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Research Article

Novel application of orange marigold flower petal (*Tagetes erecta* L.) as a dietary supplement to improve skin redness of Koi carps (*Cyprinus carpio* L.)

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ABSTRACT

In this study, orange marigold (*Tagetes erecta* L.) petal was used as a direct lutein-containing resource for the Koi carps (*Cyprinus carpio* L.) with aim to enhance redness of specific area on their skin. The petals were collected from fresh orange marigold flowers, subsequenly dried at 50 ^oC and intensively ground into fine powder. Ultraviolet-visible (UV-vis) spectroscopy was performed for an ethyl acetate-mediated extract of the dried petal sample, showing the signature absorption peaks of lutein presented in the literature. Furthermore, a total carotenoid content of 6.57 mg/g was determined using a calibration curve of commerial lutein. Redness pigmentation of the selected Koi carps was performed via mixing the orange marigold petal powder with their daily food at two weight ratios of 5 and 20 $g_{marigold\ petal}/kg_{total\ food}$, respectively, for a feeding period of six weeks. The photographs of the tested fish was recorded before and after this period and subsequently analyzed using a red-green-blue (RGB) system-based software to detect and compare possible variations in redness at the selected fish skin area. It was observed that the redness improvements of about 40% were obtained for all of the five fish fed with the food supplemented with the 20 g/kg ratio while using the 5 mg/kg ration was found to be inefficient. Importantly, the tested fish were in healthy conditions during the feeding period. These experimental results proved the great efficiency of orange marigold petal as an inexpensive and abundant agricultural alternative to costly imported carotenoid-containing feed for redness pigmentation of Koi carp in Vietnam.

Key words: Orange marigold flower, Koi carp, lutein, dietary supplement, redness

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INTRODUCTION

Koi carps are colored varieties of *Cyprinus carpio* kept in outdoor and indoor aquatic gardens for ornamental purposes [1–4]. Economic value of Koi carps is highly dependent on coloration, color patterning, and color scalation on their skin. Red, blue, yellow and black are typically the most attractive colors. Therefore, pigmentation and stabilization of the target colors for Koi via dietary resources and aquatic conditions have been of great interest to scientists [3,5–7]. For example, astaxanthin is identified as a major active component for the formation of redness on the skin of Koi and other animals. Therefore, the diet of Koi usually requires either natural plant and animal resources consisting of astaxanthin or addition with pure astaxanthin for red coloration of its skin [5,6,8,9]. In Vietnam, *Haematococcus pluvialis* algae and *Phaffia rhodozyma* yeast with high astaxanthin contents were commonly utilized; however, such dietary supplements are imported with high costs due to their unavailability in the Vietnam market.

Recent studies have proposed the carotenoid metabolic pathways which present the bioconversion of other carotenoid derivatives such as α-carotene, β-carotene, zeaxanthin, canthaxanthin and lutein into astaxanthin (Figure 1) [10–12]. Such carotenoid transformation is reasonable upon numerous reported experimental results [13,14]. Ettefaghdoost and co-workers reported beneficial effects of adding lutein to the diet on growth performance, biochemical and immuno-physiological parameters of oriental river prawns (*Macrobrachium nipponense*) [15]. On the other hand, in the work of Besen and co-worker, the goldfish juveniles (*Carassius auratus*) was fed with the diet containing lutein, the skin pigmentation was as efficient as the result of astaxanthin. The lutein supplementation of 50 mg kg^{-1} could improve survival and promote the pigmentation on the skin of goldfish juveniles [16]. Notably, both lutein and canthaxanthin were previously demonstrated to be efficient for improving skin redness of large yellow croakers (*Larimichthys croceus*) [17].

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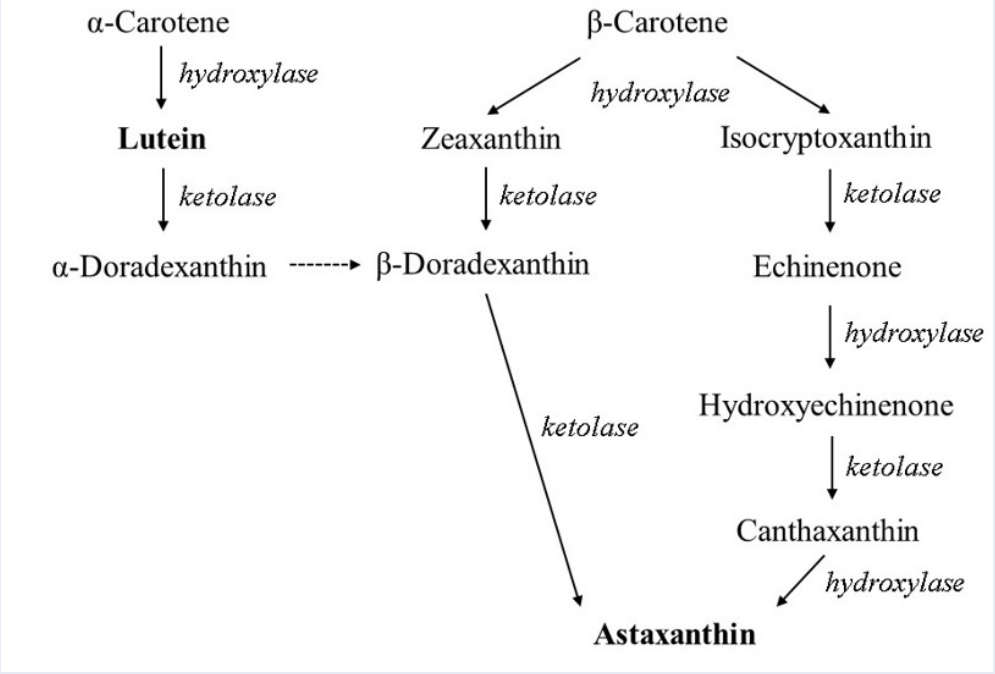

Figure 1: Proposed bioconversions of carotenoid derivatives to astaxanthin [10–12].

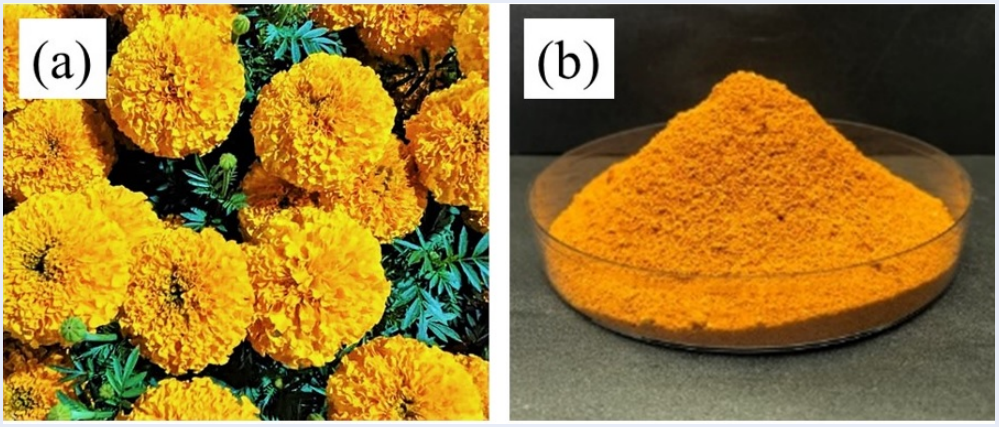

Figure 2: Orange marigold flowers (a) and powder of dried flower petals (b).

Table 1: Humidity and color of the fresh and dried marigold petals

Marigold petals	Fresh	Dried
Weight (g)	1440.0	202.4
Humidity (wt.%)	86.9	6.8
Color	Light orange	Brownish orange

Orange marigold (*Tagetes erecta* L.) is a popular and inexpensive flower planted in Vietnam and other tropical countries. Importantly, petals of orange marigold flower are considered as the richest source of lutein, which are promising alternatives to costly imported astaxanthin sources in Koi farming[18–20]. Therefore, in this study, the impact of adding orange marigold petals to the Koi diet on the red coloration would be investigated towards valorization of local agricultural products.

MATERIALS AND METHOD

Materials

Fresh orange marigold flowers were collected in Dong Thap, Viet Nam (FFigure 2a). Petals were separated from the flowers, washed with water, dried at room temperature for 1 day and 50 ^{o}C for 48 hours. Humidity and color of the fresh and dried marigold petals are presented in Table 1. The dried marigold petals were intensively ground into fine powder for further steps (Figure 2b). Commercial lutein (90%) was obtained from Thermo Scientific Chemicals.

Determination of total carotenoid content

In a typical experiment, powder of the dried marigold petals (20 g) was extracted with ethanol (96^{o}, 200 mL) for 2 hours at 70 ^{o}C[21]. The extract in ethanol was cooled and collected by vacuum filtration. Potassium hydroxide (12 g) was added to the liquid for a de-esterification reaction which was performed at 70 ^{o}C for three hours to obtain free lutein[18,21,22].

After the reaction, ethanol was removed by the first vacuum rotary evaporation at 50 ^{o}C. The resulting mixture contained free lutein, salt of fatty acids and unreacted potassium hydroxide. To remove these unexpected compounds, this mixture was washed with water (30 mL) and extracted with ethyl acetate (4 x 50 mL)[18,21,22]. To recrystallize lutein, the extract obtained upon the second vacuum rotary evaporation was dissolved in a 50-mL centrifuge tube with a minimal amount of ethanol at 60 ^{o}C and added with water until the solution turned cloudy[23,24]. The solution was subsequently cooled to room temperature and carotenoid crystals were collected by centrifugation. The recrystallized extract sample was dried at room temperature in a vacuum desiccator.

UV-vis spectroscopy was performed using a GENESYS-10S Thermo Scientific device in the wavelength range from 300 to 750 nm. Using the solution of standard lutein (90%, Thermo Scientific Chemicals) in ethyl acetate at varied concentrations, the calibration curve of lutein was obtained at the maximum absorbance wavelength of 445 nm in good agreement with the literature (Figure 3)[25]. The total carotenoid amount in the extract was calculated based on this calibration curve[26–28].

Determination of red pigmentation for Koi carps

Five healthy 8-month-old Koi carps with several red or orange areas on their skin. were selected for this investigation.

The daily diet of Koi carps in which any carotenoid derivatives disappeared to eliminate their affect was supplemented with fine powder of dried marigold petals at two weight ratios of 5 and 20 $g_{marigold\ petal}/kg_{total\ food}$. The selected fish was fed this mixed food in separate aquariums for 6 successive weeks. Photographs of their upper part were recorded under identical conditions before and after the feeding period. An ibis Paint software (ver.11.1.0) was applied to convert the obtained photographs to 24-bit ones and analyze three selected skin areas in the latter versions according to the RGB color model. In this color space, three main colors consisting of red (R), green (G), and blue (B) were used to quantify the level of red, green, and blue light in a studied color. In detail, an integer value from 0 to 255 would be represented for each of these colors with 0 being no color and 255 being full intensity of that color[29,30].

RESULTS AND DISCUSSION

Marigold petals have been indicated to be the rich source of carotenoids in which the ester form of lutein occupies 70-79%[18,31,32]. Therefore, after the extraction step, saponification with potassium hydroxide was carried out to convert the esters of lutein with fatty acids to free lutein based on the method of Boonnoun and co-workers[18,22]. The authors pointed out that saponified lutein sample from marigold petals contained contains free lutein, anhydrolutein and small amount of other impurities[18,22].

In this study, the extraction and purification procedure for orange marigold petal powder yielded free lutein in an orange solid form (Figure 4), similar to the previously reported results on this naturally occurring compound[33–35]. The UV-vis spectrum of the extract in ethyl acetate revealed three absorbance peaks at 427, 447 and 475 nm, which are spectroscopic features of lutein (Figure 5). These peaks can be useful in distinguishing lutein from other carotenoid derivatives[25,26,36,37]. Importantly, no more peaks were observed in the visible wavelength range, indeed indicating the presence of lutein in high purity in the purified marigold extract.

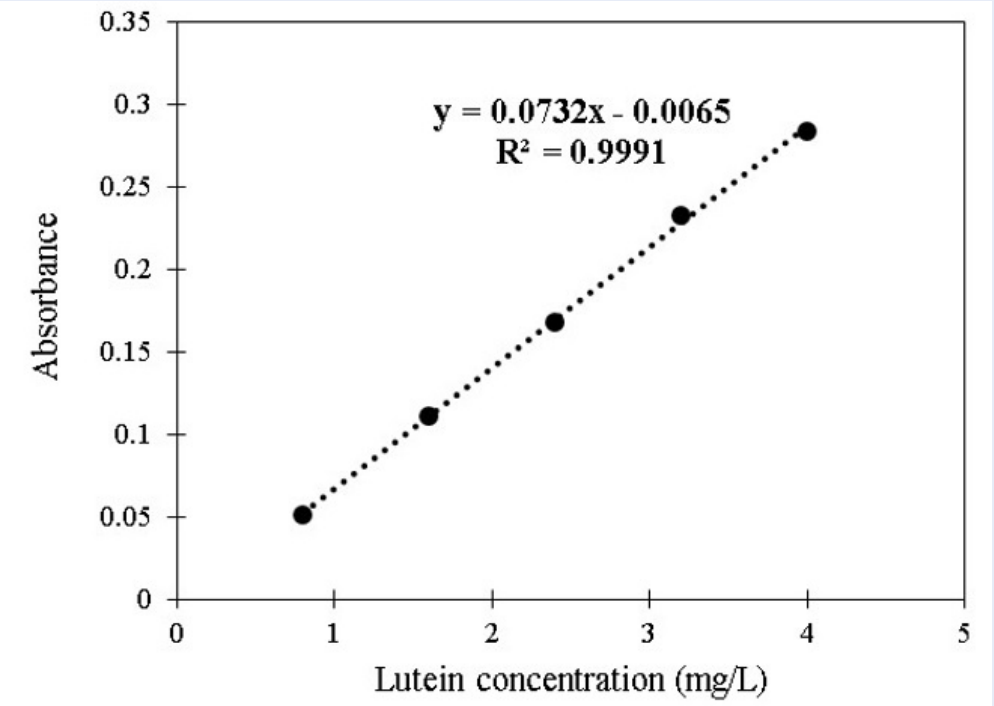

Figure 3: Calibration curve of commercial lutein.

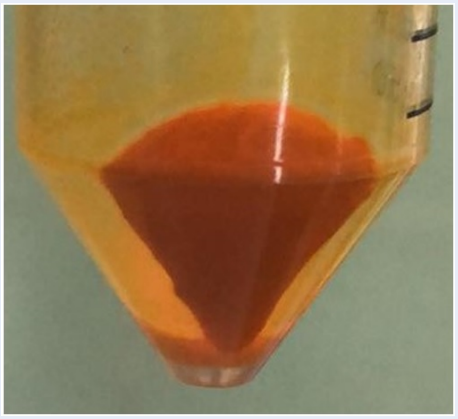

Figure 4: Marigold petal extract after the recrystallization with ethanol and water.

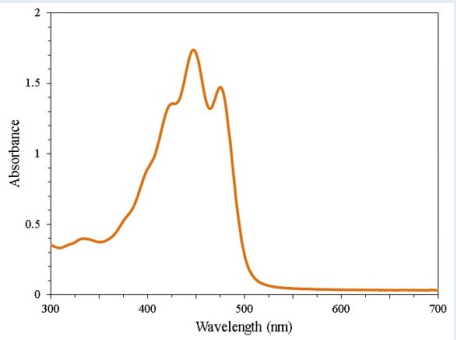

Figure 5: UV-vis absorbance spectrum of the marigold petal extract in ethyl acetate.

It should be noted that identification of carotenoids in the extract was not focused in this work; therefore, the total carotenoid content including lutein and other derivatives, if any, was preliminarily determined using the UV-vis calibration curve of lutein at 447 nm. It was found that 1 g of dry marigold petal could yield 6.57 mg of lutein within the study scope. This result was much lower than those previously reported due to the fact that the applied extract pathway was not optimized[19,22,31,32]. Furthermore, the last recrystallization step could lead to a significant loss of lutein. The factors such as age, weather, temperature, light and water source were found to have insignificant effects on the red pigmentation of the Koi carp. Notably, experimental investigation showed that instant pH adjustment could even cause disappearance of the red skin areas. On the other hand, using the carotenoids-free food for six weeks under identical enviromental conditions led to no redness improvement on the fish skin. As can be ex-

pected, decreases in redness of Koi carps by 10–20% were observed upon expanding this feeding period to six months. Carotenoid supplement is obviously essential for the development of the red color of Koi carp, consistent with the literature on fish pigmentation[6,16,38]. Although present Koi farming mostly applies astaxanthin-based supplements for this coloration, other food resources containing lutein, for example orange marigold petals, are also promising due to the suggested carotenoid transformations (Figure 1)[10–12]. To investigate the effect of orange marigold petals on red pigmentation for Koi carp skin, fine powder of dried marigold petals was initially mixed with the daily diet at 5 $g_{marigold\ petal}/kg_{total\ food}$. The resulting mixture was fed to five Koi carps for six weeks. Unfortunately, no changes in redness on the fish skin were observed, suggesting that this feeding content was too low to affect the red coloration. Therefore, no further digital photographic analysis was needed and another test should be carried out with a higher marigold amount. It should be noted that, prior to the next feeding study, the Koi carps were fed with the carotenoids-free diet for another four weeks to eliminate the effect of the first round, if any, which used 5 g of marigold petal powder per kg of total food.

In the second feed, the marigold amount was increased to 20 $g_{marigold\ petal}/kg_{total\ food}$. After six weeks, the visible improvements in the skin redness were obtained. Their images were recorded for deeper investigation (Figure 6). On these images, the RGB color model was applied to detect, quantify and compare redness of the selected skin areas before and after the study (Table 2). As expected, the values expressing the red color were significantly improved for all of the fed fish. Indeed, the fish labelled with (B), (C) and (D) showed the best average redness increase by 40-43% while this enhancement ranged from 33 to 35% for other fish.

On the other hand, the red-pigmentated area was expanded based on the preliminary observation. Importantly, the fed fish remained in healthy conditions at this feeding dose during the coloration discovery. These obtained results strongly demonstrated the great efficiency of the diet containing orange marigold petal powder in the red coloration of Koi carps. Interestingly, as can be detected in Figure 6, intensity and area of the black skin parts were also significantly developed due to this lutein supplementation, showing the potential impact of orange marigold petals on the formation and enhancement of other colors for Koi carps.

CONCLUSIONS

In summary, the presence of lutein with a content of 6.57 mg/g in orange marigold flower petals was demonstrated via UV-vis spectroscopy. Prior to the utilization as a dietary supplement for Koi carps, marigold petals were simply dried at 50 ^{o}C and subsequently ground into fine powder. which was mixed with the carotenoids-free food. In a 6-week feeding duration under identical conditions, the weight ratio of 5 $g_{marigold\ petal}/kg_{total\ food}$ was found to be inefficient while increasing this ratio to 20 g/kg led to remarkable redness improvements for all of the tested fish. Moreover, such feeding had no negative effects on their growth and development. These experimental results showed that orange marigold petals are indeed a highly potential dietary food for red coloration in Koi farming. Direct lutein supplementation via this local agricultural source as an alternative to expensive dietary without complicated processing was expected to reduce the cost of manufacturing and farming. The present work aims to investigate correlation between the feeding ratio and the rate of red coloration towards commercialization of the product.

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ABBREVIATION

RGB: Red-Green-Blue
UV-Vis: Ultraviolet-visible

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Ha V. Le: Conceptualization, Methodology, Writing – review & editing. **Long V. B. Pham**: Investigation, Formal analysis, Writing – original draft. **Bao N. H. Vo**: Investigation, Formal analysis. **Nhan T. H. Le**: Methodology. **Quan H. Nguyen**: Funding acquisition, Project administration, Data curation. **Phu V. Luu**: Investigation, Data curation. Formal analysis. **Thien D. Huynh**: Investigation, Data curation. **Khoa D. Nguyen**: Writing – review & editing. **Nam H. Nguyen**: Methodology, Investigation.

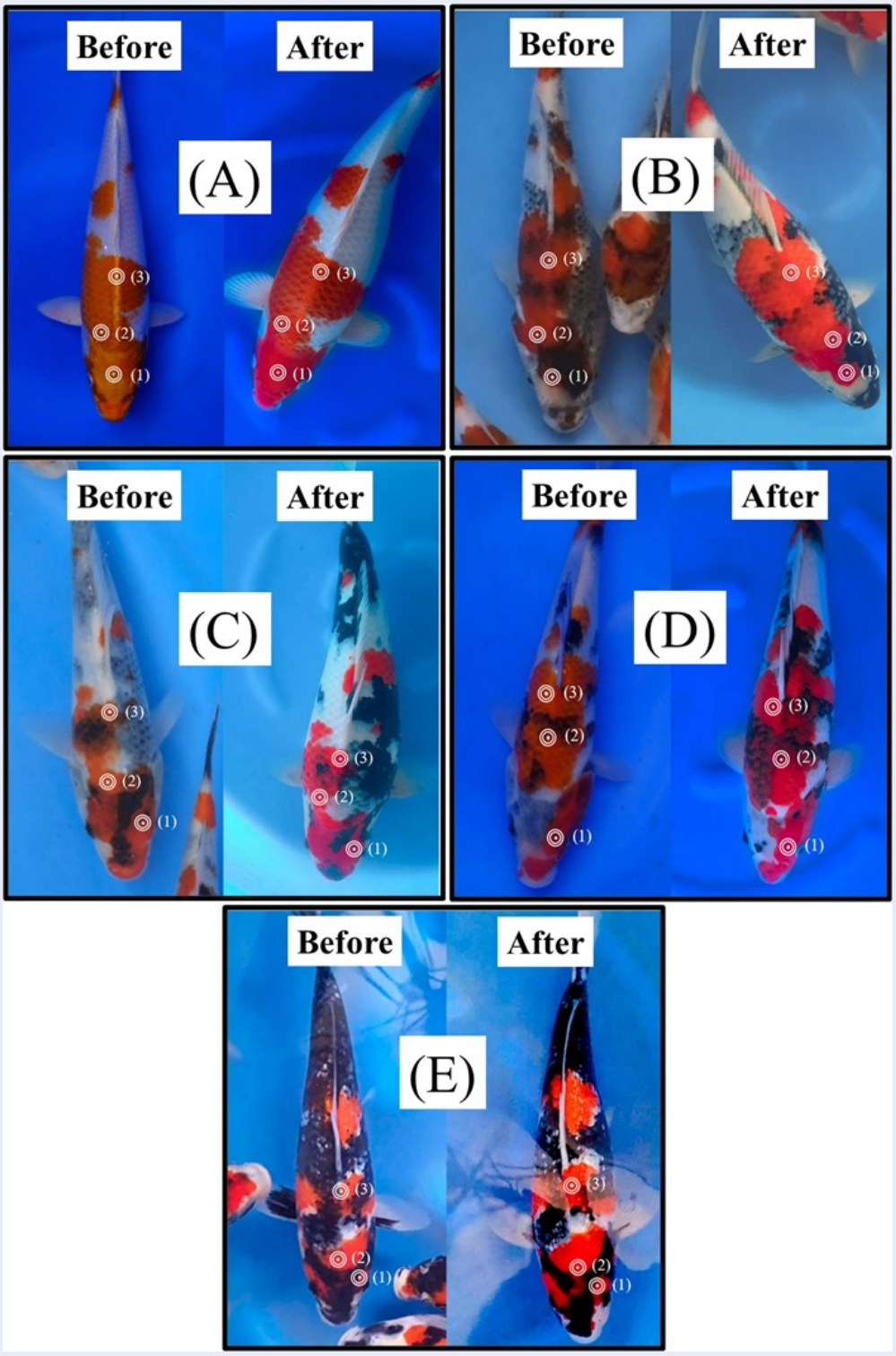

Figure 6: Photographs of five Koi carps named (A), (B), (C), (D), and (E) before and after the feed supplemented with fine powder of dried marigold petals at the weight ratio of 20 $g_{marigold\ petal}$/$kg_{total\ food}$ (the skin positions selected for redness measurement were marked as (1), (2) and (3) in the photographs).

Table 2: Measurement of redness on three skin positions of the selected Koi carps using the RGB color model before and after the feed supplemented with fine powder of dried marigold petals at the weight ratio of 20 $g_{marigold\ petal}/kg_{total\ food}$.

Skin positions*	Before	After
Koi (A)		
(1)	154	210
(2)	138	195
(3)	156	191
Average redness	149.3	198.7
Koi (B)		
(1)	103	190
(2)	164	234
(3)	165	197
Average redness	144.0	207.0
Koi (C)		
(1)	170	213
(2)	140	193
(3)	126	219
Average redness	145.3	208.3
Koi (D)		
(1)	148	182
(2)	138	202
(3)	136	187
Average redness	140.7	190.3
Koi (E)		
(1)	189	215
(2)	133	207
(3)	161	255
Average redness	161.0	225.7

*Three skin positions on the Koi carps were selected at their head, side and back.

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Bài nghiên cứu

Ứng dụng cánh hoa vạn thọ màu cam (*Tagetes erecta* L.) làm thức ăn bổ sung để cải thiện sắc đỏ trên da của cá chép Koi (*Cyprinus carpio* L.)

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TÓM TẮT

Trong nghiên cứu này, cánh hoa vạn thọ màu cam (*Tagetes erecta* L.) đã được sử dụng trực tiếp như nguồn cung cấp lutein cho cá chép Koi (*Cyprinus carpio* L.) với mục tiêu cải thiện sắc đỏ của một số vùng trên da. Cánh hoa đã được tách ra từ hoa tươi và được sấy ở 50 ^{o}C trước khi được nghiền thành bột. Phân tích quang phổ tia cực tím và khả kiến (UV-Vis) đã được thực hiện cho cao chiết lutein trong ethyl acetate từ mẫu cánh hoa khô, cho thấy những mũi hấp thu đặc trưng của lutein đã được đề cập trước đây. Hàm lượng carotenoid tổng được xác định vào khoảng 6.57 mg/g dựa trên đường chuẩn của lutein thương mại. Sự hình thành sắc đỏ của cá Koi đã được thực hiện thông qua việc bổ sung bột cánh hoa với thức ăn hằng ngày của cá lần lượt ở hai tỷ lệ khối lượng khác nhau 5 và 20 $g_{bot\ canh\ hoa}/kg_{thuc\ an\ tong}$. Hình ảnh của cá trước và sau thời gian cho ăn đã ghi lại và được phân tích trên phần mềm chuyên dụng để định lượng và so sánh những sự thay đổi về màu đỏ tại những vùng da được lựa chọn. Sử dụng tỷ lệ thức ăn 20 $g_{canh\ hoa}/kg_{thuc\ an}$ đã cho thấy sự cải thiện sắc đỏ trung bình khoảng 40% cho tất cả năm con cá được nghiên cứu trong khi tỷ lệ ăn 5 $g_{canh\ hoa}/kg_{thuc\ an}$ không hiệu quả. Đặc biệt, cá Koi vẫn duy trì tình trạng sức khỏe tốt trong suốt quá trình Các kết quả thực nghiệm này đã chứng minh hiệu quả cao của cánh hoa vạn thọ màu cam như một nguồn thực phẩm dồi dào có nguồn gốc từ nông nghiệp ở Việt Nam nhằm thay thế cho các thức ăn chứa carotenoid nhập khẩu chi phí cao cho cá Koi.

Từ khoá: Hoa vạn thọ màu cam, cá chép Koi, lutein, thức ăn bổ sung, sắc đỏ

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