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Research Article

Immunological and anti-*Eimeria* Effects of Hot Water and Methanolic Extracts of *Pleurotus sajor-caju* in Broiler

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Abstract

Present study reports the immunomodulatory and anti-*Eimeria* effects of hot water and methanolic extracts of *Pleurotus (P.) sajor-caju* (a locally grown oyster mushroom). Mushrooms were processed to obtain hot water and methanolic extracts followed by lyophilization. The lyophilized extracts were subjected to proximate analysis followed by their evaluation for immunomodulatory and anti-Eimeria activities. Immunomodulatory evaluation of these extracts in industrial broilers revealed significantly higher ($P<0.05$) cell mediated immunity through lymphoproliferative response to phytohemagglutinin-P as compared to control. These extracts also showed higher humoral immune response through elicited total Ig, IgG, and IgM titers at day 7th and 14th after primary and secondary intramuscular injections of sheep red blood cells. Further, all the groups were orally inoculated with infective dose of sporulated oocysts of *Eimeria (E.)* species (including *E. tenella, E. maxima, E. acervulina* and *E. necatrix)* followed by monitoring of per cent mortality, oocysts per gram of feces (OPG) and intestinal lesion scoring. OPG values in birds of experimental groups administered with mushroom extracts were significantly lower ($P<0.05$) as compared to those of control group. Control group showed higher mortality ratio and lesions scores as compared to groups administered with extracts. In conclusion, hot water and methanolic extracts of *P. sajor-caju* showed significant immune boosting activity in broilers and subsequent protective efficacy against Eimeria infection.

Keywords: *Mushroom extracts, Pleurotus sajor-caju, Eimeria, Immunological, Broiler*

Etlik Piliçlerde *Pleurotus sajor-caju* Sıcak Su ve Metanolik Ekstraktının İmmunolojik ve Anti-Eimeria Etkileri

Öz

Bu çalışmada *Pleurotus (P.) sajor-caju* (yerel yetişen bir istiridye mantarı) sıcak su ve metanolik ekstraktının bağışıklık düzenleyici ve anti-*Eimeria* etkileri araştırılmıştır. Mantarlardan liyofilizasyonla sıcak su ve metanolik ekstraktı elde edildi. Liyofilize edilen ekstraktlar bağışıklık düzenleyici ve anti-*Eimeria* aktivitelerini test etmek amacıyla analiz edildi. Ticari broiler tavuklarda bu ekstraktların bağışıklık düzenleyici etkisinin değerlendirilmesi sonucunda kontrol ile karşılaştırıldığında fitohemaglutinine karşı lenfoproliferatif cevap ile karakterize hücre aracılı bağışıklığın anlamlı derecede yüksek olduğu belirlendi ($P<0.05$). Bu ekstraktlar, koyun kırmızı kan hücrelerinin primer ve sekonder kas içi enjeksiyonu sonrası 7. ve 14. günlerde total Ig, IgG ve IgM titreleri ile karakterize humoral bağışıklık cevabın da yüksek olmasına neden oldu. Tüm gruplara *Eimeria (E.)* türleri *(E. tenella, E. maxima, E. acervulina* ve *E. necatrix)* enfektif dozda oral yolla inokule edildi ve sonrasında mortalite yüzdesi, her bir gram dışkıda oosit miktarı ve barsak lezyonları incelendi. Kontrol ile karşılaştırıldığında mantar ekstraktı verilen hayvanların yer aldığı deney gruplarında her bir gram dışkıda oosit miktarı anlamlı derecede düşüktü ($P<0.05$). Kontrol grubunda mortalite oranı ve lezyon skorları ekstrakt verilen gruplarla karşılaştırıldığında daha yüksekti. Sonuç olarak, *P. sajor-caju* sıcak su ve metanolik ekstraktı etlik piliçlerde bağışıklığı artıcı ve *Eimeria* enfeksiyonuna karşı koruyucu etki gösterdi.

Anahtar sözcükler: *Mantar ekstraktı, Pleurotus sajor-caju, Eimeria, Bağışıklık, Broiler*

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INTRODUCTION

Pleurotus (P.) species are one of the 25 commonly consumed species of mushrooms worldwide and are becoming popular now a day because of excellent nutritional and medicinal properties [1]. In the world, button mushroom i.e. *Agaricus bisporous* is among the leading specie as far as cultivation is concerned, followed by shiitake *(Lentinus edodes)* and oyster *(P. ostreatus)* [2]. Commercially, mushroom production is dramatically increased in the world with mainly China and Europe contributing in its production to be increased up to almost 8 million tonnes, annually [3].

Among the oyster species, *P. sajor-caju* is gaining its due share because of its reported immunomodulatory, anti-inflammatory and anti-tumor activities [4,5]. There are several important nutrients including carbohydrates, proteins, dietary fibers, minerals and vitamins present in the mushrooms due to which they are considered as essential in the dietary composition of human beings [6,7]. For the last many years, research has been focused to explore the utility of mushroom compounds as immunomodulators in different animal models [8,9]. In this regard, several types of extracts from various mushroom species were evaluated for immune modulation in different animal models [10,11]. Moreover, various biologically active metabolites including glycoproteins, hydrolytic and oxidative compounds, phenolics and lipids had also been isolated from crude extracts of *Pleurotus* spp. to determine their biological activities [12]. These studies revealed immunomodulatory effects of these active substances through enhancing mitogenicity and activation of immune effector cells including macrophages, natural killer cells and lymphocytes. These immune cells further increase the production of cytokines including tumor necrosis factor (TNF-α), interleukins (ILs) and interferons (IFNs) [13].

In this regard, water soluble proteoglycans fractions from *P. ostreatus* had been reported for enhanced cytotoxicity of NK cells and stimulation of macrophages for increased production of nitric oxide with marked decrease in the quantity of sarcoma cells in sarcoma 180 bearing mice [14]. Similarly, methanol, dichloromethane and n-hexane extracts of *Agaricus blazei* were investigated against Ehrlich tumor bearing mice and revealed stimulated lympho-proliferative activity of splenocytes and enhanced antibody production [15]. Keeping in view the well documented pharmacological activities of *P. sajor-caju* in different disease models, this study was conducted to evaluate the immunotherapeutic activity of hot water and methanolic extracts of *P. sajor-caju* against *Eimeria* infection in chickens.

MATERIAL and METHODS

Procurement and Processing of Mushrooms

Fresh mushrooms *(P. sajor-caju)* were procured from the local grower at Millet Town, Faisalabad, Pakistan and its authenticity was confirmed by the concerned Botanist from University of Agriculture, Faisalabad, Pakistan (UAF), and a specimen (Voucher No. 175) was kept in Ethnoveterinary Research Center, Department of Parasitology, UAF.

Mushroom were dried at 50°C followed by grinding and passed through sieve (2 mm) to maintain the uniformity of the dried powder. The hot water extract was prepared by the methodology described previously [16]. In brief, the dried powder (500 g) was added in water (1500 mL) at temperature of 100°C in a water bath for 2 h. More water (500 mL) was added after each hour. The mixture obtained was centrifuged at 3000 rpm for 30 min and supernatant was collected. Supernatant thus obtained was subjected to lyophilization which yielded 85 g dried extract per 500 g of dried powder. The dried extract was stored at 4°C till further use in the experiment. The methanolic extract was prepared by the methodology described by Yang and his co-workers [17]. Briefly, dried mushroom powder (500 g) was vortexed thrice with 2000 mL of 80% methanol at room temperature for 48 h. Pooled extract obtained was subjected to rotary evaporator. Concentrated extract was lyophilized which yielded 25 gm of methanolic extract. These extracts (hot water and methanolic) were subjected to proximate analysis to determine the concentrations of crude protein, fiber, ash, ether and nitrogen free extracts [18].

Experimental Design

The experimental study was conducted at Experimental Station, Faculty of Veterinary Sciences, UAF. For the purpose, day old birds (n=150; Hubbard) purchased from local hatchery were reared under standard management conditions. Birds were given free access to fresh water and withdrawal feed. The study was approved for ethical consideration and humane handling of animals by Institutional Animal Care & Use Committee and Advance Studies & Research Board of UAF. After acclimatization for 5 days, the birds were divided into 3 groups i.e. 1, 2 and 3; each containing 50 randomly selected birds. On day 7th, 8th and 9th of the experiment, the groups 1 and 2 were administered with mushroom extracts as follows:

Group 1- Hot water extract of *P. sajor-caju* administered orally at the dose rate of 200 mg/kg body weight (BW)/day using phosphate buffered saline (PBS; 2 mL) as a solvent

Group 2- Methanolic extract of *P. sajor-caju* administered orally at the dose rate of 200 mg/kg BW/day using PBS (2 mL) as a solvent

Group 3- Control administered orally 2 mL of PBS.

On day 15th of the experiment, all three groups were subdivided into two equal groups (n=25 each) i.e. 1a, 1b; 2a, 2b and 3a, 3b, respectively. The subgroups 1a, 2a and 3a were designated to evaluate immunomodulatory effects through lymphoproliferative response to phyto-haemagglutinin-P (PHA-P), antibody response to sheep

red blood cells (RBCs) and organ (spleen, bursa of Fabricius and cecal tonsils) to body weight ratios. The subgroups including 1b, 2b and 3b were shifted to another shed and challenged with *Eimeria* infection to assess the anti-*Eimeria* efficacy of the extracts.

Immunological Evaluation

Classical toe web assay was performed to quantify cell mediated immunity. In this essay, PHA-P was injected intra-dermally in the right toe web of experimental birds. Meanwhile, PBS was also injected in the left toe web of same birds. Lymphoproliferative response was observed in terms of increase in the thickness of toe web after 24, 48 and 72 h by using the following formula:

Lymphoproliferative response = (Thickness of PHA-P injected toe web - Thickness of PBS injected toe web).

Microplate haemagglutination test was used to demonstrate humoral immune response. Sheep RBCs (5%) were injected twice as T-cell mitogen in the breast muscles of experimental birds at an interval of 14 days. Immunoglobulin (total Ig, IgG and IgM) titers were determined at day 7th and 14th after primary and secondary injections of sheep RBCs [19].

Lymphoid organs including spleen, thymus, bursa of Fabricius and cecal tonsils were collected from chickens of the experimental groups after humane slaughtering on day 42 of the experiment. All the organs were weighed to determine the per cent organ to live body weight ratios [20].

Evaluation of Anti-Eimeria Effects of Mushroom Extracts

To assess the anti-*Eimeria* effect, subgroups (1b, 2b and 3b) were challenged with infective dose (6.5×10^4-7.0×10^4) of sporulated oocysts of mixed *Eimeria (E.)* species viz. *E. tenella*, *E. maxima*, *E. acervulina* and *E. necatrix* (local isolates; maintained at Immunoparasitology Laboratory, UAF) on day 15th of experiment. These groups were monitored for oocyst per gram of droppings, lesion scoring and per cent protection from day 4th to 12th post challenge with *Eimeria* species [20-22].

Statistical Analysis

Data obtained was analyzed using completely randomized design and one-way analysis of variance (ANOVA) using SPSS ver. 16. The differences among mean values were determined using Tuckey's range test and differences were considered significant at P<0.05. For immunoglobulin titers, geometric mean titers (GMT) were calculated [23].

RESULTS

Proximate Analysis

Proximate analysis revealed 29.22% and 17.59% crude protein, 11.93% and 6.50% ash contents, 1.52% and 2.39% ether extract, 57.33% and 73.52% nitrogen free extract from hot water and methanol extracts of *P. sajor-caju*, respectively. However, no fibers were found in both extracts *(Table 1)*.

Immunological Evaluation

Lymphoproliferative Response to PHA-P: Cell mediated immune response was evaluated through Toe web essay. Thickness observed in the toe web of birds belonging to experimental groups treated with hot water and methanol extracts showed significantly higher values (P<0.05) at 24, 48 and 72 h post PHA-P injection as compared to control. The results revealed that both extracts showed excellent T-cell mitogen activity *(Fig. 1)*.

Humoral Immune Response Against Sheep RBCs: Total immunoglobulins, IgG and IgM titers observed in the hot water and methanol extracts at days 7th and 14th post-primary and post-secondary injections of sheep RBCs were significantly higher as compared to control. However, the titers (total Ig, IgG and IgM) observed in the methanolic extract treated groups were highest followed by those of hot water extract *(Table 2)*.

Lymphoid Organs to Body Weight Ratios: Birds belonging to experimental and control groups were weighed and

Table 1. *Results of proximate analysis of hot water and methanolic extract*

Extract Type	Hot Water Extract (%)	Methanol Extract (%)
Crude protein	29.22	17.59
Crude fiber	-	-
Ash	11.93	6.50
Ether extract	1.52	2.39
Nitrogen free extract	57.33	73.52

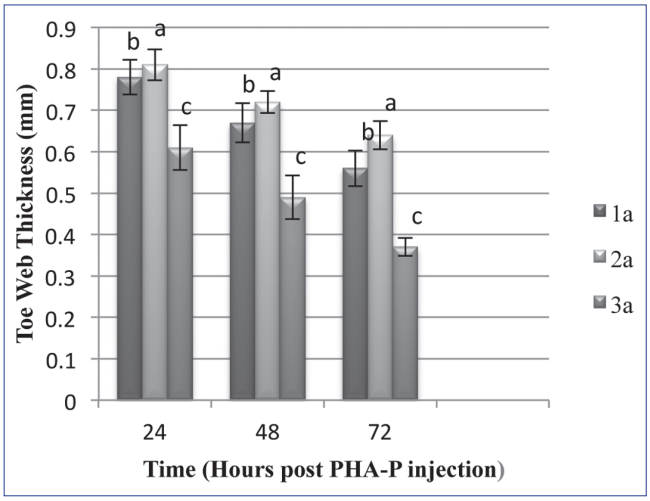

Fig 1. Lymphoproliferative response to Phytohaemagglutinin-P in mushroom extracts administered and control groups. Bars sharing different letters present a significant difference (P<0.05). Group 1a: Hot water extract of *Pleurotus sajor-caju,* Group 2a: Methanol extract of *Pleurotus sajor-caju,* Group 3a: Control

Table 2. *Anti sheep-RBCs antibody titers (Total Ig, IgG, IgM) after day 7 and day 14 post-primary and post-secondary injection of Sheep RBCs*

Total Immunoglobuln	7ppi	14ppi	7psi	14psi
Group 1a PSC/HWE	48.53	42.27	55.72	36.73
Group 2a PSC/ME	63.97	48.53	73.45	55.72
Group 3a Control	31.99	27.86	36.73	27.86
IgM				
Group 1a PSC/HWE	32.53	18.00	34.58	8.87
Group 2a PSC/ME	42.84	27.39	45.59	13.45
Group 3a Control	22.18	11.16	20.73	9.49
IgG				
Group 1a PSC/HWE	16.00	24.27	21.13	27.86
Group 2a PSC/ME	21.13	21.13	27.86	42.27
Group 3a Control	9.18	16.00	16.00	18.37

PSC/HWE - Pleurotus sajor-caju/Hot Water extract, PSC/ME = Pleurotus sajor-caju/Methanolic extract, 7ppi = day 7 post-primary injection of sheep RBCs, 14ppi = day 14 post-primary injection of sheep RBCs, 7psi = day 7 post-secondary injection of sheep RBCs, 14psi = day 14 post-secondary injection of sheep RBCs

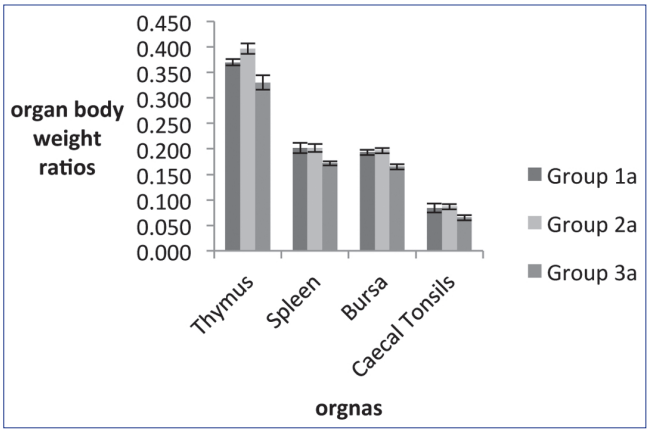

Fig 2. Organ to body weight ratios of experimental and control groups. Group 1a: Hot water extract of *Pleurotus sajor-caju*, Group 2a: Methanol extract of *Pleurotus sajor-caju*, Group 3a: Control

sacrificed to obtain lymphoid organs including thymus, spleen, bursa of Fabricius and cecal tonsils. Organ to body weight ratios of these organs were statistically non-significant (P>0.05) *(Fig. 2)*.

Evaluation of Anti-Eimeria Effects of P. sajor-caju Extracts

Post-challenge with *Eimeria* species, the birds in experimental groups administered with mushroom extracts and control group were monitored for OPG, percent protection and lesion scoring. In control group, significantly higher (P<0.05) OPG of droppings were observed as compared to *P. sajor-caju* extracts treated experimental groups. Among the mushroom administered groups, minimum OPG was observed in the methanolic extract followed by hot water extract *(Fig. 3)*.

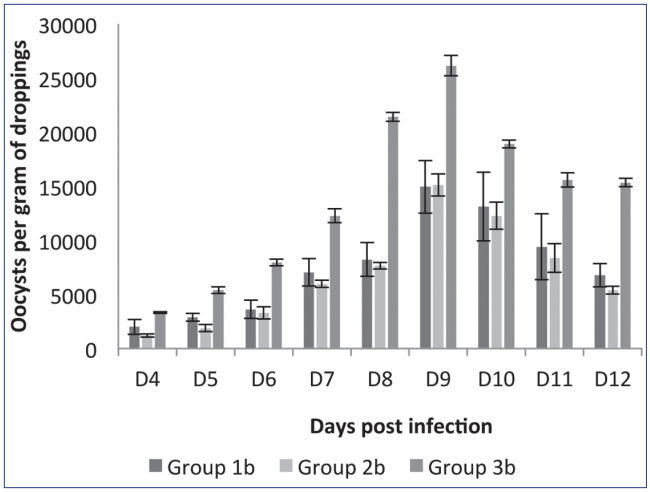

Fig 3. Oocysts per gram of droppings from day 4 to day 12 post challenge with *Eimeria* species. Group 1b: Hot water extract of *Pleurotus sajor-caju*, Group 2b: Methanolic extract of *Pleurotus sajor-caju*, Group 3b: Control

Table 3. *Lesion Scoring observed in intestines of experimental and control birds*

Groups	Lesion Scoring of the Birds				
	0	1	2	3	4
Group 1b	4	7	5	4	5
Group 2b	3	6	5	4	7
Group 3b	1	2	4	6	12

Group 1b: Birds given Hot water extract of Pleurotus sajor-caju, Group 2b: Birds given Methanol extract of Pleurotus sajor-caju, Group 3b: Control

Highest protection (45%) was observed in group treated with methanolic extract followed by hot water extract treated group (31%) and control group (24%). Birds of all the experimental groups designated for anti-*Eimeria* activities were evaluated for intestinal lesion scorings on a scale of 0 to 4. Results showed that severe lesion scores (3-4) were lesser in birds of groups administered with hot water (09) and methanolic extracts (11) as compared to those of control group (18) *(Table 3)*. It indicated the protective efficacy of *Pleurotus* extracts against adverse effects of *Eimeria* infection on the intestine of the affected birds.

DISCUSSION

Oysters *(Pleurotus)* are among the popular edible mushrooms consumed by human being worldwide. This group of mushroom is cosmopolitan for their nutritional and therapeutic properties. Several studies in the past revealed medicinal potentialities of different species of oyster mushrooms and classified them as "mushroom nutraceuticals" [14,24]. Keeping in view the medicinal properties of *Pleurotus* species, current study was designed to evaluate immuno-therapeutic activities of hot water and methanolic extracts of *P. sajor-caju* against coccidiosis in broiler.

In a recent study, proximate analysis of 10 mushrooms (*Cantharellus cibarius*, *Rusula delica var chloroides*, *Ramaria largentii*, *Hygrophorus russula*, *Amanita caesarea*, *Fistulina hepatica*, *Boletus aureus*, *Armillaria tabesceus*, *Armillaria mellea* and *Lepista nuda*) were evaluated to determine the concentration of carbohydrates (55.33-66.87), fats (2.10-6.00), proteins (21.57-34.77), and ash (5.61-9.44) [25]. In another study, nutritional composition of different *Pleurotus* species including *P. pulmonaris*, *P. floridanus*, *P. cystidiosus* and *P. sajor-caju* was demonstrated on dry weight basis. Carbohydrates, proteins, crude fat, crude fibers and ash were found in the range of 85.86-88.38%, 0.98-2.17%, 0.62-0.84%, 2.76-3.12% and 1.03-2.20%, respectively [12]. Results found in the current study have slight variations with the above-mentioned studies. It may be due to different ways and times of harvesting, different storage conditions, species differences, difference of compost used during mushroom growth, different conditions of management and other parameters including temperature, relative humidity, water availability and geographical distribution [26-28].

Mushroom extracts in the current study revealed remarkable cellular immune response in terms of increased thickness of toe web in birds treated with hot water and methanolic extracts of *P. sajor-caju*. These immunomodulatory activities may be due to activation of NK cells, higher production of interferon, enhanced complement activation, potentiation of phagocytic activities and prevention of leukocyte reduction [29,30]. In another study, after administration of *Lentinus edodes'* polysaccharides, chickens challenged and infected with Marek's disease showed significant increase in T-cell proliferation and interleukin production [31,32]. Studies in other animals including rats showed almost similar results as after administration of different mushrooms resulted in the enhancement of T-cell mediated immune responses including lymphocyte proliferation, increasing spleen thymus indexes and corrections of immunosuppression after administration of immune inhibitors such as cyclophosphamide and deltacortisone [33,34]. Moreover, mushroom extracts obtained from different mushrooms also enhanced the production of interleukin 1 and 8 [35]. Likewise, polysaccharide isolated from *Lentinus edodes* enhanced cell-mediated immune response through delayed type of hypersensitivity reaction shown through enhancement in proliferation of splenocytes and increased production of TNF-α and IFN-γ [36]. However, the actual mechanism involved in the enhancement of cellular immune response is still obscure.

Enhanced immunoglobulin production in chicken mucosa had been reported in the past after administration of different mushrooms including *Lentinus edodes*, *Tremella fuciformis* and *Fomitella fuciformis* [33,37]. This enhanced production may be due to proliferation and activation of immune effector cells including macrophages, T-helper, natural killer cells and dendritic cells. Enhanced activation and proliferation of these cells increase the production of certain cytokines such as IL-12 and IFN-γ after administration of different extracts of *Agaricus brazelliance* and *Antrodia camphorate*. These cytokines may be responsible for activation of effector phase of innate and adaptive immunity [38,39].

After challenge infection of sporulated oocysts of *Eimeria*, oocysts per gram of droppings, mortality percentage and lesion scores in experimental groups were observed. In this regard, limited research has been conducted on the effects of mushrooms particularly against *Eimeria* infection in chicken [40-42]. Birds administered with hot water and methanolic extracts of *P. sajor-caju*, showed excellent protection against coccidiosis in chicken. This might be due to polysaccharides present in the crude extracts [33-35]. Similar findings had also been reported in some previous studies [43,44]. In conclusion, results of this study revealed the immunopotentiating and anti-*Eimeria* efficacy of hot water and methanolic extracts of *Pleurotus sajor-caju*. Further studies to rule out the specific mechanism of action of these extracts for such activities and their commercial feasibility are underway in our lab.

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