



## Effect of lemon (*Citrus lemon*) pumace powder supplementation on growth performance, lipid peroxidation and protein oxidation biomarkers in some tissues of common carp (*Cyprinus carpio*)

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### ABSTRACT

The aim of this study was to evaluate the antioxidant potential of lemon pumace powder (peel and pulp), as an inexpensive and valuable source of nutrient in diet of common carp. Fish ( $60 \pm 5$  g) were divided randomly into four groups of 30 each. Group 1 fish were fed with basic diet, serving as the control. Fish in group 2 and 3 and 4 were fed the basic diet supplemented with 1.5%, 3% and 5% lemon pumace powder, respectively. Results showed that growth performance including final weight, weight gain (WG), feed conversion ratio (FCR) and specific growth rate (SGR) significantly increased as compared to control. Malondialdehyde (MDA) values of muscle significantly increased as compared to control in all treatment groups and the decreasing effect of lemon pumace powder on malondialdehyde (MDA) values of kidney and liver was only significant in group 4, when compared with the control group ( $p < 0.05$ ). Protein carbonyl contents were significantly decreased in kidney and liver in group 3 and 4 as compared to control group and protein carbonyl of muscle significantly decreased as compared to control in all treatment groups. FRAP values of liver significantly increased only in group 3 as compared to control, and FRAP values of kidney and muscle significantly increased only in group 4 as compared to control ( $p < 0.05$ ). These data suggest that supplementation of 5% lemon pumace powder to be more effective than its lower levels in strengthening the antioxidant system against oxidative stress.

### Keywords

Antioxidant, Common carp, Lipid peroxidation, Protein carbonyls

### Abbreviations

MDA: Malondialdehyde  
EPA: Eicosapentaenoic acid  
DHA: Docosahexaenoic acid  
PUFA: Polyunsaturated fatty acid  
ROS: Reactive oxygen species  
TBARS: Thiobarbituric acid

## Introduction

Fish lipid varies from that in mammalian lipid. The major difference is that lipids in fish is composed of about 40% from long chain fatty acids (14–22 carbon atoms) which are highly unsaturated. Mammalian lipids contain up to two double bonds in every fatty acid molecule, while fish depot fats have enormous fatty acids with five or six double bonds. In addition, fish oils have other PUFAs, known as 'essential', including eicosapentaenoic (EPA, C20:5n3) and docosahexaenoic (DHA, C22:6n3) acids among many others. In fact, EFSA [1] indicated that a daily consumption of 250–500mg of EPA and DHA lowers mortality risk resulting from coronary heart disorder and sudden heart failure. This confirms the past results that EPA in blood is an intensely powerful antithrombotic factor [2]. Notwithstanding, long-chain fatty acids are as vital as they are highly sensitive to degradation, including oxidation. It has been established [3, 4, 5] that food lipid oxidation, in particular of PUFA contained in fish, is rather associated with the formation of off-flavor components, quality degradation, loss of nutritional value, and even formation of anti-nutritional molecules. Oxidants are found to be reactive oxygen species (ROS) which have the potential, both directly and indirectly, to degrade all biomolecules, as proteins, lipids, DNA, and carbohydrates. ROS such as superoxide anion, hydrogen peroxide and the hydroxyl radical are produced in the process of normal metabolism via electrons leakage from the electron transport chain and at the same time using the functions of different oxidoreductase enzymes [6]. Insufficient dietary antioxidants have been followed by a decrease in antioxidative defense and increased susceptibility to oxidative stress in both mammals and fish [7, 8]. The antioxidative defense system potential of aqua-cultured fish has been reported to be very poor [9]. The antibacterial, antioxidant and anticancer effects found in citrus are due to its high content of phenolic compounds particularly limonene. Lemon (*Citrus limon*) is the third most important species of citrus in the world, behind orange and mandarin. Production of lemon and lime in 2012 in FAO countries was 11.2 million tones. Lemon contains various compounds such as calcium, potassium, magnesium, phosphorus and vitamins including A, E, C, B12, B6 and flavonoids as antioxidative agents [10]. The major portion of vitamin C located in lemon peel and pulp acts as nutraceutical for treatment of obesity, diabetes, blood lipid levels, cardiovascular diseases and cancer [11]. Lemon peels exhibit antimicrobial activities and are rich in flavonoid glycosides, coumarins,  $\beta$  and  $\gamma$ -sitosterols, and other compounds [12]. The unstable compounds are mixes of monoterpenes (limonene), sesquiterpenes and sesquiterpenoids, for

instance, aldehydes (citral), ketones, acids, liquor (linolel) and esters [13]. As common carp is one of the most economically vital cultured fish species, it is necessary to identify its dietary requirements. Here we report the effects of dietary lemon pumace powder (peel and pulp) supplementation on oxidation biomarker levels of proteins and lipids in various tissues of common carp.

## Results

Analyzed proximate composition of lemon pumace powder and diet formulation and proximate composition of the basal diet is shown in Table 2. GC-MS chromatograms of lemon essential oil is shown in Table 3. The most predominant compound of lemon essential oil was the limonene. The growth performance of common carp fed diets supplemented with varying levels of lemon pumace powder is presented in Table 4. At the end of 30 days experimental periods the survival was 100% in all groups. Fish fed with the diet supplemented with 3% and 5% lemon pumace powder did improve ( $p < 0.05$ ) growth performance including final weight, weight gain (WG), feed conversion ratio (FCR) and specific growth rate (SGR).

**Table 1**  
Ingredients of diets.

Ingredient	(g/kg)
Fishmeal	300
Soybean meal	160
Corn meal	240
Wheat flour	180
Rice bran	80
Fish oil	20
Soybean oil	20

**Table 2**  
Composition and proximate analysis of lemon pumace powder

Composition	(%)
Crude protein	8.4
Moisture	5.9
Ash	6.7
Dry matter	94.1

**Table 3**

GC Analysis of lemon essential oil

Component	Retention time(min)	Area sum,%
3-methylheptane	4.16	0.01
1,3-dioxolane	4.32	0.72
Cis-1-ethyl-3-methyl-cyclopentane	4.51	1.42
n-octane	4.75	8.26
P-cymenene	5.65	4.13
linalool	6.92	1.12
fenchol	8.19	1.29
$\alpha$ -pinene	8.48	0.61
camphene	8.96	0.38
decane	10.71	2.08
m- cymenene	11.58	2.65
D-limonene	11.77	28.86
isoborneol	12.20	1.52
$\alpha$ -terpinene	12.82	2.18
Terpinene-4-ol	12.97	1.05
$\alpha$ - terpineol	13.90	15.65
$\beta$ -linalool	14.28	2.22
Fenchyl alcohol	14.78	1.47
Endo-borneol	16.66	1.71
$\alpha$ - terpineol	17.57	12.72
$\alpha$ - bergamotene	25.74	2.41
$\beta$ -bisabolene	27.48	3.59
$\beta$ -bisabolene	27.97	3.97

**Table 4**

Growth performance of common carp fed with various levels of lemon pumace powder

Group	Initial weight	Final weight	WG (%)	SGR (%)	FCR	Survival (%)
Control	61.12 $\pm$ 0.68	65.57 $\pm$ 0.62	7.33 $\pm$ 1.07 <sup>a</sup>	14.82 $\pm$ 2.1 <sup>a</sup>	8.7 $\pm$ 2.7 <sup>a</sup>	100 <sup>a</sup>
Treatment (1.5%)	60.21 $\pm$ 1.12	65.80 $\pm$ 1.04	9.41 $\pm$ 1.55 <sup>a</sup>	18.61 $\pm$ 2.89 <sup>a</sup>	6.1 $\pm$ 1.4 <sup>ab</sup>	100 <sup>a</sup>
Treatment (3%)	68.13 $\pm$ 1.38	81.11 $\pm$ 1.24	19.23 $\pm$ 1.64 <sup>b</sup>	43.26 $\pm$ 3.08 <sup>b</sup>	2.3 $\pm$ 0.15 <sup>c</sup>	100 <sup>a</sup>
Treatment (5%)	63.18 $\pm$ 1.15	85.27 $\pm$ 0.91	35.22 $\pm$ 1.9 <sup>c</sup>	73.64 $\pm$ 2.95 <sup>c</sup>	1.2 $\pm$ 0.88 <sup>c</sup>	100 <sup>a</sup>

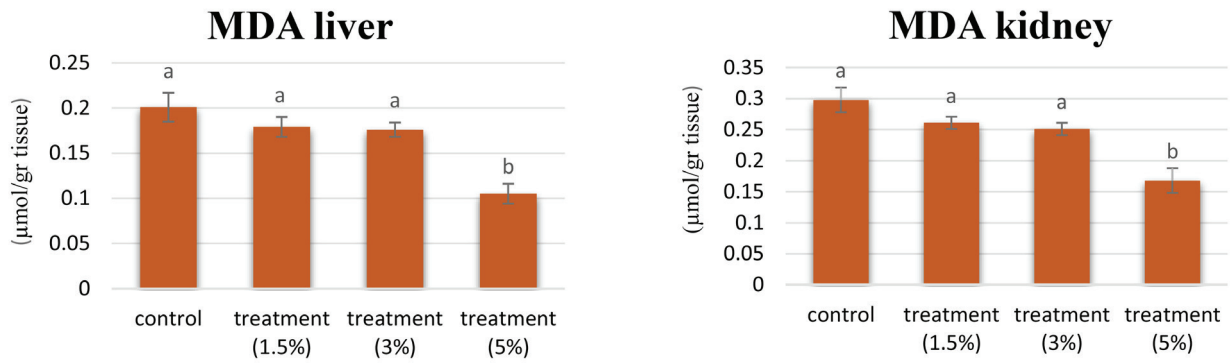
Values are mean  $\pm$  SEM of each experimental group. Mean values with different superscripts are significantly different from each other (significance level is defined as  $p < 0.05$ ). WG: weight gain, SGR: specific growth rate, FCR: feed conversion ratio.

### Biochemical assays and analysis

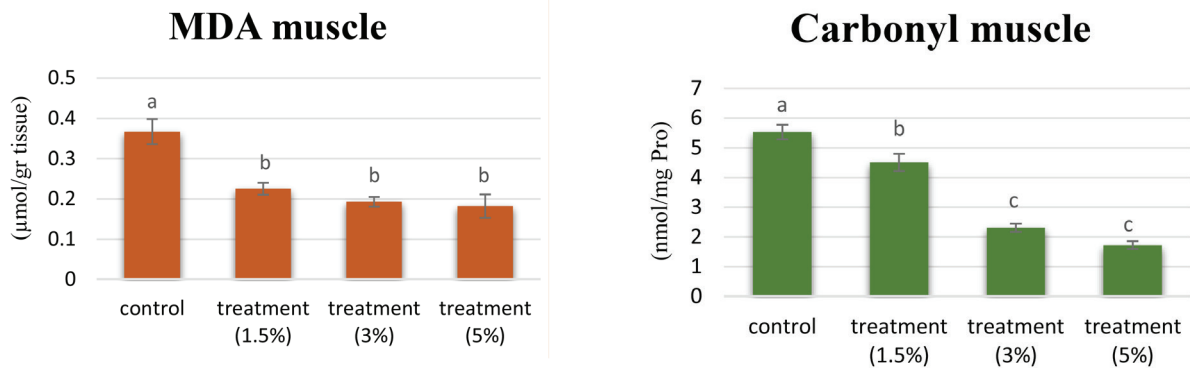
The effects of dietary lemon pumace powder supplementation on the levels of oxidation biomarkers of proteins and lipids in some tissues of common carp are presented in Figures 1-4. As shown in Figure 1, the decreasing effect of lemon pumace powder on malondialdehyde (MDA) values of kidney and liver was only significant in group 4, when compared with the control group ( $p < 0.05$ ). Muscle malondialdehyde (MDA) values significantly increased in all treatments compared to control (Figure 2). Protein carbonyl contents were significantly decreased in kidney and liver in groups 3 and 4 compared to control group, and protein carbonyl of muscle significantly decreased compared to control in all treatment groups (Figures 3 and 4). As shown in Figure 5, Group 3 showed significant increase in liver FRAP values compared to control. Meanwhile, only group 4 had major increase in kidney and muscle FRAP contents compared to control (Figures 5 and 6).

### Discussion

Potential lipid oxidation is affected by increase in PUFA content which in turn may affect color and flavor in weak storage conditions [15]. But, lipid oxidation can be avoided using dietary antioxidants. It has been clearly proved that  $\alpha$ TAc supplementation led to optimal oxidative stability [16, 17, 18]. Our results showed an antioxidative effect for natural antioxidant of lemon. These findings confirm the work of Lau et al [19] for grape seed and of Coetzee and Hoffman [17] for  $\alpha$ TC. As for green tea, a pro-oxidative effect was found, which contradicts the findings of Tang [20], who reported a clear antioxidative dose-response effect. At the same time other plant extracts, rosemary and sage extracts at 500 ppm [18], oregano and rosemary essential oils at 150 and 300 ppm [15] or at 100 and 200 ppm [16, 21], and a combination of marigold, purple coneflower, black currant and yellow bark at

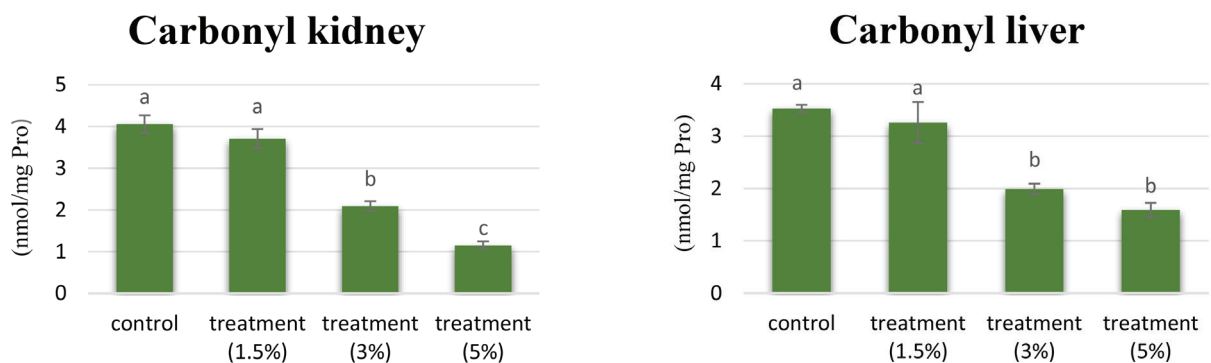


**Figure 1**  
Effect of dietary lemon pumace powder on malondialdehyde concentration in liver and kidney of common carp. Data are mean  $\pm$  SEM (n=10 in each group).



**Figure 2**  
Effect of dietary lemon pumace powder on malondialdehyde concentration in muscle of common carp. Data are mean  $\pm$  SEM (n = 10 in each group).

**Figure 3**  
Effect of dietary lemon pumace powder on protein carbonyl content in muscle and liver of common carp. Data are mean  $\pm$  SEM (n = 10 in each group).



**Figure 4**  
Effect of dietary lemon pumace powder on protein carbonyl content in kidney and liver of common carp. Data are mean  $\pm$  SEM (n = 10 in each group).

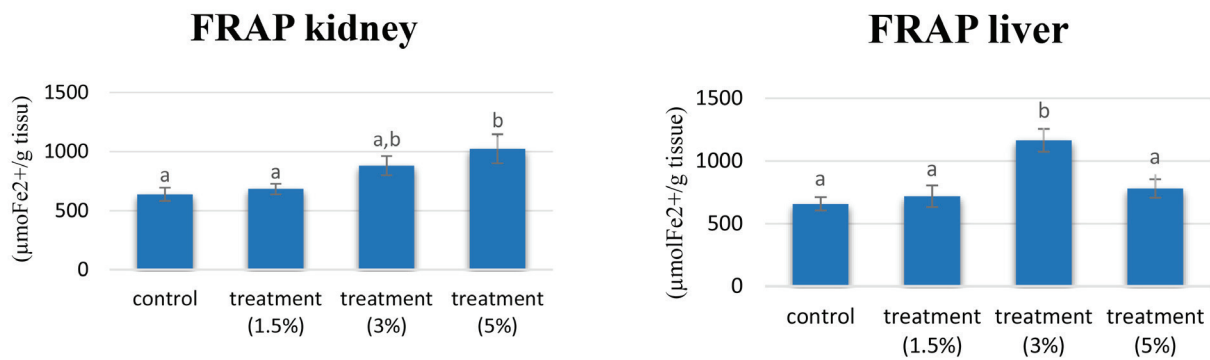
1000 ppm [16] have been shown to improve the oxidative stability of chicken meat. The antioxidant potential was also higher for lemon pumace. Radical scavaging activity of pumace in the DPPH assay was  $82 \pm 1.23 \mu\text{g/mL}$  and its IC<sub>50</sub> was  $83.061 \mu\text{g/mL}$ . The FRAP value of the lemon extract was  $92.25 \pm 2.45 \mu\text{M}$  trolox equivalents/g dry weight. The antioxidant properties and high content of flavonoids in the peels can make this waste material a good source of nutraceutical and healthy phenolic compounds, especially to be used as anti-aging product, due to the high content of polyphenols. Previous researches have proved that plant extracts improve the immune system of fish by enhancing the innate and adaptive immune response against pathogens [22, 23]. Such extracts trigger both humoral and cellular defense mechanisms [24]. Plant extract can enhance fish health status by stimulating immune potential and leukopoiesis. [25]. In mammals, many properties associated with digestive functions have been attributed to citrus fruits, such as lemon, including increased appetite or prebiotic impacts [11]. In the current study, at the end of 30 days of experimental period fish that were fed the diet supplemented with 3% and 5% of lemon pumace powder displayed significant growth including final weight, WG, FCR and SGR. This result is in line with those obtained for *O. mossambicus* fed with *citrus sinensis* peel essential oil [26] and in *Labeo victorinus* fed with citrus lemon peel essential oil [27]. Nutrient supplementation in fish diets has been identified as a cost-effective approach to enhance function of different intensive fish production mechanisms [28]. Most of the researches including changing oxidative status biomarkers in aquatic animals have focused on stress resulted from salinity variations, temperature fluctuations, hypoxia, etc. Just a few studies have evaluated the impact of varying nutritional status upon oxidative status biomarkers in fish. Most cellular structures and function components are probably to be potential targets of oxidative degradation. Some analytical methods have been extended to assess the oxidation products directly (carbonyl assay for oxidized proteins) or the resultant degradation products (malondialdehyde for lipid peroxidation) [29]. Such oxidation products may be used as biomarkers in tissue or plasma to assess the irreversible impacts of oxidative stress in animal [30, 31] and human models [32, 33]. In addition, according to the present study, dietary lemon pumace supplementation at 5% diet led to significant decrease in TBARS values in liver, kidney and muscle compared with the control group. In line with the present results, several researches have indicated decreasing impacts of garlic in lipid peroxidation in humans [34, 35, 36] and animal models [37, 38, 39, 40]. Kumar et al. [41] have illustrated an ameliorative effect of gar-

lic on lipid peroxidation in freshwater catfish *Clarias batrachus* while exposing to cadmium. In addition, some researches have indicated an enzymatic antioxidant system enhancement after garlic administration that could offer some protection against free-radical degradation [42, 43, 44]. Our results show that protein carbonyl contents significantly decreased in muscle, kidney and liver among others. Cured garlic administration inhibited the gentamicin-resulted rise in renal levels of protein carbonyl groups [45]. It has been reported that protein oxidation is associated with lipid oxidation in turkey meat [46] and fish fillet [47]. In fish, Srinivasan and Hultin [47] found a relationship between carbonyl content and TBARS values in cod when exposed to a free-radical-generating system. However, our data do not support such a possible relationship. In line with our findings, the study of Mercier et al [46] on beef showed an effect of diet on lipid oxidation but no significant dietary effect on protein oxidation. On the other hand, another study on lamb showed a significant effect of diet on meat protein oxidation that was not associated with a dietary effect on lipid oxidation [48]. However, it must be noted that carbonyl production is just a general index of protein oxidation and oxidation of other chemical groups may also occur [47].

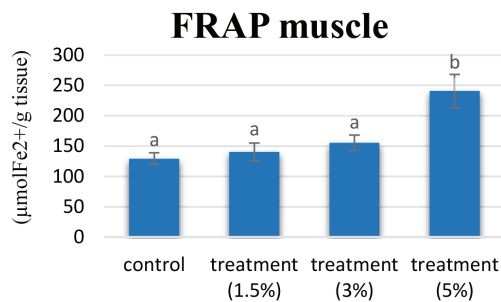
In the present research, a significant rise in FRAP value as a measure of total antioxidant status in muscle, kidney and liver tissue was found. It has been indicated that using some medicinal herbs and natural antioxidants is efficient in raising the total antioxidant status in different phases of meat preservation [49]. According to Norhaizan [50] it was found that some rice bran compounds can significantly elevate the amount of FRAP value in some cell cultures compared to the control group.

In conclusion, this research indicated that using lemon pumace powder can enhance the oxidative status by lowering the oxidation of lipids and proteins in the muscle of common carp. Thus, as a diet supplement in diet of fish can be applied to enhance the health status. In the past decades, plant-derived compounds have received a great deal of attention mainly for their contributions in food preservation, particularly for stopping lipid oxidation. As a natural product, lemon pumace with high antioxidant potential have been increasingly preferred over synthetic antioxidants due to nontoxic nature and having no health concerns. The results of the present study indicate that dietary lemon pumace powder supplementation can decrease oxidative stress to some extent using of antioxidant system improvement and lowering levels of lipid peroxidation and protein oxidation in some tissues of common carp. In addition, according to biochemical analysis results, it could be recommended



**Figure 5**

Effect of dietary lemon pumace powder supplementation on FRAP concentration in kidney and liver of common carp. Data are mean  $\pm$  SEM (n = 10 in each group).

**Figure 6**

Effect of dietary lemon pumace powder supplementation on FRAP concentration in liver of common carp. Data are mean  $\pm$  SEM (n = 10 in each group).

that the lemon doses used in this research might have no damaging impacts on organs of common carp.

## Materials and methods

### Experimental design and sampling

A commercial pellet diet was crushed and mixed with tap water before adding the correct amount of crushed lemon pumace powder and pelleting to obtain diets supplemented with 0% (control), 1.5, 3 and 5 percent lemon pumace powder. Diet formulation and proximate composition of the basal diet are shown in Table 1.

One hundred and twenty common carp (*Cyprinus carpio*), weighing  $60 \pm 5$  g, were obtained from a local farm (Mazandaran, Iran). They were divided randomly into 4 equal groups and held in four glass aquaria, each containing 250 L fresh water. Fish were acclimatized for 7 days before commencement of the experiment and were fed with a commercial pellet diet at a rate of 2% body weight per day. Physicochemical conditions of the water during

the experimental period were dissolved oxygen 5.5–6 ppm, temperature  $25 \pm 1^\circ\text{C}$ , and pH  $7 \pm 0.5$ . Photoperiod was a 12:12 light–dark cycle. Water in the aquaria was renewed every 48 h. Group 1 fish were fed with basic diet, serving as the control. Fish in groups 2, 3 and 4 were fed the basic diet supplemented with 1.5, 3 and 5% lemon pumace powder, respectively. The fish in each group were fed three times daily at 8:00, 13:00 and 19:00 throughout the experiment period (30 days). At the end of the experiment, 10 fish were selected randomly from each aquarium and anesthetized in diluted MS-222. Blood samples were taken by cardiac puncture using heparinized syringes and tubes. After plasma separation by centrifugation at  $1000 \times g$  for 20 min, erythrocyte pellet was washed three times with normal saline solution. The washed centrifuged erythrocytes were hemolyzed by the addition of an equal volume of ice-cold redistilled water and prepared plasma hemolysate aliquots were stored at  $-70^\circ\text{C}$  until analysis.

### Preparation of lemon pumace powder

Lemon pumace (*C. lemon*) was obtained from a local lemon juice factory in Mashhad, Iran. The lemon peels were dried under the shade in room temperature. After that, the dried peels were powdered using a mortar as well as with an electric blender.

### Chemical analysis

Analyzed proximate composition of lemon pumace powder was determined according to the Method of AOAC (2002). Crude protein content was determined by Kjeldahl method using an Auto Kjeldahl System (Kjeltec™ 2300, Foss, Sweden). Moisture and dry matter content, as well as protein and ash percentage were measured by AOAC (2002) method.

### GC-MS analysis (ISO 7609:1985)

GC-MS analysis was performed on Agilent Technology (Little Falls, California, USA) 6890 series gas chromatography (GC) system, equipped with 5973 mass spectrometry (MS) detector and a 7683 series auto-injector. Compounds were separated on Rtx®-Wax capillary column (30 m  $\times$  0.25 mm, film thickness 0.25  $\mu\text{m}$ ; RESTEK, Pennsylvania, USA). Helium (5N5 grade) was used as carrier gas, with a flow rate of 0.8 mL/min, and the split ratio was 60:1. Sample injection volume was 1  $\mu\text{l}$  and the injector temperature was  $230^\circ\text{C}$ . The column oven temperature was held at  $70^\circ\text{C}$  for 2 min, and then programmed to  $130^\circ\text{C}$  at  $30^\circ\text{C}/\text{min}$  and then to  $230^\circ\text{C}$  with  $10^\circ\text{C}/\text{min}$ . Finally, held at  $230^\circ\text{C}$  for 6 min

and the total run time was 20 min. An electron ionization (EI) system with ionization energy 70 eV was used for detection. The ion source temperature was set at 230°C, the interface temperature was 250°C, detector voltage was 2 kV. The mass spectrum was acquired in scan mode at a scan rate 0.98 scan/sec within a mass range of 20-800 amu. The measurement was performed in duplicate for each sample with solvent delay for 2 min.

### Antioxidant Assessment

Free Radical Scavenging Activity: 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radicals absorb at 517 nm, but upon reduction by an antioxidant compound, absorption decreases. Briefly, 50 µL of processed SPE MeOH extract or pure compound prepared at different concentrations was added to 2 mL of fresh 0.1 M solution of DPPH in methanol and allowed to react at 37 °C in the dark. After thirty minutes the absorbance was measured at 517 nm [51]. The DPPH scavenging ability as percentage was calculated as: DPPH scavenging ability =  $(A_{\text{control}} - A_{\text{sample}}/A_{\text{control}}) \times 100$ .

### Ferric Reducing Antioxidant Power

The determination of ferric reducing antioxidant power or ferric reducing ability (FRAP assay) of the extracts was performed as described by Benzie [14] with some modifications. The stock solutions prepared were 300 mM acetate buffer pH 3.6, 10 mM TPTZ (2, 4, 6-tri (2-pyridyl)-s-triazine) solution in 40 mM HCl, and 20 mM FeCl<sub>3</sub>·6H<sub>2</sub>O solution. Plant extracts or standard methanolic Trolox solutions (150 µL) were incubated at 37°C with 2 mL of the FRAP solution (prepared by mixing 25 mL acetate buffer, 5 mL TPTZ solution, and 10 mL FeCl<sub>3</sub>·6H<sub>2</sub>O solution) for 30 min in the dark. Absorbance of the blue ferrous tripyridyltriazine complex formed was then read at 593 nm.

### Chemicals

2,4-Dinitrophenylhydrazine (DNPH) and 2-thiobarbituric acid (TBA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The rest of the utilized chemicals were of analytical grade and were supplied by Sigma (St. Lewis, MO, USA) or Merck (Darmstadt, Germany).

### Biochemical assays and analysis

Tissue samples including muscle, kidney and liver were rapidly thawed and homogenized in 10 volumes (w/v) of ice-cold 0.05 M phosphate buffer (pH 7.4) for 5 min, and centrifugated at 4,000g for 15 min at 4°C; the supernatant was kept in ice until assayed. Determination of malondialdehyde (MDA) concentration was based on spectrophotometry of the pink-colored product of thiobarbituric acid reactive substances, as described by Latha and Pari. The concentration of MDA was calculated using a molar extinction coefficient value of 156,000 M<sup>-1</sup> cm<sup>-1</sup>. Carbonyl groups of proteins were detected by reaction with 2, 4-dinitrophenylhydrazine, which leads to the formation of a stable 2, 4-dinitrophenylhydrazone product (Petron et al., 2007). Resulting 2, 4 dinitrophenylhydrazones were quantified spectrophotometrically at 370 nm using a molar extinction coefficient of 22,000 M<sup>-1</sup> cm<sup>-1</sup>.

### Growth performance

All fish in different experimental groups were weighed at the end of 30 days feeding trial for estimation of growth. Growth performance parameters were calculated according to the following formulae:

Weight Gain (WG) =  $(\text{final weight} - \text{initial weight}) \times 100 / (\text{initial weight})$

Specific Growth Rate (SGR) =  $(\text{final weight} - \text{initial weight}) \times 100 / \text{days}$

Feed Conversion Ratio (FCR) =  $\text{feed given (dry weight)} / \text{total wet weight gain}$ .

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

### Statistical analysis

The data (means ± SD) were analyzed by using one way analysis of variance (ANOVA) followed by Duncan's post hoc test to compare the means between treatments and differences were considered significant when  $p < 0.05$ .

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### Author Contributions

Conceived and designed the experiments: A.S., D.S., H.B. Performed the experiments: S.S., A. S., D.S., H.B. Analyzed the data: S.S. Contributed reagents/materials/analysis tools: H.B., S.S. Wrote the paper: S.S.

### Conflict of Interest

None.

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