EFFECT OF CRUDR AND EXTRACT OF PROPPOLES AS ADDITIVES ON IMMUNOLOGICAL AND BIOCHEMICAL ON CYPRINUS CARPIO CHALLENGED WITH *AEROMONAS HYDROPHILA*

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ABSTRACT

The present study was carried out to investigate the effect of propolis [(crud propolis and Water Ethanol Extract Propolis (WEEP)] as feed additive on biochemical and immunological in common carp *Cyprinus carpio* challenged with *Aeromonas hydrophila.* A total of 60 *C. carpio*, (75±10g) were randomly stocked into five treated groups in duplicate as follows: control group (C) fish were fed basal diet without any addition of propolis; T1 fish were fed basal diet supplemented with 10g/kg crud propolis; T2, T3 and T4 fish were fed WEEP at concentrations of 2g, 4g and 8 g/kg diet.Results indicated that The highest albumin content was found in T1 which showed a significant increase (P≤0.5) compared to C+ group, and globulin content did not show significant differences between T1, T2, T3, T4, C- and C+ groups. Cholesterol level did not lead to changes in all (P > 0.05) treated groups (T1, T2, T3 and T4) when compared with the control group. The glucose level showed a significant (P≤0.05) increase in all WEEP groups (T2, T3, and T4). The highest weighted Somatic Spleen Index (SSI) was reported in T3 which showed a significant (p < 0.05) increase compared to T1. The result of nitro blue tetrazolium (NBT) assay showed non-significant differences (P > 0.5) in T1, T2, T3 and T4 compared to C and C+ groups.

Keywords: bees glue– propolis- total protein-cholesterol

مجلة العلوم الزراعية العراقية- 2025 :56 (3):1053-1059 الجبوري ومصطفى

تاثير العكبر الخام والمستخلص الايثانولي كاضافه علفيه على بعض الاختبارات المناعيه والكيموحيويه في اسماك الكارب الشائع المصابة ببكتريا *Aeromonas hydrophila*

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المستخلص

أجريت هذه الدراسة للتحقق من تأثير البروبوليس [(العكبر الخام ومستخلص العكبر المائي (WEEP)] كمادة مضافة للأعلاف على الكيمياء الحيوية والمناعية في اسماك الكارب الشائع المصابة تجريبيا ببكتريا*A. hydrophila* تضمنت التجربه استخدام 60 سمكه وزنها 75 ± 10 غم. تم توزيعها عشوائياً على خمس معاملات علاجية مكررة ، تم تغذية الأسماك على النحو التالي:مجموعة السيطرة غذيت الاسماك على عليقة اساسية دون اضافة العكبر: المعاملة الاولى غذيت على عليقه اساسية مع اضافة العكبر الخام بمعدل 10غم/كغم علف: المعاملات الثانية والثالثة والرابعة غذيت على عليقة اساسية مع اضافة مستخلص الايثانول المائي للعكبر بمعدل 2 و4 و 8 غم/كغم علف على التوالي. بعد مرور 42 يومًا من التغذية وأشارت النتائج إلى أن أعلى محتوى من الألبومين وجد في T1 والذي أظهر زيادة معنوية (P≤0.5) مقارنة بمجموعة C+ ، ولم يظهر محتوى الجلوبيولين فروق معنوية بين المجموعات T1 و T2 و T3 و T4 و C– و C+. لم يؤد مستوى الكوليسترول إلى تغيرات في جميع المجموعات المعالجة (T1) (P> 0.05 و T2 و T3 و T4) عند مقارنتها مع مجموعة السيطره. مستوى الجلوكوز أظهر زيادة معنوية (P≤0.05) في جميع مجموعات، WEEP (T2 و T3 و T4). وان أعلى مؤشر (SSI) في T3 والذي أظهر زيادة معنوية (p <0.05) مقارنة مع T1. أظهرت نتيجة مقايسة (NBT) اختلافات غير معنوية (P> 0.5) في T1 وT2 وT3 و T4 مقارنة بمجموعات السيطرة.

الكلمات المفتاحية: صمغ النحل–الكارب الشائع، العكبر، البروتين الكلي.

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INTRODUCTION

In aquaculture, immunostimulants have been shown to be beneficial for fish farmers (30). Use a wide variety of immunostimulants that may or may not need to be purified (vitamins, chitin, glucans, extracts of microorganisms, animals, and plants, by-products from other industries, etc.). The second class of immunostimulants has recently emerged and is receiving more attention due to their low cost, ease of use, and low environmental impact. In addition, they have numerous additional effects on fish physiology because it functions as a "cocktail. "It contains immunomodulators in addition to numerous nutrients and micronutrients (23). Propolis is bee glue which is a sticky resin material produce by honey bee (*Aspis millfera* l) (28). Several research has also been conducted on its efficacy against infections. Propolis's antimicrobial efficacy has generally been attributed to one of two different mechanisms: first, direct impact on pathogens through inhibition of some biochemical reactions, and second, increasing product resistance to pathogens through the enhancement of other biochemical reactions (5). It changes when mix with bee salivary enzymes are present. Its color, range from dark brown to red-green. Since it has been used in medicine for a variety of pharmacological properties. Propolis typically consists of 30 % wax, 50 % resin, and botanical conditioner. Pollen and other materials, 5% and essential and aromatic oils10% (11). Propolis contain Flavonoids, flavones, flavanones, flavanol's, Sugar, Vitamins, Minerals, Esters like Benzyl caffeate (7).is now widely used as a growth promoter in fish and poultry and as an immunostimulant in fish, poultry and other mammals (34). Aeromonas species are widely distributed in the environment, cause a wide range of diseases in both humans and fish, and have the greatest mortality rate for infectious diseases in fish (20). *Cyprinus carpio* is the most important freshwater fish aquiculture in Iraq (3). In fish, the same as in mammals, the innate immune system serves as the first line of defense, protecting them against hyperthermia stress, which has been proven to cause immunological damage and inflammatory reactions (4).Non-specific immunostimulants have recently attracted attention, since they have lower costs and are easily incorporated into the diet, along with their low impact on the environment (13). The aim of study is to determine the effect of crude and water-ethanoloic extract of propolis on biochemical and immunological parameters in common carp challenged with *Aeromonas hydrophila.*

MATERIALS AND METHODS

Crude propolis and its water ethanolic extract (WEEP): The ethanolic extract of propolis was prepared following the procedure of Eraslan *et al* (19), by using a dark sterile vial (200 ml), the propolis was cute into small portions, frozen for 6 hours, then mixed with an electric mixer to become a powder. Then, 40g of propolis was added to 50 ml of water for 24 hours and mixed by a magnetic stirrer and then added 150 ml of 99% ethanol for 48 hours using a magnetic stirrer. Then, filtrate the insoluble constituent with (clothe tulle) and evaporate at room temperature to gained powder which was applied for experimental feed formation.

Experimental Feeding

A total of 60 fish (average weight; 75 ±10 g/fish) were randomly selected from fish farm ponds near AL-Musib/Babylon. After two weeks of adaptation, fish were distributed into five treated groups in duplicate as follows: control group (C) fish were fed basal diet without any addition of propolis; T1 fish were fed basal diet supplemented with 10g/kg crud propolis; T2, T3 and T4 fish were fed WEEP at concentrations of 2g, 4g and 8 g/kg diet for a period of 42 days. Fish were given manual feed twice a day as desired dissolved oxygen 6.82±0.2 mg/L temperature 23.2±0.1°C, pH 6.8-7. 2, Daily water changes occurred in the aquariums.

Determination LD_{50} of *A. hydrophila*

the bacterial strain was cultured in Brain Heart Infusion Agar at 28 ºC for 24 h, LD_{50} according to Elwafai, *et al*. (18) (exactly at a concentration of 0.1 ml 1.57×10^6 colony forming units CFU/ml fish, 1 ml 1.57×10^8 CFU/ ml which causes high mortality 100% and no mortality0% on 0.1ml 1.57×10^4 CFU /fish of approximately.

Blood collection and biochemical analysis

Fish were aneastized using clove powder of 25mg/L (36 and 27). Blood samples were

collected from fish randomly from caudal vein puncture using plastic syringe, blood samples were transferred in to Eppendorf tubes contain gelatin and permitted to clot for tow hour. Serum was separated by centrifugation and stored at freeze (-20°C) were used for biochemical parameters (Total protein, Albumin, Globulin, sugar and Cholesterol). Biochemical profiles (albumin, total protein, globulin, glucose and cholesterol) were determined using Spectro-photometer metric using a commercial kit (Biosystems, S.A.France).

Immunological Parameters

Spleen somatic index (SSI): The spleen is an essential part of the immune system, and its proper function is required for effective pathogen resistance. It also includes a reserve of red blood cells, which may be used to compensate for the lack of oxygen in certain situations, such as stress(10).According to Abbass *et al.* (1). Spleen indices were calculated by divided spleen weight (g)/total body weight (g)×100.

Respiratory burst activity using Nitro blue tetrazolium (NBT): according to Biller *et al* (9) Reactive oxygen radical production by neutrophils during respiratory burst activity to formazan blood samples were mixed with (20 mg/10 ml) NBT in similar proportion (200 ul/200 ul) and incubated for 30 min at 25°C taken 50µl of this mixture and 1 ml of dimethyl formamide (DMF) was added to solubilize the reduced formazan product in glass tube. Then, centrifuged at 2000 rpm for 5 min, The reduced extent of NBT was measured at an optical density of 540 nm by using UV- Spectrophotometer with dimethyl formamide as the blank.

Statistical analysis

The Statistical Analysis System-SAS (31) program was used to detect the effect of various factors on the study variables. Least significant difference - LSD test (Analysis of Variance-ANOVA) was used for significant comparison between means in this study. Probability level was assessed at ≤0.05 and 0.01.

RESULTS AND DISCUSSION

Biochemical parameters: All treatments in table (1), when compared to the C- and C+ groups, produced no significant differences in total protein. In comparison to the C+ group, albumin content was not significantly (P≤ 0.05) different in any of the treated groups. There were no significant differences in Globulin between any of the treated groups (P>0.05). The A/G ratio decreased significantly (P> 0.05) in comparison to the C+ group. Blood proteins function as a buffer to maintain the body's natural osmotic pressure and hydrogen ion concentration (14) similar to (6) hen fish are exposed to stress, they disrupt their internal balance and trigger certain physiological reactions to maintain body balance and survive.. Albumin is an essential serum protein for the transport of hormones and steroids. Reduced concentrations of total protein, albumin, and globulin in C+ lead 58% increase in fish mortality from *A.hydrophilia* infection. It leads to blood loss because to the relative loss of all blood components, body cavity fluid accumulation, and liver failure, which leads to lower globulin and albumin levels and increased A/G ratios when compared to the control group. Many fish diseases have been discovered to alter albumin, globulin, and protein. Studies have shown that fish infected with *A. hydrophila* have a much lower serum protein level (25). According to Edrees et al (17) Increased protein catabolism induced through inflammation could be the reason of the challenged fish's lower protein profile. Otherwise, the negative effects of inflammation on albumin manufacturing has been shown. Compared to the control group, T3 group had the highest level of globulin and total protein in table (1). There may be abnormalities in the liver and kidney tissue causing the depletion of total protein, albumin, and serum globulin in sick fish (22). Fish's decreased appetite can be influenced by stress and infection, which can lead to an increase in serum protein (24). The A/G ratio index is a measure to monitor changes in the composition of serum or plasma Also, the T3 group have had lowest A/G ratio They tend to indicate swelling, liver disease, or a shift from albumin production to globulin production in connection with infection (2).

Table 1. Biochemical parameters of *C. carpio* after 14 days post challenged with *A.hydrophila.*

Group	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	A/G Ratio
C-	14.97 ±0.69 ab	4.35 ±0.215 ab	10.62 ±0.54	0.405 ±0.02 abc
C+	12.06 ±0.68 b	3.91 ±0.01 bc	8.15 ±0.67	0.482 ±0.04 a
T1	15.70 ±2.03 ab	4.86 ±0.33 a	10.76 ±1.77	0.470 ±0.04 ab
T2	12.93 ±0.79 ab	3.55 ±0.09 c	9.37 ±0.71	0.377 ±0.02 bc
T3	17.08 ±1.64 a	4.07 ±0.16 bc	13.01 ±1.50	0.322 ±0.03 c
T4	14.16 ±2.55 ab	3.59 ±0.32 c	10.57 ±2.23	0.357 ±0.04 c
LSD value	4.685 NS	0.679 **	5.143 NS	0.101 *
P-value	0.0498	0.0067	0.289	0.022

Means having with the different letters in same column differed significantly* (P≤0.05), ** (P≤0.01)

Glucose Level: After being exposed to *A. hydrophila* for 14 days, all WEEP groups (T2, T3, and T4) demonstrated significantly higher glucose levels (P≤ 0.05) than the C- and C+ groups. Additionally, the WEEP groups demonstrated a notable improvement over T1 (a crude propolis 10g/Kg diet). In T2, the glucose reading was at its highest Table (2). In order to maintain metabolic functions, fish have evolved a variety of strategies, including hormonal, behavioral, and metabolic responses, such as using stored glycogen in muscles and liver or metabolizing fats and proteins under stress or without food, which raise plasma levels glucose concentrations (8). The highest blood glucose levels in table (2) were 176.11, 151.46, and 155.38 g/dL in T2, T3, and T4, respectively, indicating hyperglycemia. Due to stress conditions, there may be a high concentration of glycogen in the liver during the feeding period (26), this increase may be attributed to the increased gluconeogenesis response of stressed fish in order to meet their increased energy requirements (35). One biomarker of impaired kidney and liver function, in addition to changes in lipid metabolism, could be elevated glucose levels in fish-fed diets containing 2, 4 or 8 g/kg propolis (16). The current findings are comparable to Fuat Gulhan (21) who used a variety of propolis concentrations and found that high concentrations raised glucose levels. Additionally, the current findings are consistent with Talas (33) who discovered that rainbow trout's glucose levels increased when propolis levels were high .

Table 2. glucose(g/dl) and cholesterol(mg/dl) parameters of *C. carpio* after 14days post challenged with *A.hydrophila*

Groups	Glucose (g/dl)	Cholesterol (mg/dl)
C+	61.47 ±0.60 b	141.82 ±53.49 ab
C-	54.16 ±1.44 b	129.84 ±12.14 ab
T1	97.77 ±17.189 b	104.21 ±13.05 ab
T2	176.11 ±46.61 a	169.96 ±26.57 ab
T3	151.46 ±6.80 a	186.52 ±27.07 a
T4	155.38 ±21.57 a	96.15 ±29.44 b
LSD value	44.24*	88.97 *
P-value	0.045	0.0436

Means having with the different letters in same column differed significantly. * (P≤0.05).

Cholesterol: After 14 days following *A. hydrophila* infection, the cholesterol levels of C. carpio showed no significant change (P>0.05) between the C+ and C- groups. Additionally, there were no apparent differences between the control groups and the treatment groups. But when compared to T3, T4 showed a substantial reduction (P ≤0.05) Table (2). It has been observed that propolis apparently includes benzyl caffeate, which reduces the production of lipid peroxides, propolis extract, if used in very small amounts, have anti-lipid peroxidation properties (32). When compared to the normal level of 129.84 mg/dl, the cholesterol concentration in T4 was low (96.15 mg/dl), which is harmful to fish health, this is most likely because it contains a lot of benzyls caffeate (29). Therefore, it appears to have the ability to protect kidney and liver function and prevent hyper catabolism when feeding animals with high concentrations of propolis. These results are consistent with Fuat Gulhan (21) who found that using various concentrations of propolis resulted in an increase in cholesterol, elevated glucose levels, and changes in lipid metabolism.

Spleen somatic index (SSIs): The results of the splenic index of C. carpio plants after 14 days of infection with *A. hydrophila* did not record any significant difference (P > 0.05) between C+ and C- groups and between

treated groups (T1, T2 and T4), the highest weight of SSIs was reported in T3 which exhibited significantly increased (P≤0.05) compared to T1. The spleen has an important function in hematopoiesis in vertebrates, and because of this size is a good indicator of the state of immune system activity and the incidence and severity of infections and diseases general health of the fish (15). The present data of the spleen somatic index clearly showed a significant (P≤ 0.05) increase in T3 compared to the T1 and control groups in table (3). This raise in spleen weight due to a bacterial infection and also due to the pressure when a large amount of blood was taken during the blood sampling. Also, this could be considered modifiable for fish to provide blood storage capacity to maintain spleen homeostasis after treatment of fish, and likely indicates a compensatory imbalance in the of spleen volume under stress (1).

Table 3. spleen somatic index and Nitro blue tetrazolium parameters of *C. carpio* after 14days post challenged with *A.hydrophila*

Group	Mean ± SE of Spleen weight (g)	Nitro blue tetrazolium
C+	0.192 ±0.04 b	0.011 ±0.004
C-	0.167 ±0.01 b	0.442 ±0.24
T1	0.180 ±0.03 b	0.132 ±0.02
T2	0.227 ±0.03 ab	0.201 ±0.01
T3	0.312 ±0.04 a	0.252 ±0.02
T4	0.257 ±0.02 ab	0.490 ±0.27
LSD value	0.092 *	0.443 NS
P-value	0.0328	0.236

Means having with the different letters in same column differed significantly. * (P≤0.05).

Nitro blue tetrazolium) NBT)reduction assay: drooping of nitro blue tetrazolium (NBT) by oxygen radical produced from the neutrophil cell of *C. carpio* showed non-significant (P > 0.5) differences in T1, T2, T3 and T4 compared to the C- and C+ groups. Also, there were non-significant differences (P>0.05) among all treatment groups (P>0.05) in table (3), In the present results, there were no changes between the treated groups that were fed different concentrations of propolis These results are consistent with Cuesta *etal* (13) who reported that no significant effects were seen on gilthead seabream, Sparus aurata with the use of propolis on either of the humoral parameters assayed, peroxidase content and alternative complement pathway. According to Choobkar (12) who found that rainbow trout, *Onchorhynchus mykiss* fed diet containing 1% propolis or 0.5% Pollen+ 0.5% propolis stimulation non-specific immunity including respiratory burst activity and phagocytosis. these results indicate that levels of reactive oxygen species (ROS) production cannot reach a limit that can induce respiratory burst activity in target cells.

CONCLUSIONS

In the present study, the results indicated that the crude propolis and its extract activated the immune system of of the *Cyprinus carpio* with increased in blood glucose values. The propolis extract has positive effects on biochemical profile in common carp challenged with *A. hydrophila.*

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DECLARATION OF FUND

The authors declare that they have not received a fund.

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