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COMMON CARP (*CYPRINUS CARPIO*) EXPOSURE TO WATER-SOLUBLE FRACTION (WSF) OF CRUDE OIL: EVALUATION OF PROTECTIVE EFFECTS OF DIETARY *ROSMARINUS OFFICINALIS* LEAF ON WATER QUALITY, GROWTH PERFORMANCE, OXIDATIVE STRESS, STRESS HORMONES AND HEAT SHOCK PROTEINS

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Abstract

The present study aimed to investigate the protective effects of dietary rosemary (*Rosmarinus officinalis*) leaf on water quality, growth performance, oxidative stress, stress hormones and heat shock proteins in common carp *Cyprinus carpio* (40.41±2.28 g). In a 56-day feeding trial, fish were exposed to water-soluble fraction (WSF) of crude oil (25 and 50% of the LC50 corresponding to 3.57 and 7.14 mg/l, respectively) and fed diets containing 0 and 0.1% rosemary. The results demonstrated that the concentrations of water quality parameters were within acceptable range and are sufficient to sustain common carp. The results showed a significant decrease in growth parameters and feed utilization in the WSF-exposed groups, whereas the growth parameters improved in the rosemary fed fish. The serum, liver and gill malondialdehyde (MDA), glutathione peroxidase (GPx) and superoxide dismutase (SOD) levels were significantly increased, whereas reactive oxygen species (ROS) and glutathione reductase (GR) were significantly ($P<0.05$) decreased in the WSF-exposed groups. The serum, liver and gill heat shock proteins (HSP70 and HSP90) were significantly increased in WSF-exposed groups. Similarly, the levels of triiodothyronine (T_3) and thyroxine (T_4) in serum and gill were significantly elevated in response to exposure of fish to WSF. The cortisol level in serum and gill tissues was significantly elevated in WSF fish versus the control. Acetylcholinesterase (AChE) level in serum and gill was significantly decreased in exposed groups compared with unexposed groups. In the liver, the level of T_4 and AChE was significantly ($P<0.05$) increased by exposing fish to WSF, whereas the level of T_3 showed an opposite trend. The cortisol level in liver was significantly ($P<0.05$) decreased especially in fish exposed to 50% of WSF. However, dietary supplementation of rosemary improved growth performance, feed utilization, oxidative stress markers, HSP and stress hormone parameters, especially in fish exposed to highest concentration. The results of the present study indicated that the exposure of common carp juveniles to sublethal concentration of WSF is capable of inducing homeostatic stress, leading to alterations in growth performance, heat shock proteins and stress hormone parameters of the common carp. Furthermore, inclusion of rosemary as a feed additive succeeded in improving growth performance and mitigating the deleterious effects of crude oil pollution in comparison to fish fed basal diet without rosemary supplementation.

Key words: WSF, rosemary, *Cyprinus carpio*, heat shock proteins, stress hormones, oxidative stress

Crude oil spills are contaminating the world water sources and are considered as one of the most predominant forms of water pollution (Olajuyigbe et al., 2020; Kazempoor et al., 2020; Anwar et al., 2022). Crude oil is a toxic compound mixture with a high potential, has an organic origin and is categorized as light, medium or heavy, depending on the dominant type of hydrocarbon that could have a negative impact on marine ecosystem, fisheries resource. The toxic effects of crude oil on the majority of living organisms are due to its toxic components such as polycyclic aromatic hydrocarbons (PAHs) and WSF (Sakineh Hamidi et al., 2022). Although oils are insoluble, crude oil includes a very small soluble section, called water-soluble fraction (WSF), which is a toxic and complex mixture of hydrocarbons (Dighiesh et al., 2022). Recently, changes have been reported in water quality after the introduction of water-soluble fractions of crude oil (Eriegha et al., 2019; Anwar et al., 2020, 2022). Fish have a very high capability to accumulate WSF compared to other components of petroleum (Dighiesh et al., 2019). Water accommodated fractions could be the cause of the aquatic toxicity for three reasons: first, chemical toxicity associated with dissolved materials (from oil or dispersant); second, physical effects associated with contact with oil droplets; and third, effect associated with enhanced uptake resulting from direct animal oil contact (Kazempoor et al., 2020).

Medicinal plants and herbs are considered an alternative to antibiotics (Forouzi et al., 2020) due to their being rich in vitamins E and C, glutathione, enzymes and phenolic compounds with antioxidant properties. Recently, medicinal plants and herbs such as rosemary (*Rosmarinus officinalis*) (Hoseinifar et al., 2020), thyme (*Thymus vulgaris*) (Abd El-Naby et al., 2020) and *Moringa oleifera* (Ibrahim et al., 2019) showed beneficial effects in aquaculture (Badawi et al., 2021). Previous studies have identified the most widely used medicinal herb worldwide is *R. officinalis* due to its high content of polyphenol and its antioxidant, anti-carcinogenic properties (Ahmadifar et al., 2020; Chelemal Dezfoulnejad and Molayemraftar, 2022).

Common carp, *Cyprinus carpio*, is widely distributed in eutrophic freshwater and is one of the most important cultured species in Asian and some European countries (FAO, 2020). In addition, common carp is a widely used fish for various researches such as nutritional and physiological studies. Common carp is recognized for its desirable characteristics including high growth rate, better feed conversion ratio, higher capability of using carbohydrates and plant protein sources along with relatively high resistance to variable environmental conditions and diseases (Tasa et al., 2020). Dietary supplementation of medicinal plants and herbs could be one effective way of mitigating the negative impact of WSF on fish. However, limited information is available to evaluate the effects of dietary supplementation of rosemary on growth performance, oxidative stress, stress hormones and heat shock proteins in fish reared in water contaminated with crude oil. Therefore, the present study aimed to evaluate the effects of rosemary leaf on water-soluble fraction of crude oil, water quality, growth performance, oxidative stress, stress hormones and heat shock proteins in common carp *Cyprinus carpio*.

Material and methods

Fish and experimental facilities

Ninety-six common carp (*Cyprinus carpio* L.) with an average initial body weight of 41±2.28 g/fish were obtained from Ankawa hatchery station, Erbil, Kurdistan Region, Iraq. Prior to the start of the experiment, the fish were given 2 weeks to acclimate to the experimental conditions, and during this period they were fed a maintenance diet (34% protein and 7% lipid), and were homogenized to assure an equal initial body weight for all treatments. Then, fish were randomly distributed into 12 rectangular polyethylene tanks with dimensions of 30 cm × 38 cm × 65 cm, each with 0.074 m² capacity (8 fish per tank, 2 tanks per group of fish). All tanks were supplied with aeration and water exchange daily with non-chlorinated water from deep well with the addition of water-soluble fraction of crude oil for 8 tanks in total 12 tanks. The parameters were: water salinity (0.19–0.22 ppt), rearing conditions (24±1.0°C), pH (7.0±0.50) and a photoperiod regime (12:12 h light: dark). Experimental diets were given three times daily at a ration level of ~3% of the fish body weight. Fish were weighed weekly and the amount of diet was adjusted accordingly to the weight.

Experimental design and diets

Two isonitrogenous (~380 g/kg crude protein) and isolipidic (~80 g/kg crude lipid) experimental diets were formulated (Table 1) to the NRC (2011) guidelines on the nutritional requirements for carp, with the same composition with the addition of 0.1% rosemary leaf powder supplementation for one of them (Anwar et al., 2022). The control group contained no rosemary leaf powder or WSF. There were six groups of fish, three groups of fish exposed to 0%, 25% and 50% of sublethal concentrations of WSF and fed control diet without rosemary supplementation and other three groups of fish exposed to 0%, 25% and 50% of sublethal concentrations of WSF and fed diet supplemented with 0.1% of rosemary leaf powder. Diets were manufactured by initially dry mixing ingredients before homogenizing through a commercial food mixer. Diet pellets were extruded through a cold press extruder (SUNRRY, model: SYMM12, China) using a 2 mm aperture die. Feeds were subsequently dried in a dehumidifying oven for 24 h at 40°C. Test diet formulations and proximate composition are presented in Table 1.

Table 1. Formulation and proximate analysis of the experimental diets (g/kg dry weight)

Ingredient g kg^{-1}	C	R
Soybean	530	530
Corn	150	150
Fishmeal	100	100
Premix	25	25
Soya oil	50	50
Wheat flour	100	100
Wheat bran	13	12
Vitamin premix	11	11
Trypsin	1	1
Mineral premix	20	20
Rosemary	–	1
Total	1000	1000
Proximate composition (%)		
Moisture (%)	7.79±0.42	8±0.28
Protein (%)	38.67±0.24	38.67±0.24
Lipid (%)	6.46±0.35	7±0.28
Ash (%)	6.4±0.21	6.66±0.31
NFE (%)	39.4±014	37.09±0.14

Each kg of fish premix contained: $MnSO_4$, 40 mg; MgO, 10 mg; K_2SO_4, 40 mg; $ZnCO_3$, 60 mg; KI, 0.4 mg; $CuSO_4$, 12 mg; ferric citrate, 250 mg; Na_2SeO_3, 0.24 mg; Co, 0.2 mg; retinol, 40,000 IU; cholecalciferol, 4,000 IU; α-tocopherolacetate, 400 mg; menadione, 12 mg; thiamine, 30 mg; riboflavin, 40 mg; pyridoxine, 30 mg; cyanocobalamin, 80 mcg; nicotinic acid, 300 mg; folic acid 10 mg; biotin, 3 mg; pantothenic acid, 100 mg; inositol, 500 mg; ascorbic acid, 500 mg.

Nitrogen-free extracts (NFE %) = 100 - (ash + moisture + crude fat + crude protein)

C = control diet.

R = diet supplemented with 0.1% of rosemary.

Preparation of water-soluble fraction

Crude oil was obtained from the Khurmala oil field, and transported to the research laboratory of the Department of Fish Resources and Aquatic Animals, College of Agriculture Engineering Sciences, Salahaddin University. The extraction of the water-soluble fraction from the crude oil followed the protocol described by Audu and Ajima (2020). The ash was collected, retrieved and sieved. A mortar and pestle were used to crush the black ash into dust, then soaked in distilled water (Iheanacho et al., 2021). The mixture was filtered with non-absorbent cotton wool, and the filtrate from the WSF was collected and stored in a plastic container until used.

Water quality analysis

Parameters such as temperature, dissolved oxygen (DO), pH and total dissolved solids (TDS) of the experimental set-up were monitored on a periodic basis using standard methods (APHA, 1998). Total hardness, total alkalinity, nitrate (as NO_{3-}) and sulfate (SO_4^{2-}) contents were determined using the methods of FAO/SIDA (1983) and Olaifa (2012), respectively.

Fish growth performance and mortality %

To calculate the growth performance and feed utilization parameters, total fish biomass in every tank was individually weighed at the beginning and end of the trial. Weight gain (WG), feed efficiency ratio (FER), protein efficiency ratio (PER), protein productive value (PPV) and mortality %, were calculated according to Ghanei-Motlagh et al. (2021) using the following equations:

$$WG = W2–W1,$$

where *W1*: initial weight of the fish (g), and *W2*: final weight of the fish (g).

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

Protein productive value (PPV) = (protein gain (g)/ protein intake (g)) × 100.

Mortality (%) = (initial number of fish – final number of fish)/initial number of fish ×100.

Tissue sampling

Liver and gill tissues of six fish from each group were collected, cleaned and immediately mixed with saline (NaCl 0.9%). The tissues were homogenized in buffer saline (0.1 M pH 7.4). One gram of liver and gill was frozen in liquid nitrogen using pestle and mortar and stored at −80°C for determination of SOD, GPx, GR, ROS, MDA, HSP, AChE, T_3, T_4 and cortisol.

Measurement of the antioxidant enzymatic activity

The liver and gill antioxidant enzyme activities were assayed as described by Fontagné-Dicharry et al. (2014) and Iheanacho et al. (2021). Superoxide dismutase (SOD) activity was measured at 37°C by monitoring the inhibition of nitrotetrazolium reduction at 450 nm. Glutathione peroxidase (GPx) activity was assayed at 30°C by the coupled reaction with glutathione reductase (GR) using cumene hydroperoxide and hydrogen peroxide (H_2O_2) as substrates for measuring the total GPx. The GR activity was determined at 30°C by monitoring NADPH oxidation at 340 nm. The malondialdehyde (MDA) concentration in plasma was measured with some modification according to the method of Grotto et al. (2007). TBARS were formed from the reaction of MDA with 2-thiobarbituric acid in an acid environment. The absorbance of the colored complex was measured spectrophotometrically at 532 nm. Detection of reactive oxygen species (ROS) was performed by a fluorometric assay kit (MyBioSource, USA). The levels of ROS were determined in accordance with the protocol provided by the manufacturer and fluorescence was read with a fluorescence plate reader (Promega, USA) at excitation 500 nm and emission 525 nm.

Tissue HSP70 and HSP90

Liver and gill tissues of fish from each group were rapidly removed, cleaned from any extraneous materials and immediately perfused with cold physiological saline (NaCl 0.9%). The tissues were homogenized in cold phosphate buffer saline (0.1 M pH 7.4). About one gram of liver and gill was frozen in liquid nitrogen using pestle and mortar and stored at −80°C. Repeating freeze/thaw cycles was avoided. The determination of liver and gill HSP 70 concentration was made using ELISA fish Kit Cat.No: MBS007829. The determination of liver and gill heat shock protein 90 (HSP90) concentration was made using ELISA fish Kit Cat.No: MBS032328.

Serum HSP70 and HSP90

The blood was immediately put in centrifuge at 3,500 rpm for 15 minutes and the supernatant serum collected and put in labelled Eppendorf tubes stored at –80°C**.** Repeated freeze/thaw cycles were avoided. The determination of serum HSP70 concentration was made using ELISA fish Kit Cat.No: MBS007829. The determination of serum heat shock protein 90 (HSP90) concentration was made using ELISA fish Kit Cat.No: MBS032328.

Acetylcholinesterase assay

AChE activity determination was carried out using the method of Delaney and Klesius (2004). The supernatant (100 µl) was added to a test tube (1.5 ml) containing 880 µl of 0.1 M phosphate buffer (pH 7.5), 10 µl of 100 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), and 10 µl of 0.1 M acetylthiocholine chloride. The contents were then mixed and the absorbance read continuously for 1 min using a StatFax 100 spectrophotometer (USA). Three samples were assayed for each tissue. Enzyme activity was reported as millimoles of product formed per milligram protein per minute.

Hormone determinations (ng/ml)

Serum, gill and liver corticosterone (cortisol) levels were determined by ELISA Kit (MBS2700193) for cortisol. Thyroxine (T_4), triiodothyronine (T_3) hormones concentration in plasma was determined using ELISA according to the instructions of the kit included in the Cusabio technology LLC.

Statistical analysis

The data were subjected to one-way ANOVA test using SPSS program (Statistical Package for the Social Sciences, version 26, IBM Company, 2019). All data are presented as mean values ± standard error (mean±SE). Duncan test was used to determine significant differences at 0.05 levels among the treatments (Duncan, 1995).

Results

Water quality

The variations in the physico-chemical parameters of the test medium during the experiment are shown in Figure 1. The temperature of the present study ranged between 22 and 28°C and the pH was not significantly ($P \geq 0.05$) affected by exposure to sublethal concentrations of WSF, whereas temperature and total dissolved solids were increased significantly ($P<0.05$) with exposure to both sublethal concentrations of WSF in comparison with the control group.

Total alkalinity was increased significantly ($P<0.05$) with exposure to 50% sublethal concentration of crude oil WSF, while this trend was not significant with 25%. However, dissolved oxygen and total hardness were reduced significantly ($P<0.05$) with exposure to sublethal concentrations of WSF. On the other hand, dietary supplementation of rosemary modified significantly the total hardness, total alkalinity and sulfate toxicity induced by WSF in fish groups.

Growth performance

The results of Table 2 showed that the final body weight (FBW), weight gain, PER and PPV were significantly ($P<0.05$) lower in fish groups exposed to sublethal concentration of crude oil WSF. Conversely, mortality levels were increased significantly ($P<0.05$) in fish groups exposed to sublethal concentrations of crude oil WSF. On the other hand, FBW, weight gain, PER and PPV were significantly ($P<0.05$) higher in fish groups fed a diet supplemented with rosemary compared to fish groups exposed to sublethal concentration of crude oil WSF and fed the control diet. Conversely, the FI was decreased significantly ($P<0.05$) in fish groups fed a diet supplemented with rosemary and exposed to sublethal concentration of crude oil WSF.

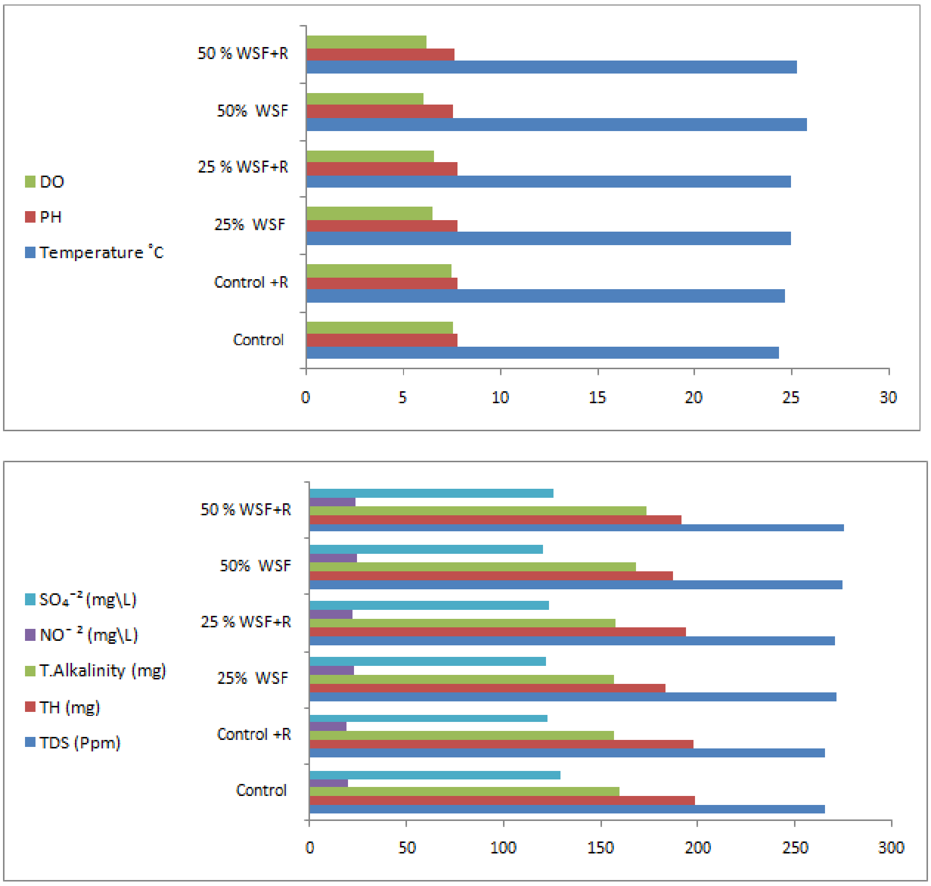

Figure 1. Effect of WSF on water quality in common carp tanks after 8-week exposure

Table 2. Growth performances and feed utilization of common carp fed the experimental diets and exposed to WSF for 8 weeks (n=2)

Parameters	C	C+R	25%WSF	25%WSF+R	50%WSF	50%WSF+R
IBW (g/fish)	41.37±0.62	41.56±0.31	42.5±1.25	40.62±1.87	41.87±1.87	41.25±1.25
FBW (g/fish)	94.94±0.8 ba	98.68±1.43 a	85.5±0.5 bc	87.21±0.88 b	80.5±0.5 c	88.74±0.41 b
Weight gain (g/fish)	53.57±1.43 a	57.12±1.12 a	43±0.75 c	46.59±0.99 c	38.63±1.37 a	47.49±0.83 ab
ADG (g/day)	0.82±0.05 b	0.90±0.09 a	0.78±0.07 c	0.77±0.04 c	0.86±0.01 a	0.84±0.08 b
FCR	2.49 a	2.45 a	1.61 c	1.68 c	1.82 b	1.74 b
Survival rate (%)	100±0.00 a	100±0.00 a	62.5±12.5 b	75±12.5 ab	62.5±12.5 b	75.00±12.5 ab
PER (g)	1.30±0.05 ab	1.38±0.10 a	1.04±0.08 c	1.29±0.04 ab	0.93±0.10 d	1.15±0.03 c
PPV (%)	16.6±0.92 b	18.2±1.31 a	11.07±1.10 cd	13.59±1.76 c	10.86±1.71 d	14.27±1.87 c

IBW: initial body weight; FBW: final body weight; ADG: average daily gain; FCR: feed conversion ratio; PER: protein efficiency ratio; PPV: protein productive value.

Data in the same row with different letters are significantly different (P<0.05).

Table 3. Heat shock proteins (HSP) of common carp fed the experimental diets and exposed to WSF for 8 weeks (n=6)

Parameters		C	C+R	25% WSF	25% WSF+R	50% WSF	50% WSF+R
HSP 70 pg	Serum	6.5±0.29 b	7.8±0.2 b	9.3±0.48 ab	10.6±1.6 ab	11.5±1.32 a	8.5±2.74 ab
	Gill	4.8±0.48 b	5.8±0.48 b	8.5±0.65 ab	7.8±0.85 ab	9.3±0.48 ab	3.5±1.9 b
	Liver	7.3±0.85 c	7.5±0.82 c	11.8±0.83 b	15.8±0.32 b	16.3±1.6 b	19.9±0.72 a
HSP 90 pg	Serum	0.06±0.04 b	1.8±0.9 b	0.7±0.03 b	1.4±0.08 b	3±0.2 a	1.9±0.04 b
	Gill	0.5±0.05 b	0.8±0.04 b	6.7±2.01 a	8.2±0.27 a	8.4±0.39 a	9.4±0.20 a
	Liver	7.3±0.85 d	7.5±0.82 d	11.8±0.83 c	15.8±0.32 b	16.3±1.65 b	19.9±0.72 a

Data are presented as mean ± S.E.

Data in the same row with different letters are significantly different (P<0.05).

Table 4. Serum antioxidant enzyme activities of common carp fed the experimental diets and exposed to WSF for 8 weeks (n=6)

Parameters	C	C+T	25%WSF	25%WSF+R	50%WSF	50%WSF+R
MDA nm/mg	7.68±0.1 d	7.60±0.24 d	8.69±0.04 c	9.05±0.06 bc	9.35±0.09 b	10.16±0.16 a
SOD nm/mg	6.88±0.04 e	6.79±0.02 f	7.16±0.01 d	8.02±0.02 c	8.92±0.03 b	9.21±0.01 a
GPx nm/mg	85.3±0.27 f	87.2±0.38 e	90.7±0.18 d	93.35±0.11 c	97.9±0.18 b	98.9±0.27 a
ROS nm/mg	288.82±0.98 a	279.95±3.51 b	198.42±1.79 d	239.07±2.81 c	134.18±1.26 f	174.86±1.99 e
GR nm/mg	41.13±0.13 a	41.75±0.38 a	30.13±0.54 d	37.30±0.97 bc	36.23±0.59 dc	34.78±0.73 d

Data are presented as mean ± S.E.

Data in the same row with different letters are significantly different (P<0.05).

MDA: malondialdehyde; SOD: superoxide dismutase; GPx: glutathione peroxidase; ROS: reactive oxygen species; GR: glutathione reductase.

Table 5. Gill and liver antioxidant enzyme activities of common carp fed the experimental diets and exposed to WSF for 8 weeks (n=6)

Tissue	Parameters	C	C+R	25%WSF	25%WSF +R	50%WSF	50%WSF+R
Gill	MDA nm/mg	7.73±0.06 d	7.62±0.06 d	8.65±0.08 c	9.45±0.13 b	9.06±0.2 b	10.1±0.18 a
	SOD nm/mg	6.88±0.04 e	6.81±0.05 e	7.16±0.01 d	8.96±0.03 b	8.02±0.02 c	9.19±0.01 a
	GPx nm/mg	70.25±1.69 e	87.42±0.88 d	92.35±0.66 c	93.22±0.49 bc	95.22±0.64 b	98.57±0.37 a
	ROS nm/ mg	304±2.73 b	315.75±2.68a	284.25±4.55 c	280.5±3.79 cd	288.62±5.45 c	269.5±2.34 d
	ROS nm/ mg	28.05±0.21a	28.7±0.18 a	26.67±0.57 b	25.90±0.47 b	26.77±0.36 b	24.65±0.17 c
Liver	MDA nm/mg	7.50±0.13 d	7.65±0.25 d	8.68±0.08 c	9.33±0.08 b	9.72±0.17 ab	10.11±0.08 a
	SOD nm/mg	6.88±0.04 e	6.81±0.05 e	7.16±0.01 d	8.96±0.03 b	8.02±0.02 c	9.19±0.01 a
	GPx nm/mg	85.12±0.34 f	86.92±0.49 e	90.86±0.16 d	93.40±0.29 c	95.70±0.73 b	98.12±0.34 a
	ROS nm/ mg	245.0±2.64 b	256.5±4.08 a	226.37±4.08 c	211.37±0.82 d	224.12±2.53 c	199.0±1.88 e
	ROS nm/ mg	30.70±0.76 a	29.82±0.48 a	26.07±0.54 bc	24.42±0.83 cd	27.16±0.49 b	23.25±0.45 d

Data are presented as mean ± S.E.

Data in the same row with different letters are significantly different (P<0.05).

MDA: malondialdehyde; SOD: superoxide dismutase; GPx: glutathione peroxidase; ROS: reactive oxygen species.

Table 6. Serum hormones and acetylcholinesterase in common carp fed the experimental diets and exposed to WSF for 8 weeks (n=6)

Parameters	C	C+R	25% WSF	25%WSF+R	50%WSF	50%WSF+R
Cortisol ng/ml	77.17±1.45 c	77.95±0.8 c	98.32±1.96 a	80.23±1.29 d	89±1.65 b	72.51±0.59 d
AChE 10^{-3}	2.08±0.07 b	2.36±0.01 a	1.67±0.085 c	1.32±0.04 d	1.21±0.06 de	1.1±0.04 e
T_3 ng/ml	0.57±0.04 b	0.61±0.02 b	0.85±0.02 a	0.71±0.06 b	0.9±0.04 a	0.9±0.05 a
T_4 ng/ml	0.92±0.06 c	0.85±0.03 c	0.95±0.02 bc	1.06±0.02 ab	1.07±0.06 ab	1.17±0.02 a

Data are presented as mean ± S.E.
Data in the same row with different letters are significantly different (P<0.05).
AChE: acetylcholinesterase; T_4: thyroxine; T_3: triiodothyronine.

Table 7. Gill and liver stress hormones and acetylcholinesterase in common carp fed the experimental diets and exposed to WSF for 8 weeks (n=6)

Tissue	Parameters	C	C+R	25%WSF	25%WSF+R	50%WSF	50%WSF+R
Gill	Cortisol ng/ml	57.5±0.73 c	56.45±0.95 c	63.22±0.43 a	60.52±0.63 b	62.6±0.38 a	63.85±0.40 a
	AChE 10^{-3}	1.20±0.07 ab	1.15±0.06 ab	1.45±0.21 a	1.10±0.04 b	1.35±0.06 ab	1.47±0.09 a
	T_3 ng/ml	0.60±0.040 c	0.52±0.02 c	0.90±0.04 b	0.80±0.040 b	1.10±0.04 a	0.87±0.04 b
	T_4 ng/ml	0.80±0.04 c	0.85±0.02 bc	0.92±0.085 bc	0.95±0.08 bc	1.40±0.04 a	1.02±0.04 b
Liver	Cortisol ng/ml	80.77±0.59 a	74.68±1.77 b	78.11±1.32 ab	76.20±3.99 ab	63.11±0.26 c	58.05±0.41 c
	AChE 10^{-3}	3.17±0.29 ab	2.97±0.176 ab	3.27±0.41 a	1.78±0.21 cd	2.41±0.22 bc	1.32±0.06 d
	T_3 ng/ml	0.053±0.002 ab	0.05±0.005 ab	0.04±0.004 bc	0.03±0.004 c	0.06±0.010 a	0.04±0.001 bc
	T_4 ng/ml	0.72±0.06 d	0.81±0.02 cd	0.85±0.11 cd	1.01±0.03 bc	1.1±0.08 b	1.5±0.10 a

Data are presented as mean ± S.E.
Data in the same row with different letters are significantly different (P<0.05).
AChE: acetylcholinesterase; T_3: triiodothyronine; T_4: thyroxine.

Heat shock protein (HSP)

The results of serum, gill and liver heat shock proteins of common carp exposed to sublethal concentrations of crude oil and fed diets supplemented with rosemary are shown in Table 3. The HSP70 and HSP90 values of fish were significantly (P<0.05) increased in common carp exposed to both doses of WSF. On the other hand, dietary supplementation of rosemary to fish exposed to 50% of WSF resulted in a significant (P<0.05) decrease in the HSP90 value. Whereas, the value of HSP70 did not significantly (P≥0.05) changed with dietary supplementation of rosemary to exposed and unexposed fish. The levels of HSP70 and HSP90 in gill and liver were significantly (P<0.05) and progressively increased in response to fish exposed to both doses of WSF. On the other hand, dietary supplementation of rosemary to fish that were exposed to both doses of WSF resulted in a significant (P<0.05) increase in the HSP70 and HSP90 values in the liver, while it did not significantly (P≥0.05) affect the values of HSP70 and HSP90 in fish gill.

Oxidative stress markers

Results for the antioxidant parameters are given in Table 4. The values of MDA, SOD and GPx were significantly (P<0.05) increased by exposing fish to WSF and increased the level of concentration. On the contrary, the ROS and GR values were significantly (P<0.05) reduced by exposing fish to crude oil WSF.

The levels of MDA, SOD, GPx and ROS were significantly (P<0.05) increased with the addition of rosemary to diets of fish exposed to both doses, whereas the SOD and ROS levels showed an opposite trend with the addition of rosemary to unexposed fish. Dietary supplementation of rosemary to 25% of crude oil WSF resulted in a significant increase in GR level. Antioxidant response analyses for SOD, MDA, GPx, GR and ROS in the gill of common carp exposed to WSF and fed experimental diets are shown in Table 5. The gill SOD, GPx and MDA activities were substantially (P<0.05) increased by exposing fish to WSF, whereas the gill ROS and GR values were significantly (P<0.05) decreased. The level of MDA, SOD and GPx were significantly (P<0.05) increased with the addition of rosemary to the diets of exposed fish. However, the ROS level was significantly (P<0.05) reduced with the addition of rosemary to the diets of exposed fish and significantly (P<0.05) increased with the addition of rosemary to the diet of unexposed fish. Furthermore, dietary supplementation of rosemary to fish exposed to 50% of WSF resulted in a significant (P<0.05) decrease in GR level.

The results of liver antioxidant activities were presented in Table 4. The level of GPx, MDA and SOD were significantly (P<0.05) increased by exposing fish to WSF, whereas the ROS and GR levels showed an opposite trend. On the other hand, the levels of MDA, SOD and GPx were significantly (P<0.05) increased with the addition of rosemary to the diets of exposed fish. However, the ROS and GR levels were significantly (P<0.05) reduced with the addition of rosemary to the diets of exposed fish. Whereas, the level of GR was increased by the addition of rosemary to the diets of unexposed fish.

Stress hormones

Serum hormone levels of common carp juveniles fed the experimental diets and exposed to WSF are presented in Table 6. The corticosteroid, and T_3 and T_4 levels were significantly (P<0.05) elevated in exposed fish compared with unexposed fish. Dietary supplementation of rosemary caused a significant (P<0.05) reduction in cortisol value in exposed fish. The value of T_3 was significantly (P<0.05) decreased in fish exposed to 25% of WSF and fed a diet supplemented with rosemary. However, dietary supplementation of rosemary did not significantly (P≥0.05) change the T_4 level in exposed and unexposed groups of fish.

The results of gill and liver stress hormones of common carp exposed to sublethal concentrations of crude oil and fed diets supplemented with rosemary are shown in Table 7. The T_3, T_4 and cortisol levels were significantly (P<0.05) increased in the gill of exposed fish compared with unexposed fish. Dietary supplementation of rosemary resulted in a significant (P<0.05) decrease in T_3 and T_4 in fish exposed to 50% of WSF. The level of T_4 was significantly (P<0.05) increased with exposing fish to crude oil WSF, whereas the level of T_3 showed an opposite trend. The level of cortisol hormone was significantly (P<0.05) decreased with exposing fish to 50% of WSF. Dietary supplementation of rosemary resulted in a significant (P<0.05) decrease in cortisol in the unexposed group of fish and T_3 in fish exposed to 50% of WSF, while a significant (P<0.05) increase was reported for T_4 level.

Acetylcholinesterase

The levels of AChE in serum, gill and liver in common carp juveniles fed the experimental diets and exposed to WSF are presented in Tables 6 and 7. The AChE level was significantly decreased (P<0.05) in exposed groups compared with unexposed groups. Dietary supplementation of rosemary caused a significant (P<0.05) reduction in AChE level in exposed fish.

Discussion

The present study showed that the concentrations of dissolved oxygen (DO), temperature and pH are sufficient to sustain common carp. It was shown that the temperature ranged between 20 and 28°C, the best growth of common carp was recorded at 26°C according to Anwar et al. (2022), and the level of pH was not significantly changed with different levels of WSF. In contrast, Eriegha et al. (2019) found a significant reduction in pH level after the introduction of water-soluble fractions of crude oil. In addition, Awoyinka et al. (2011) observed a significant reduction in pH of water following the exposure to crude oil. The differences in the results may be attributable to the i) different concentrations of WSF, ii) different types of petroleum, and iii) different ways of preparing WSF. The dissolved oxygen level in the present study decreased with an exposed system of WSF. Similar results were also reported by Edema and Asagba (2007) who observed similar reductions of DO after exposure to the different levels of WSF. The reduction of DO may be attributed to the oil film formation that reduces the dissolution of atmospheric oxygen (Awoyinka et al., 2011). The present study has demonstrated that exposure of common carp juveniles to a sublethal concentration of WSF caused a significant decrease in growth performance and feed conversion ratio. The present results are consistent with Fakolujo et al. (2018) who reported that the sublethal levels of WSF had adverse effects on the growth performance of African catfish juveniles. The reduction of growth performance and feed utilization efficiency could be attributed to different scenarios: i) the effects of the soluble fractions of crude oil as PAH and total petroleum hydrocarbons (TPH) which probably negatively affect the fish eco-physiology (Hodson et al., 1997); ii) the depression of FCR may be due to the inability of exposed fish to convert feed consumed into required body protein (Sunmonu and Oloyede, 2006); iii) the presence of crude oil fractions affects gut microbiota population and inhibits the growth of positive bacteria (Onwurah et al., 2013). However, inclusion of rosemary in common carp diet exposed and unexposed to WSF yielded significant improvements of growth performance and feed utilization in juvenile common carp. The present findings are consistent with Hassan et al. (2018), Ayoub et al. (2019) and Badawi et al. (2021) who reported that tilapia fed diet supplemented with rosemary displayed highly significant increase in growth performance, as did other species of fish such as common carp (Yousefi et al., 2019). In contrast, Mahmoud et al. (2019) and Johnson-Ashun (2018) found negative effects in growth performance and feed utilization when ginger and garlic were used as feed supplement in tilapia diets. The present study could be attributed to different scenarios as follows: i) the presence of bioactive compounds in rosemary improved growth performance (Hosseini et al., 2020), ii) the presence of bioactive compounds enhanced appetite, iii) the bioactive compounds of rosemary changed the composition, diversity and/or activity of the population of beneficial bacteria in the gut microbiota while inhibiting pathogenic bacteria in aquatic species (Ahmadifar et al., 2020; Hosseini et al., 2020), iv) these bioactive compounds may produce bile and stimulate the secretion of digestive enzymes by the pancreas, which in turn cause the digestion of nutrients (Chelemal Dezfoulnejad and Molayemraftar, 2022), v) the herbal bioactive extracts affect gut morphology and its structure that may improve the digestion and absorption of nutrients (Zeynali et al., 2020), and vi) the protective effects of herbs such as black seed against immune depression, oxidative stress and metabolism dysfunction induced by glyphosate that consequently boost the growth performance (Yousefi et al., 2021) .

Heat shock proteins (HSPs) play a vital role in the protection against various stressors (Zhang et al., 2015). In the present study the gill and liver HSP70 and HSP90 were significantly and progressively increased in response to exposure of fish to WSF. The present results are consistent with Jiang et al. (2016) and Moniruzzaman et al. (2017) who found significant increase in the HSP70 and HSP90 levels in the liver of common carp exposed to Cd, Cu, Zn, and Pb. However, the present study showed that inclusion of rosemary to fish exposed to WSF resulted in a significant increase in gill and liver HSP70 value. The present findings are consistent with Zhang et al. (2015) and Cheng et al. (2018) who found that dietary supplementation of fructooligosaccharides and astaxanthin to the diets of blunt snout bream and pufferfish exposed to high ammonia and temperature stress significantly increased the HSP70 and HSP90. In contrast, Kumar et al. (2016) pointed out that dietary supplementation of water-soluble vitamin pyridoxine to the diets of milkfish exposed to endosulfan led to a significant reduction in the liver and gill HSP 70 levels.

Cortisol is among the most frequently measured indicators of the fish stress response (Baker et al., 2013). In the present study, cortisol in serum, T_3 and T_4 levels was significantly elevated in fish exposed to WSF compared to the control without WSF; however, inclusion of rosemary caused significant reduction in cortisol and T_3 in fish exposed to WSF. The results agree with Mirghaed et al. who found that inclusion of cineole significantly decreased serum cortisol level in *Oncorhynchus mykiss* under crowding stress (Mirghaed et al., 2018) and *Cyprinus carpio* exposed to ambient ammonia (Mirghaed et al., 2019). Moreover, Shourbela et al. (2020) pointed out that dietary supplementation of *Moringa oleifera* aqueous extracts to the diets of Nile tilapia exposed to hypoxic stress led to a considerable decrease in serum cortisol level. In contrast, Kim et al. (2016) and Rajabiesterabadi et al. (2020) found decreases of T_4 and T_3 levels with exposure of larval zebrafish and common carp to WSF and copper.

Acetylcholinesterase is the primary enzyme responsible for the hydrolytic metabolism, and a key biomarker of the neurotoxicity in fishes (Kumar et al., 2017; Sahu et al., 2018). In the present study, the serum AChE level was significantly increased with WSF groups compared to the control groups. On the other hand, the results of the present study demonstrated that the dietary supplementation of rosemary caused a significant reduction in AChE in fish exposed to WSF. These results agree with Rao (2004) who observed a significant increase in acetylcholinesterase enzyme of tilapia (*Oreochromis mossambicus*) exposed to monocrotophos (MCP). Conversely, De la Torre et al. (2002) observed a substantial rise in AChE activity in the brain of common carp subjected to a heavily contaminated water body. Moreover, inclusion of rosemary led to a significant decrease in AChE levels in the gill and liver of fish exposed to WSF. The present results are consistent with Rafieepour et al. (2019) who found that inclusion of oregano extract significantly decreased the level of AChE in the liver of rainbow trout exposed to organ-phosphorus pesticide, diazinon. Conversely, Muthappa et al. (2014) and Singh et al. (2019) found that dietary supplementation of lipotropes and synbiotic in Indian major carp diet exposed to pesticide and low pH stress, respectively, led to a significant increase in AChE level.

Conclusion

In conclusion, the present research provides better understanding of the effects of WSF on fish and how they adapt to environmental contaminants in polluted water. The present study shows that dietary rosemary supplementation is beneficial for common carp health exposed to WSF. They are capable to mitigate stress responses caused by WSF toxicity. Feeding common carp with diet supplemented with both antioxidants improves their protection against toxicity by its relieving effect on hematology, immunity and antioxidants. In addition, dietary supplementation of both antioxidants modulated the transcription of HSP70 and HSP90 in common carp exposed to WSF.

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