see an increase in the value of the color in each treatment with the average value in the treatment of K: 199, A: 207. , B: 214 and C: 221. Until the 60th day an increase in color with an average value that is K: 205, A: 232, B: 242 and C: 253. The best treatment of zeaxanthin addition toward red color in skin of kohaku koi fishes is on the concentration of 12 mg / kg of artificial feed (P <0.01).

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