



SILYMARIN, *SILYBUM MARIANUM*, SUPPLEMENTED WEANING DIET BOOSTED SURVIVAL, GROWTH, ANTIOXIDANT STATUS, AND FATTY ACIDS PROFILE OF SEABASS, *DICENTRARCHUS LABRAX*

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Abstract

To sustain normal development, high survival, and rapid growth, marine fish larvae require a diet rich in polyunsaturated fatty acids, which could decrease the risk of reactive oxygen species accumulations. Consequently, a 60-day feeding experiment was conducted to determine the effect of silymarin (SM) supplementation in weaning diets on the growth performance, survival, antioxidant enzyme activities, and fatty acids profile of European seabass (*Dicentrarchus labrax*) larvae. Four isonitrogenous and isolipidic diets were investigated using SM at levels of 0, 200, 400, and 600 mg kg⁻¹ (SM_{0.00}, SM₂₀₀, SM₄₀₀, and SM₆₀₀, respectively). The findings showed that, in a dose-dependent manner, increasing dietary levels of SM enhanced survival, growth, and feed utilization. In the SM₆₀₀ group, the weight gain, survival, and feed conversion ratio (FCR) improved by 123.21, 11.66, and 38.72%, respectively, compared to the control group. The dose-response analysis demonstrated a strong positive correlation (R²=0.96) between SM levels and weight increase, and a strong negative correlation (R²=0.88) between SM levels and FCR. The antioxidant enzyme activities of larvae given SM-enriched diets were significantly greater than those of the control group. Compared to the control group, the CAT and SOD improved by 81.77 and 5.08% in the SM600 group. In addition, the saturated fatty acid content decreased while the unsaturated fatty acid content increased, particularly in the SM600 group. The results indicate that supplementing the micro diet of European seabass larvae during weaning with SM at a dose of 600 mg kg⁻¹ increases growth, survival, antioxidant status, and fatty acid profiles.

Key words: silymarin, *Dicentrarchus labrax*, oxidative stress, growth, weaning, fatty acids profile

Aquaculture, as a facility for the production of aquatic animals, plays a critical role in the availability of high-quality animal protein and the conservation of endangered species (Mansour et al., 2022 a; Vasilyeva et al., 2019). The bottleneck of marine aquaculture is providing larvae in adequate quantity and quality for commercial fish farms (Froehlich et al., 2022). Among the exogenous factors that influence larvae growth and survival, the digestibility and absorption of exogenous diets, particularly during the larval phase, are highly important (Liu et al., 2015). In comparison to juvenile and adult fish, the larval stage is crucial for the development of organs, body systems, and physiological processes (Mazurais et al., 2011; Owatari et al., 2018). The larval digestive tract is not fully developed during the early weeks of life, including an underdeveloped digestive system, insufficient digestion, and absorption of exogenous nutrients, which could contribute to the high mortality rates in the

larval stage (Liu et al., 2015). The digestive processes have been regarded as a significant constraint on the use of artificial feeds for fish larvae. As a result, the larval industry needs to develop some additives that enhance digestion (Yao et al., 2020).

Proper nutrition is a significant factor in stimulating growth, health, and immunological responses in farmed fish species (Vandeputte et al., 2019). In marine fish larvae nutrition, a high percentage of polyunsaturated fatty acids (PUFAs) is highly recommended for maintaining larval development, survival, growth, and general quality (Abdel-Rahim et al., 2019). However, the dietary PUFAs are highly susceptible to oxidative stress (Mejri et al., 2021), moreover, due to the fast growth rate of larvae, the high accumulation rate of reactive oxygen species (ROS) (Mao et al., 2020), necessitates the incorporation of antioxidant substances in the larvae weaning diets. Also, dietary inclusion of PUFAs is crucial for marine fish larvae

in captivity, as demonstrated by over 30 years of research. Incorporating these PUFAs into the diet of marine fish larvae has various good impacts, including improved growth and survival, and immune system responses due to their role in the synthesis of eicosanoids such as prostaglandins and leukotrienes (de Mello et al., 2022).

Nowadays, the use of natural antioxidants compounds, including medicinal plants and plant extracts is extensively studied (Fayed et al., 2019; García-Beltrán et al., 2020; Mansour et al., 2020 a, b, 2022 b, 2018). These plants have a rich phytochemicals profile that can be used as an efficient antioxidant, growth promoters, and alternative to antibiotics (Owatari et al., 2018; Van Hai, 2015). In addition, phytochemicals, including flavonoids, tocopherols, cinnamic acid, and folic acid carotenoids, which have a strong significant antioxidant capacity, can inhibit the formation of ROS, scavenge free radicals, and reduce the dangers of oxidative stress (Citarasu, 2010; Hassaan et al., 2019). Furthermore, the medicinal plant's active ingredients can improve nutrient digestibility, absorption, and assimilation by promoting the secretion of digestive enzymes, maintaining a healthy intestinal microbiota, and enhancing immunological function (Jia et al., 2013).

Milk thistle (*Silybum marianum*; SM) is a biennial flowering herb of the family Asteraceae, its fruits and seeds extract are a rich source of flavonoids (Abenavoli et al., 2018), that has been approved for commercial sale all over the world (Marceddu et al., 2022). SM extract is primarily constituted of four active flavonolignans: isosilybin A, isosilybin B, silybin A, silybin B, silychristin A, silychristin B, and taxifolin, accounting for around 50–70% of the total phytochemicals (Tajmohammadi et al., 2018). It also contains a considerable amount of flavonoid taxifolin (Valková et al., 2021). The biological activities of SM include hepatoprotective (Denev et al., 2020), anticancer (Choe et al., 2020), anti-inflammatory (Fanoudi et al., 2020), immunomodulatory (Valková et al., 2021), neuroprotective (Badreddine et al., 2020) and lactogenic agent (Valková et al., 2021) in several animal species. The SM dietary supplementation had favorable effects described in mammals and birds on enhancing physiological and nutritional status over the last few decades (Doostkam et al., 2022; El Hassanen et al., 2021; Janocha et al., 2021; Stastnik et al., 2020).

The effects of adding SM to aquatic species feed revealed that it enhanced the growth performance and antioxidant capacity of Prussian carp (*Carassius auratus gibelio*), turbot (*Scophthalmus maximus* L.), and large yellow croaker (*Larimichthys croceus*) (Wang et al., 2019; Yao et al., 2020; Yi et al., 2012). In addition, SM enhanced the hepatoprotective effects of common carp (*Cyprinus carpio*) (Jia et al., 2013), boosted the lipid metabolism of grass carp (*Ctenopharyngodon idella*) and strengthened the immune system of rainbow trout (*Oncorhynchus mykiss*) (Ahmadi et al., 2012; Xiao et al., 2017). Meanwhile, studies on fish are very rare especially in the larval stages as a critical life period.

Since European seabass is the most significant marine species farmed in Egypt and in the Mediterranean Sea (Lotfy et al., 2021; Mansour et al., 2022 a, b). However, there is an essential need to improve seabass hatcheries' capacity, by improving the quantity and quality of produced larvae. Therefore, this study aims to evaluate the effect of SM as a dietary supplement in the weaning micro diet (WMD) of European seabass larvae on survival, growth performance, feed conversion ratio, antioxidant status, and whole-body fatty acids profile.

Material and methods

Larval rearing facilities

The current research was conducted at the Marine Fish Hatchery, Kilo-21, the General Authority for Fish Resources Development (GAFRD), in collaboration with both the Marine Fish Laboratory (MFL), Faculty of Agriculture, Saba Basha, Alexandria University, and Fish Rearing Lab, National Institute of Oceanography and Fisheries NIOF, Egypt for doing the biochemical analysis.

European seabass *D. labrax* larvae at 16 days hatch (16 DPH) were stocked in the experimental weaning glass aquaria (each was 35-liter water volume). In twelve aquaria, larvae were initially stocked at a density of 14 larvae per liter. The larvae were fed the experimental diets for 60 days after four days of acclimatization at 20 DPH. Using artificial light and an adjustable timer, the photoperiod was adjusted to a fixed daily lighting cycle (12 h light: 12 h dark) until the trial period ended. From 300 to 600 lux, the light intensity increased with larval age. Throughout the trial period, a daily water exchange rate of 290 ml/min/aquarium was maintained. As fish biomass rose, the amount of artificial aeration generated by an air blower and distributed through very fine air bubble diffusers was gradually increased to maintain an even distribution of larvae throughout the aquaria and an oxygen concentration of more than 6 ppm. Larvae were maintained at a temperature of $16 \pm 2^\circ\text{C}$, a pH of 6.8, a salinity of 38 ± 1 ppt, and an ammonia concentration of 0.002 mg/L. The authors confirm that they have followed the guidelines of the Declaration of Helsinki and were approved by the institutional animal care and use committee of Alexandria University with approval No: AU: 19/22/06/05/3/22.

Experimental diets and feeding protocol

Four experimental weaning micro diets (WMD) with varying concentrations of *S. marianum* (SM; Legalon silymarin® 140 mg, Madaus GmbH Co, Köln, Nordrhein-Westfalen, Germany) were tested. The weaning micro diets were supplemented with SM at levels of 0.0, 200, 400, and 600 mg SM kg⁻¹ (SM_{0.0}, SM₂₀₀, SM₄₀₀, and SM₆₀₀, respectively). The used doses were selected based on the results of the previous study using SM extract as dietary supplementation on the diet of grass carp (*C. idellus*), which revealed a linear dose-dependent effect with 200 mg SM kg⁻¹ as the maximum used level (Xiao et al., 2017).

Table 1. Feed formulation (%) and chemical composition (%) of the basic experimental larval micro weaning diet (WMD) used to feed European seabass (*Dicentrarchus labrax*)

Ingredients (%)	SM _{0.0}	SM ₂₀₀	SM ₄₀₀	SM ₆₀₀
Fishmeal	36.5	36.5	36.5	36.5
Fish oil	5.0	5.0	5.0	5.0
Powdered milk	27.0	27.0	27.0	27.0
Poultry egg	23.0	23.0	23.0	23.0
Zymogen ¹	4.0	4.0	4.0	4.0
CMC ²	3.0	3.0	3.0	3.0
Vit. and min. premix ³	1.0	1.0	1.0	1.0
Ascorbic acid	0.5	0.5	0.5	0.5
<i>Silybum marianum</i> (mg)	00	200	400	600
Chemical analysis (%)				
Moisture	6.08	6.06	6.03	6.02
Crude protein	51.40	51.45	51.50	51.48
Crude fat	22.78	22.82	22.86	22.88
Crude ash	8.50	8.52	8.55	8.50
Crude ash non-soluble in HCl	2.70	2.71	2.72	2.71
Calcium	1.50	1.53	1.54	1.52
Phosphorus	1.40	1.45	1.49	1.50
Fiber	0.02	0.02	0.021	0.02

¹Zymogen: pepsin: 166 mg, protease: 8330 (NF units), lipase: 1330 (NF units), amylase: 8330 (NF units), hemicellulose: 66 mg, dimethylololsiloxane: 33 mg, Ox bile: 33 mg, vitamin B₁: 42 mg. Nile Co. for pharmaceutical and chemical industries. Cairo Egypt.

²Vitamin and minerals mixture of the micro diet / 2.5 kg premix: vitamin A, 12 million IU; vitamin D₃, 2 g; vitamin E, 10 g; vitamin K, 2 g; vitamin B₁, 1 g; vitamin B₆, 1.5 g; vitamin B₁₂, 10 mg; vitamin B₂, 4 g; pantothenic acid, 10g; nicotinic acid, 20 g; folic acid, 1000 mg; biotin, 50 mg; choline chloride, 500 g; I, 1 g; iron, 30 g; Mn, 55 g; Zn, 55 g; selenium, 1 g. (Phaezer Co.). Vitamin and minerals mixture of the commercial diet, 2.5 kg premix: vitamin A, 20000 IU; vitamin D₃, 2500 IU; vitamin E, 700 mg; vitamin C, 2000 mg; I, 5 mg; Mn, 50 mg; Zn, 50 mg; selenium, 0.3 mg; Cu, 6 mg; Co, 1 mg; ethoxyquin, 120 mg; BHA, 30 mg; propyl gallate, 30 mg.

³CMC: Carboxymethyl cellulose.

Table 2. Feeding protocol of European seabass (*Dicentrarchus labrax*) larvae fed different concentrations of *Silybum marianum* (mg kg⁻¹) during the weaning period

Experimental period (dph)	Range (average) amount of WMD (g/day)	Enriched Artemia (pcs/ml) ¹	WMD size (μm)	Number of meals/day (WMD: Artemia)
20–27	0.6–0.8 (0.7)	7	100–200	4:2
28–34	0.8–1.0 (0.9)	10	200–300	4:2
35–41	1–1.2 (1.1)	15	300–400	5:1
42–48	1.2–1.4 (1.3)	10	300–400	5:1
49–55	1.5–1.7 (1.6)	5	300–400	5:1
56–62	1.8–2 (1.9)	–	400–500	6:0
63–69	2.1–2.3 (2.2)	–	400–500	6:0
70–76	2.4–2.6 (2.5)	–	400–500	6:0
77–80	2.8–3 (2.9)	–	400–500	6:0

¹Artemia was enriched with Easy DHA (product of INVE®, Aquaculture SA, Belgium).

The basic weaning diet was developed using standard feed ingredients according to El-Dahhar et al. (2021). Briefly, the ingredients (Table 1) were milled through a screen (0.6 mm holes diameter) before mixing, then homogenized well in a food grinder (model 5 NFGA; KitchenAid, St. Joseph, MI 49085, USA). The zymogen®, vitamin and mineral premix, and SM was incorporated into the mixture and homogenized well. The fish oil (5%) was added in emulsified form using 0.7% phosphatidyl choline

(El-Dahhar and El-Shazly, 1993). Boiling water was added at a rate of 50% for pelting after mixing. The diets were then pelletized in the meat grinder (KitchenAid model 5 NFGA, St. Joseph, MI 49085, USA), dried in a force air oven, crushed, and crumbled many times through screens ranging from 100 to 500 μm. Each mesh-sized micro diet was packed in plastic bags and stored at –20°C in a deep freezer. The formulation of the WMD and its chemical analysis are listed in Table 1.

The larvae were fed the experimental diets starting at 20 DPH. The WMD and live food (newly hatched artemia) were manually fed six times each day with 90 min intervals between each meal from 8:00 AM to 5:00 PM. Table 2 illustrates the feeding quantity, micro diet size, and the number of meals per day. Each week, three samples of larvae were inspected under a binocular light microscope (B-159, Optika, Ponteranica, Italy) to measure feed acceptance and gut fullness. At 80 DPH, the experiment was terminated.

Newly hatched artemia, *Artemia urmiana*, was produced by the standard protocol of artemia hatching following cyst decapsulation (Sorgeloos et al., 1986). The newly hatched nauplii are transferred to the enrichment tank at a density of 30–50 nauplii ml⁻¹. The enrichment medium consists of 0.6 g Easy DHA (INVE® Aquaculture SA, Belgium) emulsified in 1 liter filtered water (35–40°C) for 5 min and then cooled to room temperature. Artemia was developed in the enrichment emulsions for 24 h and gathered using a plankton net and rinsed three times with seawater at room temperature (Adloo et al., 2012).

Larval growth, survival, and feed utilization

At the end of the experiment, all fresh live fish were weighed (using high precise digital scale – 0.001 g accuracy). Weight and length was determined to the nearest digit number for all fish in the aquaria. The feed conversion ratio (FCR) and protein efficiency ratio (PER) data were estimated using artificial diet consumption to the nearest precise quantity. Using an appropriate siphon, the leftover feed was sucked up from the bottom of larval rearing tanks, dried and subtracted from the total amount provided.

It was hard to estimate the actual consumption of artemia to adjust the realistic FCR based on actual dry feed and live food consumption (artemia). The following equations were used:

$$\text{Weight gain (mg fish}^{-1}\text{): } WG = W_t - W_0$$

where: W_0 = the initial mean weight of fish (mg); W_t = the final mean weight of fish (mg).

$$\text{Average daily gain (mg fish}^{-1}\text{ day}^{-1}\text{): } ADG = W_t - W_0/n$$

where: n = duration period.

$$\text{Specific growth rate (\% day}^{-1}\text{): } SGR = 100 \times [(\ln W_t - \ln W_0)/\text{days}]$$

where: $\ln$ = natural logarithm.

$$\text{Survival (\%)} = 100 \times (\text{final number of fish}/\text{initial number of fish}).$$

$$\text{Feed conversion ratio, FCR} = \text{feed intake (mg) as dry weight} / \text{weight gain (mg)}.$$

Protein efficiency ratio, PER = weight gain (mg)/protein intake (mg).

Antioxidant status assay

At the end of the experiment, larval samples ($n = 3$; 10 fish per aquaria) were stored at -20°C to evaluate

antioxidant enzyme activities. A homogenizer was used to homogenize the whole larval sample (Hettich model EBA 12R, Germany) in 0.25 M sucrose buffer. The homogenate was centrifuged at $3000 \times g$ for 10 min., then the supernatant was collected and used to determine the following parameters: catalase (CAT), superoxide dismutase (SOD), and thiobarbituric acid reactive substance (TBARS).

Catalase activity (CAT, U mg⁻¹ tissue) was measured in the body homogenate (Luck, 1974). Briefly, a 10 ml of tissue homogenate sample was added to 1.25 ml of freshly prepared buffer containing 50 ml of H₂O₂ 10 ml⁻¹ Na-K phosphate buffer (0.15 M, pH 7, El-Gomhouria Co., Egypt). The difference in absorbance was recorded after 20 s (A1) and after 80 s (A2) of incubation at 240 nm against air. The CAT value was calculated as $A1 - A2/0.0008$.

Superoxide dismutase (SOD; U mg⁻¹ tissue) was evaluated according to Misra and Fridovich (1972). Briefly, 20 ml of tissue homogenate was added to 940 ml sodium carbonate buffer (pH 10.2, 0.05 M) and 40 ml epinephrine (30 mmol l⁻¹ dissolved by adding 30 ml of HCL). The inhibition of epinephrine auto-oxidation in the alkaline medium to adrenochrome was recorded after 30 and s at 480 nm. A control was prepared as 960 ml sodium carbonate buffer and 40 ml epinephrine.

$$\text{The percent of inhibition (\%)} = 100 - [(DA \text{ control} - DA \text{ sample}) / DA \text{ control}] \times 100$$

$$\text{SOD activity in tissue (U ml}^{-1}\text{)} = \% \text{ inhibition} \times 3.75.$$

The TBARS (nmol g⁻¹ tissue) were measured according to the method of Tappel and Zalkin (1960). Briefly, the larval tissue homogenate 1 ml of the sample was added to 2 ml trichloroacetic acid (25%), properly mixed, and centrifuged at 3000 rpm for 20 minutes. 2 ml of supernatant was mixed with 1 ml TBA and incubated for 15 min at 100°C, then cooled, and the absorbance at 532 nm was determined.

Fatty acids profile analysis

Lipid extraction was conducted using whole-body larvae mixed samples of the three replicates of each treatment (10 fish per aquaria). Briefly, in a 250 ml centrifuge tube, add 2 g of the WMD or larvae fish, 16 ml of water, 40 ml of methanol, and 20 ml of chloroform. Macerate for two minutes; add 20 ml of chloroform and macerate for 30 seconds; repeat with 20 ml of water and macerate for 30 seconds. Centrifuge the mixture for 10 minutes at $2000 \times g$. Remove the lower chloroform layer and filter into a dry weighing flask using coarse filter paper. Evaporate the chloroform until it reaches the drypoint specified in Pearson's Chemical Analysis of Food (Horwitz, 1982).

Methylation of lipid: Add 5 ml of methanolic sulphuric acid (1 ml of concentrated sulphuric acid and 100 ml of methanol) and 2 ml of benzene to a tube holding 50 mg of lipid, close the tube tightly, and set it in a 90°C water bath for 1.5 hours. Allow cooling slightly before adding

8 ml of water and 5 ml of petroleum ether, shaking rapidly, and separating the ethereal layer in a dry tube, and evaporating to a state of dryness.

Analysis of lipid: the analysis was carried out by gas chromatography (GC) using HP (Hewlett Packard) 6890 series GC equipped with flame ionization detector (FID) under the following conditions: an analytical glass column DB-32 (50%-cyanopropyl-methylpolysiloxane), 30 m, 0.32 mm ID, 0.25 µm film thickness. Detector temperatures 240°C, injector temperature 220°C, injection volume 3 µl, split ratio 50:1 and carrier gas: nitrogen, gas flow: 1 ml/min.

Statistical analysis

The data were presented as means $\pm$ standard errors (S.E.) and analyzed with one-way ANOVA at a 95% confidence level, using SPSS version 22 (SPSS Inc., IL, USA) and when a significant variation was detected, a subsequent analysis of Duncan's multiple range test was performed. The regression analysis was conducted to find the pattern of dose-response of larvae to increasing levels of supplementation.

Results

Growth performance and feed utilization

Growth performance and feed utilization of seabass larvae fed SM supplemented weaning diets for the 60-day larval rearing were summarized in Table 3. The final BW of PL fed SM supplemented diets increased significantly by 25.0, 93.0, and 117.6% for the groups SM₂₀₀, SM₄₀₀, and SM₆₀₀, respectively, as compared to the PLs in the control group (SM_{0.00}). Also, the ADGs and SGRs increased significantly in the groups SM₂₀₀, SM₄₀₀, and SM₆₀₀ by 26.18, 97.56, and 123.21 and 7.2, 21.1, and 25.0%, respectively, as compared to the SM_{0.00} group. The final length increased significantly by 40.9, 90.9,

and 95.7%, respectively, for the SM₂₀₀, SM₄₀₀, and SM₆₀₀ groups, compared to the SM_{0.00} group. The SM concentration had a significant effect on the survival of *D. labrax* larvae. The survival rates of the groups SM₂₀₀, SM₄₀₀, and SM₆₀₀ increased by 4.5, 9.5, and 11.7%, respectively, as compared to the SM_{0.00} group.

The dietary SM supplementation demonstrated a significant beneficial effect on the feed utilization parameters of *D. labrax* larvae. Whereas the increase in SM level improved the quantity of feed intake by 9.3% in SM₂₀₀ group and up to 36.6% in SM₆₀₀ group. The recorded value of FCR was reduced by 13.5, 39.7, and 38.7% for the groups SM₂₀₀, SM₄₀₀, and SM₆₀₀, respectively, compared with the SM_{0.00} group. The PER value improved by 15.6, 67.3, and 63.5% for the groups SM₂₀₀, SM₄₀₀, and SM₆₀₀, respectively, compared with the SM_{0.00} group. The dose-response study of SM as dietary supplementation in the weaning diet of seabass showed a linear improving effect on weight gain and FCR in a strong correlation ($R^2 = 0.95$ and 0.88, respectively) (Figure 1).

Antioxidant status

The influence of SM dietary supplementation on antioxidant enzyme activities in European seabass PLs tissue homogenate was presented in Table 4. The SM concentration exhibited a significant influence on *D. labrax* larvae antioxidant enzymes. The TBARS (nmol/g tissue) values dropped as the SM levels increased from 11.7% at M₂₀₀ to 29.6% at M₆₀₀. The CAT activity increased by 45.4, 68.4, and 81.8%, respectively, in the SM₂₀₀, SM₄₀₀, and SM₆₀₀, compared to the SM_{0.00} group. With an increase in SM levels, a non-significant ($P > 0.05$) increase in SOD value was observed compared to the SM_{0.00} group by 1.6, 5.5, and 5.1% at SM₂₀₀, SM₄₀₀, and SM₆₀₀, respectively. The effect of increasing dietary supplementation of SM on antioxidant status (CAT and TBARS) was in a dose-dependent manner with a strong significant correlation ($R^2 = 0.93$ and 0.98, respectively) (Figure 2).

Table 3. Growth performance and feed utilization of European seabass (*Dicentrarchus labrax*) larvae fed different concentrations of *Silybum marianum* (mg kg⁻¹) during the weaning period

Parameters	Treatments			
	SM _{0.0}	SM ₂₀₀	SM ₄₀₀	SM ₆₀₀
Final weight (mg fish ⁻¹) ¹	60.02 $\pm$ 0.80 d	75.03 $\pm$ 1.08 c	115.85 $\pm$ 6.80 b	130.60 $\pm$ 3.28 a
Gain (mg fish ⁻¹)	57.3 $\pm$ 0.8 d	72.3 $\pm$ 1.1 c	113.2 $\pm$ 6.8 b	127.9 $\pm$ 3.3 a
Average daily gain (mg fish day ⁻¹)	0.96 $\pm$ 0.01 d	1.21 $\pm$ 0.02 c	1.89 $\pm$ 0.11 b	2.13 $\pm$ 0.05 a
Specific growth rate (% fish day ⁻¹)	5.17 $\pm$ 0.02 d	5.54 $\pm$ 0.02 c	6.26 $\pm$ 0.10 b	6.46 $\pm$ 0.04 a
Final length (cm)	2.08 $\pm$ 0.01 c	2.93 $\pm$ 0.04 b	3.97 $\pm$ 0.09 a	4.07 $\pm$ 0.06 a
Survival (%)	68.55 $\pm$ 0.21 d	71.61 $\pm$ 0.26 c	75.03 $\pm$ 0.26 b	76.54 $\pm$ 0.38 a
Feed intake (mg fish ⁻¹)	170.22 $\pm$ 1.4 d	186.02 $\pm$ 1.5 c	201.11 $\pm$ 3.5 b	232.5 $\pm$ 1.5 a
Feed conversion ratio ²	2.97 $\pm$ 0.04 a	2.57 $\pm$ 0.06 b	1.79 $\pm$ 0.12 c	1.82 $\pm$ 0.06 c
Protein intake (mg fish ⁻¹)	87.5 $\pm$ 0.71 d	95.6 $\pm$ 0.77 c	103.4 $\pm$ 1.80 b	119.5 $\pm$ 0.78 a
Protein efficiency ratio ³	0.655 $\pm$ 0.01 b	0.757 $\pm$ 0.02 b	1.096 $\pm$ 0.07 a	1.071 $\pm$ 0.03 a

Values with different letters in the same row differ significantly ($P < 0.05$).

¹Initial weight = 2.7 mg/larvae.

^{2,3}The feed conversion ratio and protein efficiency ratio were estimated using only dry feed (WMD).

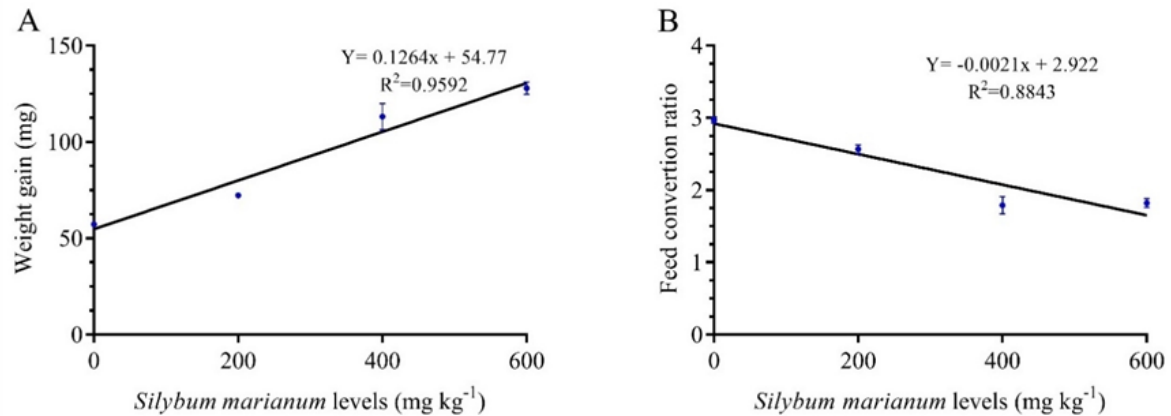


Figure 1. Fit linear regression model of increasing dietary supplementation levels and weight gain (A), and feed conversion ratio (B)

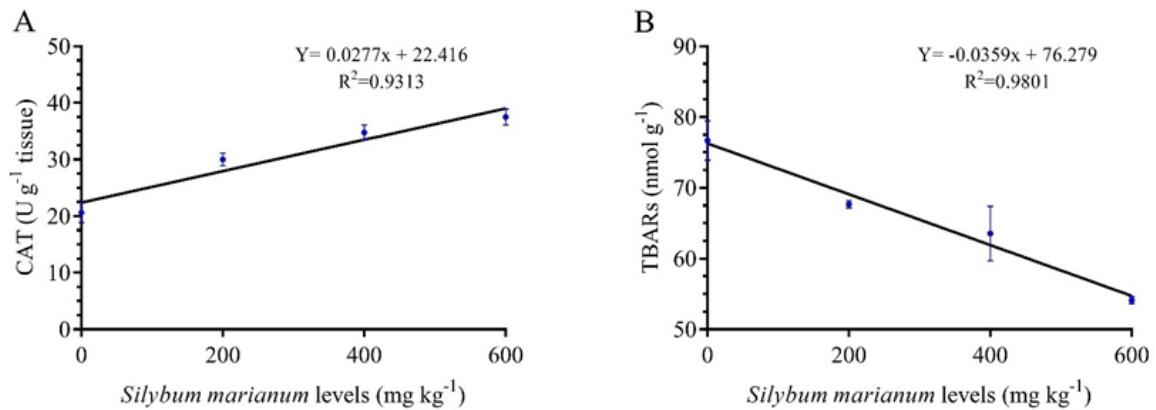


Figure 2. Fit linear regression model of increasing dietary supplementation levels and catalase activity (A), and thiobarbituric acid reactive substances (B)

Table 4. Antioxidant enzyme activities in body homogenate of European seabass (*Dicentrarchus labrax*) larvae fed different concentrations of *Silybum marianum* (mg kg⁻¹) during the weaning period

Items	SM _{0.0}	SM ₂₀₀	SM ₄₀₀	SM ₆₀₀
TBARS (nmol g ⁻¹ tissue)	76.67±2.82 a	67.69±0.52 ab	63.59±3.85 ab	54.11±0.52 b
Catalase (U g ⁻¹ tissue)	20.63±1.81 c	30.00±1.15 b	34.75±1.59 ab	37.50±1.44 a
Superoxide dismutase (U g ⁻¹ tissue)	52.19±2.19	53.00±3.69	55.07±4.38	54.84±2.30

Values with different letters in the same row differ significantly (P<0.05).

TBARS: thiobarbituric acid reactive substance.

Fatty acids profile

The fatty acid composition of seabass post-larvae fed SM-supplemented diets was summarized in Table 5. There was an increase in polyunsaturated fatty acid content (PUFA) in fish fed SM diets. Unsaturated fatty acid concentrations in the SM diets increased significantly with increasing SM levels, reaching a maximum (54.31%) in the SM₆₀₀ group compared to 52.18% in the SM_{0.00} group (Figure 3). Compared to the SM_{0.00} group, the saturated fatty acid level of the groups SM₂₀₀, SM₄₀₀ and

SM₆₀₀ decreased by 2.3%, 2.9%, and 4.9%, respectively (Figure 3). The SM₆₀₀ group demonstrated the greatest fatty acid profile improvement. Only in groups given SM, arachidonic acid (ARA) was detected (Figure 4). The SM₂₀₀ group contained the highest concentration of eicosapentaenoic acid (EPA), at 1.07%, compared to the other groups (0.53–0.64%) (Figure 4). In SM-fed groups, nervonic acid (C24:1, n-9, cis-15-tetracosenoic acid) was identified at a higher concentration (15.59–16.41%) than in the control group (13.79 %) (Table 5).

Table 5. Fatty acids profile (% dry weight) of micro diet (WMD) and European seabass (*Dicentrarchus labrax*) larvae fed different concentrations of *Silybum marianum* (mg kg⁻¹) during the weaning period

Fatty acids profile	Feed	Seabass PLs			
		SM _{0.0}	SM ₂₀₀	SM ₄₀₀	SM ₆₀₀
(C4:0) Butyric acid (%)	0.150	0 c	0.028 b	0.119 a	0 c
(C6:0) Hexanoic acid (%)	0.490	0.264 a	0.076 b	0.074 b	0.060 c
(C8:0) Octanoic acid (%)	0.435	0.247 a	0.072 b	0.035 d	0.054 c
(C10:0) Decanoic acid (%)	1.147	0.2149 a	0.0485 d	0.123 b	0.099 c
(C11:0) Undecanoic acid (%)	0.029	0	0	0	0
(C12:0) Dodecanoic acid (%)	1.837	0.446 a	0.086 c	0.328 b	0.338 b
(C13:0) Tridecanoic acid (%)	0.006	0.097 a	0	0.018 b	0
(C14:0) Myristic acid (%)	9.537	3.119 b	3.579 a	3.224 b	3.553 a
(C14:1) Myristolic acid (%)	0.288	0.186 a	0.045 b	0.180 a	0.189 a
(C15:0) Pentadecanoic acid (%)	0.954	0.478 a	0.095 c	0.478 a	0.427 b
(C15:1) Cis-10-Pentadecenoic acid (%)	0.165	0.210 b	0.050 c	0.292 a	0.232 a
(C16:0) Palmitic acid (%)	31.606	20.288 b	22.97 ab	22.93 ab	23.07 a
(C16:1) Palmitoleic acid (%)	4.332	0.35 c	0.34 c	3.50 b	3.79 a
(C17:0) Heptadecanoic acid (%)	1.332	0.744 a	0.192 b	0.020 c	0.730 a
(C17:1) Cis-10-Heptadecenoic acid (%)	0.111	0.204 a	0.033 c	0.101 b	0.097 b
(C18:0) Stearic acid (%)	9.053	15.456 a	12.267 b	12.371 b	11.043 c
(C18:1) n9t Oleinic acid (%)	24.036	2.572 b	3.512 a	3.301 a	2.644 b
(C18:2) n6c linolic acid (%)	0.046	7.193 a	1.192 b	0.332 c	0.149 c
(C18:2) n6t linolelaidic acid (%)	0.292	0.337 b	0.080 d	0.615 a	0.097 c
(C18:2) n9t Elaidic acid (%)	1.234	21.103 c	24.272 a	22.938 b	23.07 b
(C18:3) n3a-linolenic acid (%)	1.084	1.677 a	1.234 d	1.245 c	1.549 b
(C18:3) n6 γ-linolenic acid (%)	0.089	0.111 a	0	0.068 b	0.056 c
(C20:0) Arachidic acid (%)	0.010	0.290 b	0.064 c	0.308 a	0.292 b
(C20:1) cis-11-Eicosenoic acid (gadoleic acid) (%)	1.350	0.113 d	1.289 a	1.199 b	0.873 c
(C20:2) cis-11,14-Eicosadienoic acid (%)	0.007	0.236 b	0.052 c	0.039 d	0.248 a
(C20:3n3) cis-11,14,17-Eicosatrienoic acid (ETA; %)	0.075	0.252 d	3.066 b	3.244 a	0.348 c
(C20:3n6) cis-8,11,14-Eicosatrienoic acid (%)	0.114	3.041 b	0.794 c	0.195 d	3.315 a
(C20:4n6) Arachidonic acid (%)	0	0.025 c	0.042 b	0.043 b	0.060 a
(C20:5) cis-5,8,11,14,17-Eicosapentaenoic acid% (EPA; %)	1.367	0.312 d	0.567 b	0.528 c	0.647 a
(C21:0) Heneicosanoic acid (%)	0.042	0.536 b	0.452 c	0.861 a	0.034 d
(C22:0) Behenic acid (%)	4.221	5.222 c	6.173 a	5.021 d	5.780 b
(C22:1) Erucic acid (%)	0.060	0	0.042 b	0.042 b	0.052 a
(C22:2) cis-13,16-Docosadienoic acid (%)	0.045	0	0	0.035 a	0
(C22:6n-3) cis-4,7,10,13,16,19-Docosahexae-noic acid% (DHA; %)	0.013	0.42 d	0.98 c	1.31 b	1.56 a
(C23:0) Tricosanoic acid (%)	0.005	0.262 a	0.074 b	0	0
(C24:0) Tetracosanoic acid (%)	0.027	0.022 b	0.083 a	0.088 a	0.087 a
(C24:1) cis-15-Tetracosenoic acid (%)	4.412	13.892 d	16.076 a	14.788 c	15.41 b

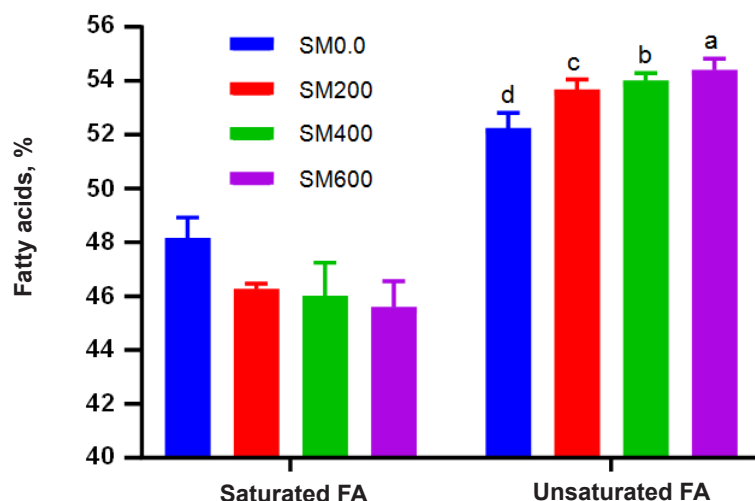


Figure 3. Saturated and unsaturated fatty acid profiles of *D. labrax* larvae fed different concentrations of *Silybum marianum* (mg kg^{-1}) during the weaning period of 60 days

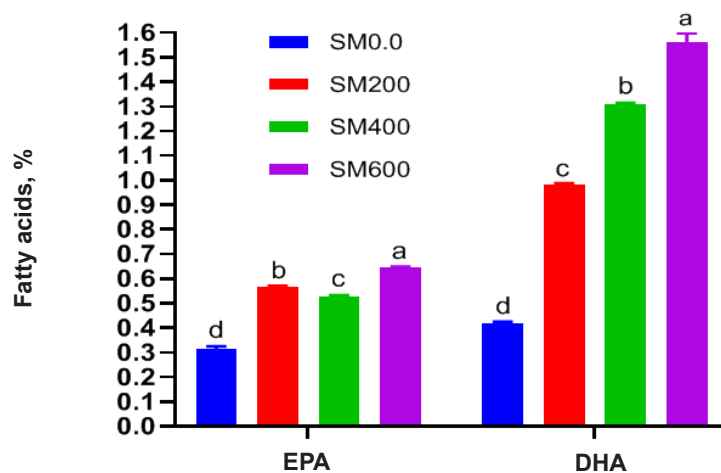


Figure 4. Eicosapentaenoic acid (EPA; %) and docosahexaenoic acid (DHA; %) of *D. labrax* larvae fed different levels of *Silybum marianum* (mg kg^{-1}) during the weaning period of 60 days

Discussion

The marine fish larvae weaning diets could contain a high level of polyunsaturated fatty acids to cover the requirement of fast-growing larvae and maintain fish survival, development and reduce abnormalities (Ahmadi et al., 2012; Betancor et al., 2011; Carvalho et al., 2022). Accordingly, dietary supplementation of antioxidant substances is highly recommended to eliminate the ROS from the body of high metabolic rate larvae and protect organelles, cell membranes, and valuable nutrients from oxidative damage. The therapeutic properties of SM have been well recognized for many years (Choe et al., 2020; Tajmohammadi et al., 2018). SM extract is rich in phytochemicals extracted from milk thistle seeds that serve as an antioxidant, growth promoter, appetite stimulant, and immunostimulant in fish due to the bioactive components, such as essential oils, pigments, flavo-

noids, and phenolics (Hassaan et al., 2019; Owatari et al., 2018).

Fish growth depends on nutritional digestion and absorption, disease resistance, and organ development, which depends in part on intestinal health (Mazurais et al., 2011). In the present study, a significant increase in *D. labrax* growth performance (FW, WG, and SGR) occurred with increasing the amount of dietary SM supplementation in the weaning diets. FW of larvae in T4 (SM₆₀₀) exceeded by more than 100% compared to the control diet (SM_{0.00}). These findings can be expounded by that higher levels of SM added to the diet led to better growth and appetite stimulation, which resulted in increased feed intake, and consequently its reflection on the improvement of growth parameters. Moreover, the improved growth indices presented here can be attributed to the bioactive components present in SM, which not only improve feed efficiency but also influence protein reten-

tion as well as the high content of flavonolignans, a component that plays as an antioxidant and stimulates protein synthesis (Banaee et al., 2011; Owatari et al., 2018). In addition, dietary SM seed upregulates the transcription level of the growth hormone gene in the pituitary gland of Nile tilapia (Hassaan et al., 2019). Moreover, SM contains sugars, vitamins (A, B₁₂, E, C, and K), as well as apigenin, tyramine, and fixed oils of 20–30% that could participate in improving larvae growth performances (Tajmohammadi et al., 2018). The current findings are consistent with Hassaan et al. (2019), who reported that the addition of SM seeds resulted in better growth performance of Nile tilapia (*O. niloticus*) fingerlings, whereas an increase in FW of more than 28% occurred with the addition of SM by 7.5 g kg⁻¹ compared to the control diet. In the same context, Jia et al. (2013), Xiao et al. (2017) and Owatari et al. (2018) reported that SM extract improved growth performance and feed utilization efficiency in common carp, grass carp, and Nile tilapia, respectively. In addition, dietary SM at dose of 10 g/kg diet improved growth performance of African catfish (*Clarias gariepinus*) exposed to high fluoride (73.45 mg FL L⁻¹) (El-Houseiny et al., 2022).

On the contrary, the addition of dietary SM seeds at a rate of 5–10% did not improve the growth performance of common carp, *C. carpio* L. (Al-Jubouri et al., 2021). Furthermore, the addition of SM to the diet of *C. auratus gibelio* at a level of 4 mg kg⁻¹ diet did not influence growth performance or feed consumption (Yi et al., 2012). These contradictory findings may be attributable to differences in the applied levels of SM, which is very high in the first study or very low in the second study compared to our findings, also fish growing stages which are marine larvae with different nutritional requirements.

In the present study, the best survival rate was obtained with T4 (SM₆₀₀), outperforming T1 (SM_{0.00}) by more than 9%. In this study, improved survival in T4 is consistent with the increased survival stated for Nile tilapia and showed notably more than 6%, with the addition of SM (7.5 g kg⁻¹) compared to the control diet (Hassaan et al., 2019). The current findings of improving survival rate with increasing SM supplementation levels revealed that 600 mg kg⁻¹ diet was not the toxic levels of SM. Wang et al. (2019) reported that the chronic toxicity of SM is very low meanwhile using an overdose of SM could result in a number of undesirable effects. However, its specific mechanism of acute toxicity remains poorly understood.

Feed utilization was improved in a dose-dependent manner in the present study. Fish fed diets supplemented with SM had the lowest FCR and greatest PER compared to the control, and there is a positive strong correlation between increasing SM intake and lower FCR. These findings may be attributable to the availability of bioactive components in SM, which not only enhanced the palatability but also improved feed efficiency and affected protein retention. In this context, plant extracts active components can enhance digestibility and nutrient

availability, resulting in increased protein synthesis and feed utilization (Citarasu, 2010; Elkaradawy et al., 2021; Fayed et al., 2019; Mansour et al., 2022 b). These findings are consistent with those of Hassaan et al. (2019) and Yao et al. (2020), who reported that the lowest FCR values were observed with increasing levels of SM in the diets.

The non-enzymatic and enzymatic antioxidant defense systems of fish provide protection against oxidative stress damage (Martínez et al., 2005). Antioxidant defense systems include antioxidant enzymes that represent protection against oxidative damage in tissue (Van Hai, 2015). The presence of TBARS is an indicator of free radical accumulation. Herein, the addition of SM supplementation to the weaning diet of seabass showed a significant downward curve in TBARS levels in the fish homogenate. This finding concurs with Wang et al. (2019), who reported that adding SM to a diet at 400 mg kg⁻¹ reduced malondialdehyde (MDA) as an indicator of lipid peroxidation by about twice compared to the control diet. Similar results were reported for seabass larvae fed a diet supplemented with 0.8 mg kg⁻¹ selenium and showed a significant increase in the glutathione peroxidase and reduced the MDA (El-Dahhar et al., 2016).

In the present trial, SOD and CAT enzymes showed higher activity in fish fed on SM supplemented diets compared to the control. These results demonstrated that adding SM at optimized dietary levels could improve the antioxidant enzymes in seabass, as both CAT and SOD were increased in fish fed SM-supplemented diets. The enhancing effects of SM on the antioxidant enzymes have been previously documented in Nile tilapia by increasing the activities of CAT and SOD (Hassaan et al., 2019). Our findings agree with those of Wang et al. (2019) and Elabd et al. (2016), who demonstrated that the antioxidant capacity of Japanese seabass, *Lateolabrax japonicus*, and Nile tilapia was enhanced by supplementation with a Chinese herbal medicine mixture, as evidenced by increasing CAT, SOD activities, and total antioxidant capacity. Similarly, Subash and Jayanthi (2010) found that the phenolic substances have beneficial effects on the CAT enzyme activity under oxidative stress. The higher activity of CAT enzyme was observed in fish fed diet supplemented with palm fruit extract compared to the control diet (Nazeri et al., 2017; Xiao et al., 2017). Furthermore, Banaee et al. (2011) found that SM extract acts as a hepatoprotective agent against oxidative stress in fish. On the other hand, SM supplementation (100 or 400 mg kg⁻¹) had no effect on the enzymatic activity of CAT and SOD in Nile tilapia and turbot in a different environment (Owatari et al., 2018).

Regarding the changes of larvae body fatty acids profile in association with dietary SM, the fatty acid content of *D. labrax* larvae is highly affected by the addition of SM to the weaning diet. The data indicated an improvement of fatty acids profile as a trend of increasing polyunsaturated fatty acids content and decreasing saturated fatty acids in larvae fed SM diets. The most important

and essential unsaturated fatty acids for marine fish are DHA and EPA which were recorded at higher levels in SM₆₀₀ groups (0.65 and 1.56%, respectively). A similar finding indicates that the highest content of PUFAs was recorded in Atlantic herring fed the SM-supplemented diet compared to the control (Domiszewski et al., 2011; Zapletal et al., 2012). In addition, SM improved the fatty acid profile in broiler chickens by increasing the polyunsaturated fatty acid content compared to the saturated ones (Zaker-Esteghamati et al., 2021). Moreover, micronized SM co-supplementation with n-3 PUFA stimulated triglyceride, cholesterol, and non-esterified fatty acids lowering effect in mice fed a diet supplemented with SM (Poruba et al., 2019). Grass carp (*C. idellus*) fed diet supplemented with SM showed an increase in the expression of hepatic *HMGCR* gene, which regulates lipid synthesis and *CYP7A1* gene, which stimulated bile acid synthesis, accordingly leading to better fatty acids absorption and metabolism (Xiao et al., 2017).

In the current study, the improvement of PUFA levels with dietary SM could be attributed to the strong antioxidant properties of SM as a rich source of different flavonoid compounds (Tajmohammadi et al., 2018; Valková et al., 2021). Moreover, the current result revealed an improving activity on oxidant/antioxidant balance with increasing dietary SM (Figure 2), this could protect the PUFAs from oxidative damage. In addition, the co-supplementation of micronized SM and n-3 PUFA increased the expression of genes affecting lipid homeostasis (Poruba et al., 2019).

In addition, the findings herein could be interpreted by considering the rich content of *S. marianum* from flavonolignans and about twenty-three fatty acids (Gök et al., 2021). The predominant fatty acids in SM are linoleic acid with an average area of 39–44%, followed by oleic acid (20.18–36%), palmitic acid (9–14.6%), and stearic acid (6–7.5%) (Gök et al., 2021; Majidi et al., 2021). The lack of presented literature on the effect of SM on fatty acids content in fish hampered the comparison of our results with others.

Conclusion

On the basis of the obtained data, it is possible to conclude that supplementing seabass, *Dicentrarchus labrax*, larvae weaning diets with silymarin (SM) increased final weight by 117.6%, feed conversion ratio by 38.7% and survival by 11.7%, with 600 mg SM kg⁻¹ diet compared to the control. The dietary supplementation of SM reduced the risk of oxidative stress in fast growth larvae by significant increase of antioxidant enzyme activities and decrease of thiobarbituric acid reactive substances. The fatty acids profile analysis showed the increase of unsaturated fatty acids against the saturated fatty acids with increasing SM supplementation. The dietary levels of 600 mg SM kg⁻¹ diet is recommended for improving growth and quality of seabass larvae. The beneficial effects of SM may be attributed to the content of highly active compounds such as flavonolignans contents. Further

studies on marine fish larvae are required to determine and characterize the mode of action of SM on the health status of fish.

Credit author statement

Shimaa A. Shahin: Experimental design, experimental conduction, culturing fish, samples collection, data analysis, and writing manuscript, Abdallah Tageldein Mansour: Conceptualization, methodology, review and editing the manuscript, Mohamed M. Abdel-Rahim: Experimental conduction, culturing fish, samples collection, and writing manuscript, Alaa A. El-Dahhar: Experimental design and revising the manuscript, Mohammed F. El Basuni: Samples collection, data validation, visualization, software, Ashraf. I. G. Elhetawy: Experimental conduction, culturing fish, samples collection, and writing manuscript.

Ethical Statement

The authors confirm that they have followed the guidelines of the Declaration of Helsinki and approved by the institutional animal care and use committee of Alexandria University with approval No: AU: 19/22/06/05/3/22.

Data availability statement

The data that support the findings of this study are available from the authors upon reasonable request.

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Conflict of interest

There is no conflict of interest among authors and organizations.

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