

Evaluation of Antibacterial Properties of *Eucalyptus spp* and *Plelargonium roseum* Extractsin Common carp, *Cyprinus carpio* and Their Effectson Blood Indices

¹Mehrak Mohamadi, ²Abbas Ali Zamini and ²Habib Vahabzadeh

¹Department of Aquaculture, Research Branch, Islamic Azad University-Guilan Science and Rasht, Iran

²Department of Fishery and Aquaculture, Faculty of Natural Resource,
Islamic Azad university-Lahijan Branch, Lahijan, Iran

Abstract: Two plant extracts were screened for evaluation of their antimicrobial activities against bacterial and fungal pathogens in Common carp, *Cyprinus carpio*. For this purpose, fish weighing 25.3±5.6 g were exposed to *Eucalyptus spp* and *Plelargonium roseum* extracts at different doses of 50, 100 and 150 mg/l for a period of 3 weeks. At the end of the experiment, bacterial and fungal CFU, hematocrit (Htc), hemoglobin (Hb), white blood cell (WBC), red blood cell (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), neutrophils, lymphocytes, monocytes, eosinophils, cortisol, glucose and total immunoglobulin were measured. Significant decrease of fungal and bacterial CFU for *Eucalyptus Spp* and *Plelargonium roseum* was observed in groups treated with 50, 100 and 150 mg/l. The obtained results showed that these extracts have some immunostimulatory effects on some indices such as MCV, MCH, MCHC and WBC especially at 150 mg/l. The results suggest that extracts of *Eucalyptus spp* and *Plelargonium roseum* can improve immune response in common carp.

Key words: CFU · *Cyprinus carpio* · *Eucalyptus spp* · *Plelargonium roseum*

INTRODUCTION

The increased productivity in fish culture has been accompanied by stressful conditions and problems related to fish disease. Enhancement of the immune system seems to be the most promising method for preventing fish diseases [1]. Nowadays, the concerns about possible antibiotic residues and antibiotic resistance have aroused great caution in the usage of antibiotics. Use of chemical materials such as malachite green, formalin and sodium chloride [2] in aquaculture systems to control fish diseases, parasites and other pests leads to high levels of residues in the animals [3]. Numerous reports declared that chemical drugs in aquaculture industry have been replaced by herbal medicine [4,5]. Recently medicinal plants from different families have been used for monitoring of aquaculture ponds against bacterial, viral and fungal diseases; because they are safe, easily biodegradable, inexpensive, locally available and extracts are easily prepared [2, 3, 6]. Studies have shown

astimulating role of plant extracts in the fish immune system challenged with bacterial, parasitic and fungal agents [4,7-10]. Therefore, plants extract as single compounds or as mixed preparations are known to have an important role in control of diseases due to their antioxidant and antimicrobial properties [11]. *Eucalyptus spp*. (Family: *Myrtaceae*) originates from Australia. It grows now in almost all tropical and subtropical areas and is cultivated in many other climates. Much research has been conducted on the medicinal properties of *Eucalyptus spp*. *Pelargonium roseum* Willd (*P. radula*) is a species originated from South-Africa and cultivated in Reunion, Egypt, Algeria, Morocco, China, Spain and in Southern France. Based on some studies on the *Eucalyptus* (*Myrtaceae*), it has been used to control different diseases derived from microbial infections. The development of bacterial resistance to presently available antibiotics has necessitated the need to search for new antibacterial agents [12]. Several compounds have been extracted from *Eucalyptus* as biological sources such as

uglobals, grandinol, macrocarpals, eucalymin, robostadial, 1, 8-eineole and eucalypton with antimicrobial and remedial properties [13-16]. According to recent studies extract of *Eucalyptus* have shown an enormous antimicrobial activity; as far as, antimicrobial activity of *Eucalyptus* oil on 22 bacteria and 12 fungi via disc diffusion method was studied by some researchers and it was also applied to produce new chemical compounds [15,17]. Moreover, essential oils of *Pelargonium* species were found to have pharmacological activity and Geraniumessential oils are tested for antibacterial and antifungal activity [17]. Common carp is an omnivorous fish seeds mainly in the benthic region of the pond. They have been serving as staple food for people [18]. To date limited information is available concerning herbal medicines and treating abnormalities of the immune system and balancing immune function [19-24]. Since there is no information about the effect of extracts of *Eucalyptus spp* and *Plelargonium roseum* on antimicrobial agents and immune system in Common carp. So objectives of the present investigation were to evaluateantimicrobial and antifungal effects of above mentioned extracts in *C. carpio* and their effects on blood parameters.

MATERIALS AND METHODS

Experimental Fish: The Juveniles of common carp, *C. carpio* were obtained from Aquaculture Research Center of Islamic Azad University,Guilan, Iran. A total of 160 fish (average body weight: 25.3 ± 5.6 g) was stocked in eight 20 L aquariums with constant water flow and aeration for adaptation. Fish were acclimated to experimental conditions for one week. Then, the healthy fish were divided into eight groups (Two control treatments and six experimental groups) with 8 fish per each treatment. The physical and chemical parameters were kept nearly constant (mean \pm SE): temperature 22.0 ± 1.8 °C; pH 7.5 ± 1.3 ; dissolved oxygen 6.5 ± 0.6 mg. Feeding was suspended 48 h prior to testing and the animals were not fed during the experiments.

Preparation and Testing of the Plant Extracts for Antibacterial and Antifungal Activities: Bacterial and fungal suspensions were sampled from skin and gills at the end of the experiment to determine colony-forming units (CFU) ml⁻¹. The quantitative enumeration of fungi and bacteria as colony-forming units (CFU/ ml⁻¹) was performed. The samples were homogenized in 90 mL of distilled water solution for 30 min in an orbital shaker.

Serial dilutions were made and 0.1-mL aliquots were inoculated in triplicate onto the media dichloran rose Bengal chloramphenicol agar (DRBC) for estimating total culturable fungi and bacteria and dichloran 18% glycerol agar(DG18) that favors xerophilic fungus development. The plates were incubated at 25°C for 5 to 7 days. Only plate's containing 100 to 1000 CFU were used for counting, with results expressed as CFU/ ml⁻¹ of sample. In this study *Eucdlyptus Spp* and *Plelargonium roseum* extracts were purchased from Barij essence pharmaceutical Company, Tehran, Iran. Experimental groups were exposed to doses of 50, 100 and 150 mg/l of *Eucdlyptus Spp* and *Plelargonium roseum* extracts for 3 weeks (3 days per week). The experiments were carried out in static systems. Temperature, pH and dissolved O₂ were monitored continuously.

Hematological Indices: At the end of the study period, blood samples were collected by piercing in the ventro-lateral side of the caudal peduncle with 2 mL heparinized disposable syringe and transferred to anticoagulant tubes for hematological analyses. Hematocrit was determined using microhematocrit capillaries filled with blood, centrifuged at 3,000 rpm for 5 min and expressed as percentage of total blood volume. Hemoglobin was measured with a spectrophotometer at 540 nm absorbance using the cyanmethemoglobin method [25]. Determination of red blood cells (RBC) and white blood cells (WBC) counts were performed with Neubauer chambers, using Rees diluting solution (1 g brilliant cresyl blue, 31.3 g sodium citrate, 10 ml formalin (37%) and 1,000 ml distilled water). Differential leukocyte count was performed with blood smears stained with Giemsa solution. The smears were examined by light microscopy (Olympus, Tokyo, Japan) under oil immersion at $\times 100$ magnification. Erythrocyte indices including mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated according to Stoskopf (1993) [26]. Plasma total immunoglobulin (Ig) content was determined following the method of Puangkaew *et al.* [27].

Biochemical Indices: Serum cortisol levels were measured by ELISA reader (SCO Elisa Reader, type MPR 01, Germany) according to the manufacturer's instructions (Monobind, Inc., USA). Glucose was measured spectrophotometrically (Biochrom, Libra S12, England) using standard kits (Pars Azmoon Company, Iran).

Statistical Analysis: All data were subjected to a one-way analysis of variance (ANOVA) after confirmation of normality and homogeneity of variance. Significance of the differences between means was tested using Duncan's multiple range test ($P < 0.05$). All assays were performed in triplicates and data were shown as mean \pm SE for each treatment.

RESULTS AND DISCUSSION

The antimicrobial effects of *Eucalyptus Spp* and *Plelargonium roseum* extracts were shown in Fig 1-8, respectively. Significant difference was observed between control fish and treatment groups in terms of CFU ($p < 0.05$). Significant decrease of bacterial CFU for *Eucalyptus Spp* and *Plelargonium roseum* was measured in groups treated with 50, 100 and 150 mg/l (Fig 1-4). The results showed that the *Eucalyptus Spp* and *Plelargonium roseum* have a lethal effect on colony forming of bacteria at high concentrations (100 and 150 mg/l). In line with these results a study done by Abouhosseini Tabari *et al.* [28], eucalyptus essence was the most effective against *E. coli*. Mahboubi [29] in a study on antimicrobial activity of essence of eucalyptus showed a favorable antimicrobial effect against *Aspegilus flavus*, *Vibrio cholera* and *Staphylococcus aureus*. Meanwhile, among 20 species which were studied for antibacterial effect ethanol extract of eucalyptus, a favorable antibacterial effect against *Staphylococcus aureus* resistant was observed [30]. With regard to bactericidal effects of herbal extracts and essences, it has been generally accepted that one important characteristic of these components is their hydrophobic nature which leads to distribution of them in lipid parts of cell wall or bacterial mitochondria and cause disruption in their structure and permeation. Many ions and other vital cell components leave microbial cells and leads to cell death [31,32]. As depicted in Fig 5-8, fungal CFU for *Eucalyptus Spp* and *Plelargonium roseum* was significantly lower in doses of 100 and 150mg/l ($p < 0.05$) and the amount of CFU in the control group was higher than other groups. Similar results were also obtained by some investigators, they demonstrated the inhibitory effects of different plants on some fungi in the *saprolegniaceae* family such as *Artemisia verlotorum* and *Antolina etrusca* extracts against *S. ferax* [33], *Blumea balsamifera*, *B. mollis*, *Eupatorium triplinerve*, *Guizotia abyssynica* and *Tagetes erecta* essential oils against *S. ferax* [34].

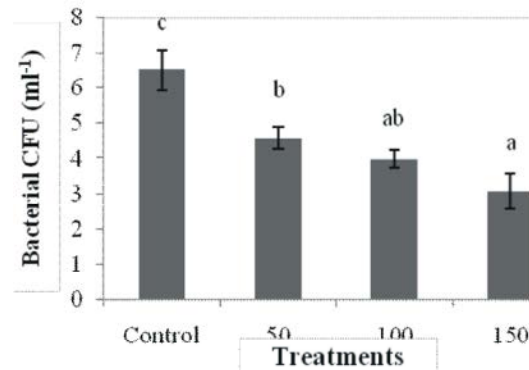


Fig. 1: Bacterial (CFU/ml⁻¹) of skin in common carp, *Cyprinus carpio* exposed to *Eucalyptus Spp* extract. Data are expressed as mean \pm SE. Mean values bearing same superscript are not statistically significant ($p > 0.05$).

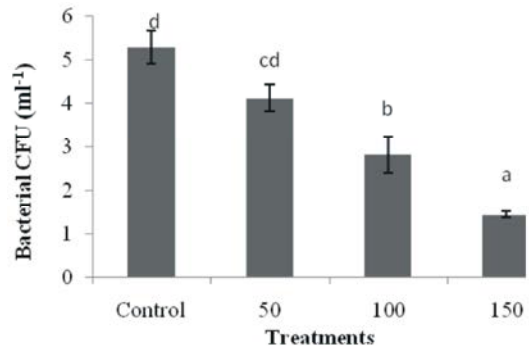


Fig. 2: Bacterial (CFU/ml⁻¹) of gills in common carp, *Cyprinus carpio* exposed to *Eucalyptus Spp* extract. Data are expressed as mean \pm SE. Mean values bearing same superscript are not statistically significant ($p > 0.05$).

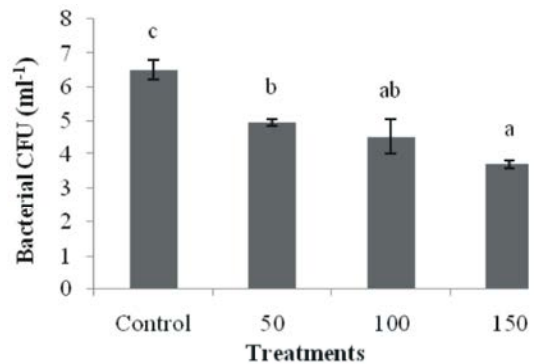


Fig. 3: Bacterial (CFU/ml⁻¹) of skin in common carp, *Cyprinus carpio* exposed to *Pelargonium roseum* extract. Data are expressed as mean \pm SE. Mean values bearing same superscript are not statistically significant ($p > 0.05$).

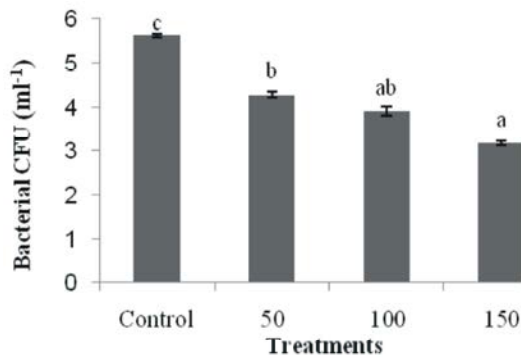


Fig. 4: Bacterial (CFU/ml⁻¹) of gills in common carp, *Cyprinus carpio* exposed to *Pelargonium roseum* extract of. Data are expressed as mean ± SE. Mean values bearing same superscriptare not statistically significant (p>0.05).

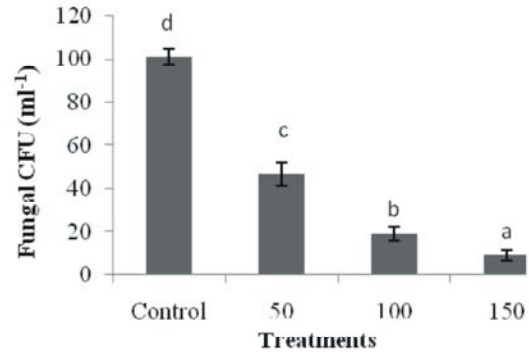


Fig. 7: Fungal (CFU/ml⁻¹) of skin (mean ± SE) in common carp, *Cyprinus carpio* exposed to *Pelargonium roseum* extract. Data are expressed as mean ± SE. Mean values bearing same superscriptare not statistically significant (p>0.05).

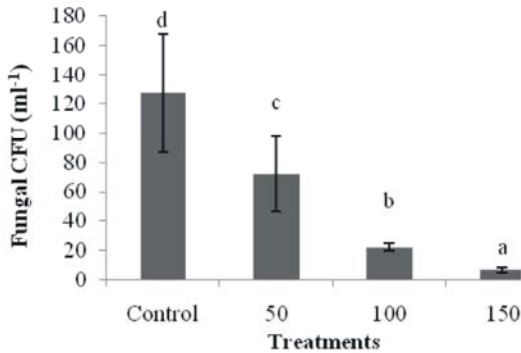


Fig. 5: Fungal (CFU/ml⁻¹) of skin in common carp, *Cyprinus carpio* exposed to *Eucalyptus Spp* extract. Data are expressed as mean ± SE. Mean values bearing same superscriptare not statistically significant (p>0.05).

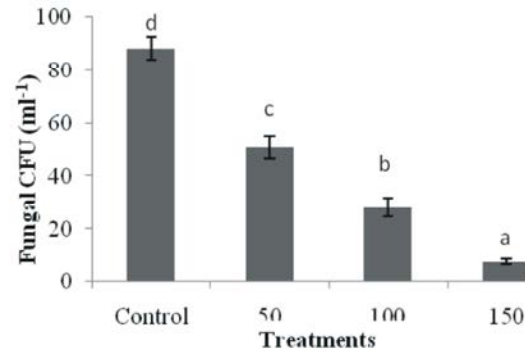


Fig. 8: Fungal (CFU/ml⁻¹) of gills (mean ± SE) in common carp, *Cyprinus carpio* exposed to *Pelargonium roseum* extract. Data are expressed as mean ± SE. Mean values bearing same superscriptare not statistically significant (p>0.05).

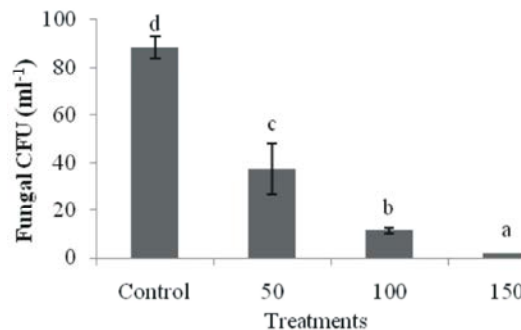


Fig. 6: Fungal (CFU/ml⁻¹) of gills (mean ± SE) in common carp, *Cyprinus carpio* exposed to *Eucalyptus Spp* extract. Data are expressed as mean ± SE. Mean values bearing same superscriptare not statistically significant (p>0.05).

In this experiment, there was significant differences in all hematology indices (p<0.05) except monocytes and eosinophils (Tables 1, 2). The control fish showed less MCV, MCH, MCHC, WBC and neutrophil. These parameters were significantly higher in dose of 150 mg/l for *Eucalyptus Spp* and *Pelargonium roseum* as well (p<0.05). The control group showed maximum RBC, Hb, Htc and lymphocytes (Tables 1, 2). Since, blood is a physiological factor of organisms; haematological tool have been used as a diagnostic parameter for investigation of disease and physiological disorders [35]. Some recent studies have addressed the hematological changes related to different plant extract [36]. In coincidence with the present findings, a clear decrease in Hb content in a study on the effect of leaf extract of

Table 1: effect of *Eucalyptus spp* extract on the hematological indices of *C. carpio*

Haematology indices	<i>Eucalyptus spp</i> extract			
	Control mg/l	50 mg/l	100 mg/l	150 mg/l
MCV(fl)	477.7 ± 5.51 ^a	474.5 ± 4.61 ^a	474 ± 5.37 ^a	506.7 ± 15.5 ^b
MCH (pg)	98 ± 1.22 ^{ab}	93.2 ± 0.48 ^a	93.25 ± 1.03 ^a	100.25 ± 2.78 ^b
MCHC (g/dl)	20.5 ± 0.29 ^b	19.25 ± 0.25 ^a	19.3 ± 0.2 ^a	19.5 ± 0.28 ^a
WBC (×10 ⁴ mm ³)	41.6 ± 16.7 ^a	89.5 ± 6.38 ^b	123.6 ± 6.44 ^c	125 ± 15.4 ^c
RBC (×10 ⁶ mm ³)	96.4 ± 18.6 ^c	62.70 ± 38.02 ^a	56.3 ± 17.8	77 ± 21.3 ^b
Hb (g/dl)	9.48 ± 0.15 ^c	5.88 ± 0.36 ^a	5.28 ± 0.2 ^a	7.75 ± 0.16 ^b
Hematocrit (%)	46 ± 0.41 ^c	29.75 ± 1.75 ^a	26.75 ± 0.95 ^a	39 ± 0.71 ^b
Neutrophils (%)	35.5 ± 2.1 ^a	40.5 ± 0.65 ^{ab}	46 ± 3.03 ^b	57.25 ± 1.11 ^c
Lymphocytes (%)	62.25 ± 2.3 ^c	57.75 ± 0.8 ^c	50.75 ± 2.78 ^b	39.5 ± 74.2 ^a
Monocytes (%)	1.5 ± 0.2	1.25 ± 0.4	2.5 ± 0.2	2.25 ± 0.4
Eosinophils (%)	1 ± 0.4	1 ± 0.2	1 ± 0.2	1 ± 0.4

Values sharing different superscript letters are significantly different.

Table 2: Effect of *Plelargonium roseum* extract on the hematological indices of *C. carpio*

Haematology indices	<i>Plelargonium roseum</i> extract			
	Control mg/l	50 mg/l	100 mg/l	150 mg/l
MCV (fl)	477.7 ± 5.51 ^a	473.2 ± 4.87 ^a	467.5 ± 3.95 ^a	536.5 ± 37.86 ^b
MCH (pg)	98 ± 1.22 ^{ab}	94.25 ± 0.25 ^a	94 ± 1.08 ^a	98.25 ± 1.89 ^b
MCHC (g/dl)	20.5 ± 0.29 ^b	19.5 ± 0.29 ^a	19.75 ± 0.25 ^{ab}	20.25 ± 0.25 ^{ab}
WBC (×10 ⁴ mm ³)	41.6 ± 16.7 ^a	88 ± 5.8 ^b	122.7 ± 57.6 ^c	149.5 ± 618 ^d
RBC (×10 ⁶ mm ³)	96.4 ± 18.01 ^b	65.9 ± 19.7 ^a	70.6 ± 5.1 ^a	63.1 ± 13.5 ^a
Hb (g/dl)	9.48 ± 0.15 ^b	6.23 ± 0.17 ^a	6.68 ± 0.54 ^a	6.23 ± 0.25 ^a
Hematocrit (%)	46 ± 0.41 ^b	31.25 ± 1.11 ^a	33 ± 2.35 ^a	30.25 ± 1.03 ^a
Neutrophils (%)	35.5 ± 2.1 ^a	55 ± 3.14 ^b	54.75 ± 1.93 ^b	56 ± 1.22 ^b
Lymphocytes (%)	62.25 ± 2.2 ^b	41.75 ± 2.6 ^a	40.75 ± 2.1 ^a	40.25 ± 1.5 ^a
Monocytes (%)	1.5 ± 0.2	3 ± 0.4	3.75 ± 0.2	2.75 ± 0.2
Eosinophils (%)	1 ± 0.4	0.5 ± 0.2	0.75 ± 0.2	1 ± 0.2

Values sharing different superscript letters are significantly different.

Table 3: effect of *Eucalyptus spp* extract on the immunological indices of *C. carpio*

Immunology indices	<i>Eucalyptus spp</i> extract			
	Control mg/l	50 mg/l	100mg/l	150 mg/l
Total immunoglobulin(mg ml ⁻¹)	135.5 ± 13 ^b	66±12.43 ^a	111.25±14.99 ^a	145.5 ± 10.56 ^b
Glucose (mg/dl)	138.25 ± 3.2 ^b	33.75 ± 3.71 ^a	56.5 ± 8.27 ^a	42.75 ± 5.68 ^a
Cortisol (ng/ml)	42.5 ± 8.47 ^a	19.75 ± 3.68 ^a	42.5 ± 14.5 ^a	127.5 ± 11.18 ^b

Values not sharing identical superscript letters are significantly different.

Table 4: Effect of *Plelargonium roseum* extract on immunology indices in *C. carpio*

Immunology indices	<i>Plelargonium roseum</i> extract			
	Control mg/l	50 mg/l	100 mg/l	150 mg/l
Total immunoglobulin (mg ml ⁻¹)	135.5±13	122.75±8.67	151.25±12.1	158.25±20.74
Glucose (mg/dl)	138.25±3.2 ^b	70±6.75 ^a	47.5±10.68 ^a	52.25±8.96 ^a
Cortisol (ng/ml)	42.5±8.47 ^a	156.25±36.79 ^b	158.5±23.51 ^b	166.75±21.49 ^b

Values not sharing identical superscript letters are significantly different.

Khaya senegalensis onchannel catfish, *clarias gariepinus* was reported [37]. Contrary, some researchers reported that the hematological parameters such as hemoglobin and RBC are significantly higher in groups treated with herbal immunostimulants over control group [38-40]. The findings of the present study is in agreement with the work of Sahu *et al.* [38] and Subeena Begum and Navaraj [39], who reported that WBC counts are higher in *Labeo rohita* fingerlings treated with *Mangifera indica* when compared to control. Further, these observations also verify the findings of other investigations by Gopalakannan and Arul [41] who found that there is an increase in the WBC count in the common carp after exposure to chitin extract. On the other hand, Sudagar and Hajibeglou [40] stated that after feeding the common carp with plant extracts and challenging with *Aeromonas hydrophila* in *Cyprinus carpio*; all experimental groups showed higher hemoglobin, WBC and RBC compared to the control ($p < 0.05$). These alterations could possibly be due to activation of the immune system in the presence of herbal extracts, which in turn may be an adaptive response of the organism resulting in a more effective immune defense [42]. Hematocrit level is an indicator for fish health which gives clue on fish health and explains abnormalities caused by immunostimulants [43]. The increase of hemoglobin and hematocrit levels have been showed after exposure to black cumin seeds in rats [44-46]. Also, in another research done by Dorucu *et al.* [43] results are similar and there was a statistically significant increase ($P < 0.05$) in hematocrit level in 5% black cumin seeds group compared with the control and the other experimental groups. Phagocytosis and killing activity by phagocytic cells such as blood neutrophils play an important role in non-specific defense mechanisms against pathogenic agents [47,48]. These cells adhere to tissue surface by producing adhesive proteins that facilitate migration in problematic zones [49]. Two herbal extracts (*Astragalus membranaceus* and *Lonicera japonica*) significantly enhanced phagocytic cells in *Oreochromis mossambicus* [50], which is similar to our findings about neutrophil. This enhancement could be due to the enlightening immune response by a suitable immunostimulants [39]. The results of the immunological parameters were shown in Tables 3 and 4. Significant increase in cortisol in the plant extracts (*Eucalyptus Spp* and *Ptelargonium roseum*) treated groups was found ($p < 0.05$) and dose of 150 mg/l was most effective. Fish exposed to extract of *Eucalyptus Spp* and *Ptelargonium roseum* showed a significant reduction in glucose ($p < 0.05$).

The enhancement of glucose in fish blood helps to satisfy the increased energy demand during stress, allowing the fish to react to stressors [51]. So, decline of glucose suggests that the herbal treatment as immunostimulants improved the immune system. The increment in blood glucose might result from an increase in plasma catecholamine and corticosteroid hormones [52]. The decrease of glucose levels due to herbal extract is consistent with other studies in rat [44-46]. The findings of our study agree with many workers; Citarasu *et al.* [4] and Sahu *et al.* [38] who found that glucose levels are reduced in the aquatic animals affected by herbal immunostimulants.

In conclusion, the use of plant extracts for antibacterial and antifungal protection of fish is highly effective and decreased fungal and bacterial contamination. Considering their immunostimulant effect they could be recommended for use with farmed fish to control pathogenic microorganisms.

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