Dietary phytoimmunostimulant Persian hogweed (*Heracleum persicum*) has more remarkable impacts on skin mucus than on serum in common carp (*Cyprinus carpio*)

Hoseinifar, Seyed Hossein ; Zoheiri, Fazel; Lazado, Carlo Cabacang

Published in:
Fish and Shellfish Immunology

Link to article, DOI:
[10.1016/j.fsi.2016.10.025](https://doi.org/10.1016/j.fsi.2016.10.025)

Publication date:
2016

Document Version
Version created as part of publication process; publisher's layout; not normally made publicly available

[Link back to DTU Orbit](#)

Citation (APA):
Hoseinifar, S. H., Zoheiri, F., & Lazado, C. C. (2016). Dietary phytoimmunostimulant Persian hogweed (*Heracleum persicum*) has more remarkable impacts on skin mucus than on serum in common carp (*Cyprinus carpio*). *Fish and Shellfish Immunology*, 59, 77-82. <https://doi.org/10.1016/j.fsi.2016.10.025>

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Accepted Manuscript

Dietary phytoimmunostimulant Persian hogweed (*Heracleum persicum*) has more remarkable impacts on skin mucus than on serum in common carp (*Cyprinus carpio*)

Seyed Hossein Hoseinifar, Fazel Zoheiri, Carlo C. Lazado

PII: S1050-4648(16)30657-X

DOI: [10.1016/j.fsi.2016.10.025](https://doi.org/10.1016/j.fsi.2016.10.025)

Reference: YFSIM 4253

To appear in: *Fish and Shellfish Immunology*

Received Date: 20 July 2016

Revised Date: 7 October 2016

Accepted Date: 13 October 2016

Please cite this article as: Hoseinifar SH, Zoheiri F, Lazado CC, Dietary phytoimmunostimulant Persian hogweed (*Heracleum persicum*) has more remarkable impacts on skin mucus than on serum in common carp (*Cyprinus carpio*), *Fish and Shellfish Immunology* (2016), doi: 10.1016/j.fsi.2016.10.025.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Full length article

Dietary phytoimmunostimulant Persian hogweed (*Heracleum persicum*) has more remarkable impacts on skin mucus than on serum in common carp (*Cyprinus carpio*)

Seyed Hossein Hoseinifar ^a, Fazel Zoheiri ^a, Carlo C. Lazado ^{b*}

^a Department of Fisheries, Faculty of Fisheries and Environmental Sciences, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran

^b Technical University of Denmark, DTU Aqua, Section for Aquaculture, The North Sea Research Centre, DK-9850 Hirtshals, Denmark

*Corresponding author:

Carlo C. Lazado

Technical University of Denmark

The North Sea Research Centre

9850 Hirtshals, Denmark

Tel: + 45 35 88 32 00

Fax: + 45 35 88 32 60

E-mail: carlolazado@yahoo.com

Abstract

Immunostimulation through the use of sustainable and eco-friendly dietary additives is one of the current prophylactic strategies in fish husbandry. Plant-based immunostimulants are highly considered for this intent, both for their scientific and practical advantages. Persian hogweed (*Heracleum persicum*) is a flower-bearing herb that possesses interesting pharmacological importance due to its bioactive compounds. It is commonly used as a spice, food additive, dietary supplement and traditional remedy. The present study evaluated the potential of *H. persicum* as a dietary phytoimmunostimulant in common carp (*Cyprinus carpio*). The powder form of *H. persicum* was incorporated in the basal diet at three different inclusion levels: 2.5, 5 and 10 g Kg⁻¹. The basal diet (0 g Kg⁻¹ of *H. persicum*) served as control. Experimental diets were administered to the fish for a period of 8 weeks. At the termination of the feeding experiment, impacts on fish immunity and performance were evaluated.

Inclusion of *H. persicum* in the diet significantly elevated several immunological factors such as immunoglobulins, lysozyme, protease and alternative complement activities in carp. Interestingly, the changes were more pronounced in the skin mucus than in the serum. Performance was significantly improved in the fish groups that received the candidate phytoimmunostimulant. Specifically, final weight, weight gain, specific growth rate and feed conversion ratio were significantly improved in the fish that received dietary *H. persicum* at inclusion levels 5 g Kg⁻¹ and higher. This study demonstrated the potential of Persian hogweed as a candidate dietary phytoimmunostimulant in carp, impacting

mainly the skin mucosal defenses. The study supports the current trend in the exploration of sustainable plant-based dietary supplements that are capable of boosting the immunological defenses of farmed fish.

Keywords: aquaculture; fish health; immunostimulant; mucosal immunity; Persian hogweed

1. Introduction

Immunomodulation is one of the major prophylactic strategies being advanced in aquaculture [1]. The approach lies on the fundamental concept that modulating the immunological defenses of fish potentiates the capability of the organism to respond more effectively in an event of danger (*i.e.* pathogen, stress insult), thereby conferring them resistance and protection [2]. Dietary supplements, particularly those with immunomodulatory properties, have been explored for this purpose.

An immunostimulant is a naturally occurring substance that has a modulatory effect upon the immune system particularly by increasing the resistance of the host against diseases that in most cases are caused by pathogens [2, 3]. In the last decade, several substances have been identified as potential immunostimulants in fish. However, many candidate immunostimulants cannot be used due to high production cost and limited effectiveness [4]. The interest has been diverted to the use of plant-based products, in which many have been customarily used in traditional human medicine. Phytoimmunostimulants have little side effects, are easily degradable, and are abundantly available, making them as cheap and sustainable alternatives to conventional microbial-based immunostimulants [5].

Heracleum persicum, commonly known as Golpar or Persian hogweed, is a plant belonging to the Apiaceae family and is considered native to Iran [6, 7]. This flower-bearing herb grows about 150 to 200 cm high with bristly haired stems of up to 50 cm in thickness. They are widely distributed in Iran, but grow best in moist and nutritious areas

70 especially in northern mountainous regions with an altitude ranging from 1500-2500 m.
71 Traditionally, they are being used as a spice, food additive and supplement. They are also
72 widely known by the locals as a carminative agent. Pharmacological studies indicate that
73 *H. persicum* contains a number of bioactive compounds, many of which have been
74 previously suggested possessing antioxidant, anticonvulsant, analgesic, anti-inflammatory
75 and immunomodulatory properties [7, 8].

76 Cyprinid species are one of the major farmed fish species on a worldwide scale
77 (<http://www.fao.org>), and among these, the common carp (*Cyprinus carpio*) is the third
78 most important [9]. Like any other aquaculture species, carp farming is faced with
79 incessant threats, especially from infectious pathogens. Measures that can improve their
80 disease resistance are to be adopted to support its sustainable production. Extracts from
81 *H. persicum* have been earlier described possessing inhibitory activity against several fish
82 pathogens [4], making this herb a prospective dietary supplement to improve the health
83 of cultured fish. However, their immunomodulatory functions are yet to be investigated in
84 fish. Hence, this study characterized the effects of dietary *H. persicum* on immunity and
85 performance of rainbow trout. The impacts on both systemic and cutaneous mucosal
86 immunity were investigated following dietary administration of this candidate
87 phytoimmunostimulant.

2. Materials & methods

2.1. Preparation of plant powder and experimental diets

The whole part of Persian hogweed (*Heracleum persicum*) was collected from Qazvin (Qazvin province, Iran) during their flowering period (July–September). Botanists from the Botany Department at Golestan University (Golestan, Iran) were requested to properly identify the collected plants. The whole plant was air-dried at room temperature and powdered as previously described [4]. The plant powder was incorporated in the basal diet (Table 1) at three different inclusion levels: 2.5, 5 and 10 g kg⁻¹. The basal diet (*i.e.* 0 g kg⁻¹ of *H. persicum* powder) served as the control feed. The experimental diets were prepared as detailed elsewhere [10, 11]. The dried pellets were stored in plastic bags at 4 °C until delivery.

2.2. Fish and experimental conditions

The feeding experiment was performed at the Aquaculture Laboratory of Department of Fisheries, Gorgan University of Agricultural Sciences and Natural Resources (Gorgan, Iran). Common carp (*Cyprinus carpio*) were procured from a private fish farm (Mazandaran Province, Iran). The fish stock was free of disease outbreak history. Upon their arrival, they were acclimated to the laboratory conditions for 2 weeks. The following physico-chemical parameters were monitored and maintained: water temperature (26 ± 1.0 °C), dissolved oxygen (7.2 ± 0.01 mg L⁻¹), pH (7.6 ± 0.2) and natural photoperiod (12L:12D). Fish with an average weight of 23.5 ± 0.2 g were randomly stocked into a 150-L

tank at a density of 30 fish per tank. Twelve tanks were used in total, with three replicate tanks allocated to each of the experimental diet group.

The experimental diets were delivered manually to apparent satiation twice a day (09:00 and 15:00) over an 8-week period. All fish handling procedures employed in the study were in accordance with the internationally accepted principles on animal experimentation.

Table 1. Dietary formulation and proximate composition of the basal diet (%)

Ingredients	
Fish meal	40.0
Wheat flour	21.0
Soybean meal	13.5
Gluten	5.5
Soybean oil	6.0
Fish oil	6.0
Mineral premix [*]	3.0
Vitamin premix [*]	2.0
Binder [†]	2.0
Anti-fungal [‡]	0.5
Antioxidant [§]	0.5
<i>Proximate composition (% dry matter basis)</i>	
Dry matter	91.12
Crude protein	36.81
Crude lipid	11.33
Ash	3.50
Fiber	11.58

^{*} Details of the premix [10].

[†] Amet binder™, Mehr Taban-e- Yazd, Iran

[‡] ToxiBan antifungal (Vet-A-Mix, Shenandoah, IA)

[§] Butylated hydroxytoluene (BHT) (Merck, Germany)

2.3. Immunological assays

2.3.1. Collection and preparation of skin mucus and serum

Fish were not fed 24 h prior to sample collection. Three fish (*i.e.* 9 fish per experimental feed group) were taken from each tank and were anaesthetized using clove powder (500 mg L⁻¹) dissolved in water. Thereafter, the anaesthetized fish was transferred into a polyethylene bag containing 10 mL of 50 mM NaCl (Sigma, Steinheim, Germany). Skin mucus was collected by gently rubbing the fish inside the plastic in a downward motion for 1–2 min as described earlier [12, 13]. The collected mucus sample was immediately transferred to a sterile 15 mL tubes and centrifuged at 1500 *g* at 4 °C for 10 min (5810R Eppendorf, Engelsdorf, Germany). The supernatant was collected and kept at -80 °C until analysis.

Blood was drawn from the caudal vein and was allowed to clot for 12 h at 4 °C. Thereafter, serum was collected by centrifuging the clotted blood at 5000 *g* at 4 °C for 5 min (International Model CL, International Equipment Co., Needham, MA, USA). The serum samples were stored at -80 °C until further analysis.

2.3.2. Immunological factors in skin mucus

The total Ig content in skin mucus was determined following a method described by Siwiki and Anderson [14]. Briefly, the total protein level of the samples were measured by Bradford protein assay [15]. Immunoglobulin molecules were precipitated down by a 12 % polyethylene glycol solution (Sigma) and the protein content of samples

were measured again. The difference in protein content was considered as the total Ig content of skin mucus.

Lysozyme activity in skin mucus was measured by a turbidimetric assay [16]. Briefly, 50 μL suspension of Gram-positive bacterium *Micrococcus luteus* (Sigma) (0.3 mg mL^{-1} of lyophilized cells dissolved in 40 mM sodium phosphate buffer, pH 6.5) was mixed with 50 μL of the mucus sample. The reaction mixture was incubated at 30 $^{\circ}\text{C}$ and the reduction in absorbance at 450 nm was measured after 0 and 15 min in a microplate reader. A unit of lysozyme activity was defined as the amount of enzyme that caused a decrease in absorbance of 0.001 per minute.

Protease activity in skin mucus was measured by the azocasein hydrolysis method described earlier [12], with minor modifications. Briefly, 100 μL of skin mucus was mixed with 100 μL 0.7 % azocasein solution (Sigma) and thereafter incubated at 30 $^{\circ}\text{C}$ with constant agitation for 19 h. Then, 4.5 % trichloroacetic acid was added to stop the reaction and the supernatant was obtained by centrifuging the reaction mixture at 15 000 g for 5 min. The resulting supernatants were pipetted to a 96-well flat bottom plate that was earlier seeded with 100 μL 1 N sodium hydroxide (NaOH) per well. The optical density (OD) was measured at 450 nm. Protease activity was expressed relative to a positive control (mucus replaced with trypsin solution of 5 mg mL^{-1} ; 100% protease activity).

2.3.3. Immunological factors in serum

Serum total Ig and lysozyme activity were determined similarly as described above for skin mucus samples.

Serum alternative complement pathway hemolytic activity (ACH50) was determined following a method described previously using the New Zealand rabbit red blood cells (RaRBC) [17]. The volume of serum complement yielding 50 % haemolysis (ACH50) was measured and used to calculate the complement activity.

2.4. Fish performance

Fish performance was evaluated by determining weight gain, specific growth rate (SGR), feed conversion ratio (FCR) and percentage survival (%) after the 8-week feeding period.

- Weight gain (g) = W_2 (g) – W_1 (g)
- Specific growth rate (SGR) = $100 (\ln W_2 - \ln W_1) / T$
- Feed conversion ratio (FCR) = feed intake (g) / weight gain (g)
- Survival rate = $(N_f / N_i) \times 100$

Where W_1 is the initial weight, W_2 is the final weight, T is the number of days in the feeding period, N_i is initial number of fish and N_f is final number of fish.

2.5. Statistical analysis

The homogeneity of variance and normality of the data were confirmed by Leaven and Kolmogorov-Smirnov tests. Then, data were subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests to determine significant difference at $P < 0.05$. SPSS statistical package version 16.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

3. Results

3.1. Skin mucus immune responses

Dietary supplementation of *H. persicum* significantly impacted the skin mucosal immune responses of common carp (Figs. 1-3). Skin mucus total Ig increased significantly in the fish fed with diets containing 5 and 10 g Kg⁻¹ of *H. persicum*, but not at inclusion level of 2.5 g Kg⁻¹. The increase in the skin mucus Ig level in the two experimental groups was at least 30 % relative to the control group (Fig. 1).

Inclusion of the candidate phytoimmunostimulant also affected the activity of skin mucus lysozyme (Fig. 2). A significant increase of at least 60 % compared with control was documented in the group fed with 5 g Kg⁻¹ of *H. persicum*. However, this significant elevation of skin mucus lysozyme was not observed in other experimental feed groups.

Proteolytic activity in skin mucus was significantly influenced following feeding with diets enriched with powder *H. persicum* (Fig. 3). A remarkable significant increase in skin mucus protease activity was observed in all treatment groups, where the highest

202 increment was noted at inclusion level of 5 g Kg⁻¹. The percentage increase ranged from
 203 75 – 200 % relative to control.

204 **Figure 1.** Total immunoglobulin levels in the skin mucus of common carp (*Cyprinus carpio*)
 205 fed with diets supplemented with Persian hogweed (*H. persicum*) at different inclusion
 206 levels for 8 weeks. Statistical differences ($P < 0.05$) between experimental groups are
 207 indicated with different letter notations. The values are mean $\pm$ S.E of 9 individual fish.
 208
 209

210 **Figure 2.** Lysozyme activity in the skin mucus of common carp (*Cyprinus carpio*) fed with
 211 diets supplemented with Persian hogweed (*H. persicum*) at different inclusion levels for 8
 212 weeks. Statistical differences ($P < 0.05$) between experimental groups are indicated with
 213 different letter notations. The values are mean $\pm$ S.E of 9 individual fish.
 214
 215

Figure 3. Protease activity in the skin mucus of common carp (*Cyprinus carpio*) fed with diets supplemented with Persian hogweed (*H. persicum*) at different inclusion levels for 8 weeks. Statistical differences ($P < 0.05$) between experimental groups are indicated with different letter notations. The values are mean $\pm$ S.E of 9 individual fish.

3.2. Serum humoral immune responses

Immunological factors in the serum were also influenced by dietary supplementation of *H. persicum*, however the changes were not as pronounced compared with the observations in skin mucus (Figs 4-6). Serum total Ig level was significantly elevated in fish fed with 10 g Kg⁻¹ of the candidate phytoimmunostimulant (Fig. 4). The increment was noted to be at least 20 % compared with control. There were no significant changes in the groups fed with 2.5 and 5 g Kg⁻¹, respectively.

The activity level of serum lysozyme was unaltered following feeding with *H. persicum*, regardless of the inclusion level (Fig. 5).

Serum alternative complement pathway hemolytic activity (ACH50) was minimally influenced by the dietary manipulation (Fig. 6). A significant change was only

233 observed in the group that received a diet enriched with the candidate
 234 phytoimmunostimulant at a level of 10 g Kg⁻¹ compared with control. ACH50 activity in
 235 this group was elevated by around 8 % relative to control. In addition, the level of ACH50
 236 in the three groups that received *H. persicum*-enriched diets was not significantly different
 237 from one another.

238
 239
 240 **Figure 4.** Total serum immunoglobulin levels of common carp (*Cyprinus carpio*) fed with
 241 diets supplemented with Persian hogweed (*H. persicum*) at different inclusion levels for 8
 242 weeks. Statistical differences ($P < 0.05$) between experimental groups are indicated with
 243 different letter notations. The values are mean $\pm$ S.E of 9 individual fish.

Figure 5. Serum lysozyme activity in common carp (*Cyprinus carpio*) fed with diets supplemented with Persian hogweed (*H. persicum*) at different inclusion levels for 8 weeks. Statistical differences ($P < 0.05$) between experimental groups are indicated with different letter notations. The values are mean $\pm$ S.E of 9 individual fish.

Figure 6. Serum alternative complement pathway haemolytic activity (ACH50) fed with diets supplemented with Persian hogweed (*H. persicum*) at different inclusion levels for 8 weeks. Statistical differences ($P < 0.05$) between experimental groups are indicated with different letter notations. The values are mean $\pm$ S.E of 9 individual fish.

3.3. Fish performance

Fish that received *H.persicum*-supplemented diets at levels of 5 and 10 g kg⁻¹, respectively, had significantly higher final weight compared with the control group. Weight gain in these groups was at least 18 % higher than the group receiving the basal diet (Table 2). Performance indicators such as SGR and FCR were significantly altered as well in groups receiving diets enriched with at least 5 g Kg⁻¹ of *H. persicum*. SGR in fish fed with 5 and 10 g Kg⁻¹ of the candidate phytoimmunostimulant was at least 12.3 % higher than the unsupplemented group. On the other hand, the FCR in these two groups were at least 18 % better than the control. All groups registered 100 % survival rate after an 8-week feeding period.

Table 2. Changes in the performance indicators of common carp (*Cyprinus carpio*) following feeding with diets supplemented with *H. persicum* powder at different inclusion levels. Values are presented as mean $\pm$ SE.

	Persian hogweed (g Kg ⁻¹)			
	0	2.5	5	10
Final weight (g)	50.24 $\pm$ 3.17 ^a	53.23 $\pm$ 1.02 ^{ab}	55.41 $\pm$ 1.06 ^b	55.93 $\pm$ 2.01 ^b
Weight gain (g)	26.86 $\pm$ 3.31 ^a	29.73 $\pm$ 1.28 ^{ab}	31.83 $\pm$ 1.07 ^b	32.55 $\pm$ 1.90 ^b
SGR	1.55 $\pm$ 0.13 ^a	1.66 $\pm$ 0.06 ^{ab}	1.74 $\pm$ 0.04 ^b	1.78 $\pm$ 0.06 ^b
FCR	2.37 $\pm$ 0.11 ^a	2.15 $\pm$ 0.10 ^{ab}	1.96 $\pm$ 0.05 ^b	1.90 $\pm$ 0.08 ^b
Survival rate (%)	100	100	100	100

Values in a row with different letter denote significant difference ($P < 0.05$)

4. Discussion

The present study demonstrated the potential of Persian hogweed (*H. persicum*) as a dietary phytoimmunostimulant in common carp (*C. carpio*). Immune responses were remarkably modulated in fish that received diets supplemented with *H. persicum* powder. In particular, both the serum humoral and skin mucosal responses were altered in the fish fed with the dietary supplement, where the latter exhibited more marked changes.

Fish have interestingly complex defense system, with the integument providing the first line of defense [18]. In particular, the skin mucus is a critical component of the integumentary system and is characterized by a collection of potent defense molecules [13, 19]. The present study demonstrated that the dietary administration of *H. persicum* could modify some key defense molecules in the skin mucus of carp. Secretory immunoglobulins play important roles in the maintenance of mucosal homeostasis [20], and the candidate dietary phytoimmunostimulant could potentiate their level in the skin mucus of carp by at least 30 % especially at inclusion levels 5 g kg⁻¹ and higher when compared with control group. Lysozyme (also known as N-acetylmuramide glucanohydrolase or muramidase) is a ubiquitous bactericidal enzyme and by far the most studied defense molecule in fish skin mucus [20]. It was observed that the effect of dietary *H. persicum* in the skin mucus lysozyme activity demonstrated a ceiling effect-like tendency. Significantly elevated level of skin mucus lysozyme was only noted in the group fed with 5 g Kg⁻¹ of *H. persicum*; no significant change was noted in concentration higher

than this. Skin mucus proteases play a protective function against pathogenic invasion either by direct action through cleaving their proteins or indirectly by hampering their colonization and invasion mechanisms [21, 22]. Regardless of the inclusion level, dietary *H. persicum* significantly elevated the activity of skin mucus proteases where the highest increment was observed at inclusion level of 5 g Kg⁻¹. This noteworthy effect in mucosal proteolytic activity following dietary *H. persicum* supplementation could be considered beneficial for the protective function of the skin mucosa. Further, this is an interestingly remarkable effect because besides the protective role of proteases mentioned above, they have been shown as well influencing the production of other innate immune components present in fish mucus such as complement, immunoglobulins or antibacterial peptides [23-25], hereby adding to the immunomodulatory potential of this candidate phytoimmunostimulant.

Aside from the immune molecules in the skin mucus that provide the first line of defense, soluble mediators of immunity are constantly present as well in fish circulatory system as serum factors. It is interesting to highlight that the effect of dietary *H. persicum* in serum was not as striking compared with the changes observed in the skin mucus. Generally, significant changes were only observed at the highest inclusion level. From the three immune parameters studied in the serum, total immunoglobulins and alternative haemolytic complement activity were distinctly enhanced in the abovementioned inclusion level. While immunoglobulins identify and neutralize pathogens, the alternative complement system is composed of soluble plasma proteins, which is antibody

independent, that play key roles in the opsonization and killing of pathogens [26]. It could be speculated that the potentiation of the recognition and presentation capability for foreign antigenic factors was the main immunomodulatory impact of dietary *H. persicum* in the serum. This deduction could be fully substantiated with more mechanistic approaches in future studies. Further, the minimal changes in serum immune parameters posit a possibility that the immunostimulatory functions of dietary *H. persicum* were higher towards the mucosal rather than the systemic arms of the host immunity.

We could implicate the bioactive compounds that have been previously characterized from this plant as major contributors in the observed immunomodulatory activities of dietary *H. persicum*. Furanocoumarins such as pimpinellin, isopimpinellin, bergapten, isobergapten and sphondin are some of the bioactive compounds being attributed to the immunomodulatory activity attributed to *H. persicum* [7, 8]. In addition, major constituents of its essential oils including hexyl butyrate, octyl acetate, hexyl 2-methylbutanoate and hexyl isobutyrate, have been documented with anti-inflammatory and analgesic properties [27]. It is likely that these compounds that have been previously characterized and tested in mammalian models may also be the same compounds endowing the immunostimulatory features observed in the current study. To explore the definitive underlying mechanism of these beneficial actions, it is essential to extract and characterize the bioactive components of *H. persicum* in future studies. On the other hand, it is a practical advantage that even the crude powder extract of the plant could elicit significant immunomodulation in carp. It is highly favourable in aquaculture that *H.*

persicum could be used as a phytoimmunostimulant without undergoing tedious and expensive extraction and purification procedures which eventually present significant production cost making its use impractical.

Besides their key functions as modulators of immunity, immunostimulants are also considered as growth promoters. In the present study, fish fed with diet supplemented with *H. persicum* powder demonstrated enhanced performance as indicated by higher final weight and weight gain, as well as improved SGR and FCR values. Observed changes indicate that in order to elicit significant improvement in growth performance, the candidate phytoimmunostimulant must be included in the basal diet of carp at least 5 g Kg⁻¹ or higher. The growth promoting function of plant-based immunostimulant had been previously shown in a carp study, where traditional Chinese medicinal plants increased body weight of fish by at least 16 % [28]. It could be possible that the growth-promoting property of the phytoimmunostimulant may be through stimulation of appetite, modulation of the digestive enzymatic physiology, supply of beneficial vitamins and minerals or by providing substrate for microbial activity in the gut. These mechanisms have been proposed in several earlier studies in fish [5, 29, 30].

In conclusion, the present study revealed the immuno-nutritional benefits from dietary Persian hogweed (*H. persicum*), hence, designating the herb as an interesting candidate phytoimmunostimulant to improve the health status of carp. Remarkably, the inclusion of the crude powder form of the plant in the diet stimulated both the skin mucosal and serum humoral defense molecules. The extent of stimulation was more

prominent in the soluble factors in the skin mucus than in the serum. The results support the exploration of traditional medicinal plants as effective, sustainable and relatively cheap dietary supplements capable of enhancing the immunological robustness of farmed fish.

Acknowledgements

The author would like to thank the research affairs of Gorgan University of Agricultural Sciences and Natural Resources (GUASNR) for financing this research. The technical assistance of the staff at the Aquaculture Laboratory of GUASNR is also appreciated.

References

1. Magnadottir B. Immunological control of fish diseases. *Marine Biotechnology*. 2010 12:361-79.
2. Caipang CMA, Lazado CC. 9 - Nutritional impacts on fish mucosa: immunostimulants, pre- and probiotics In: Peatman BH, Beck E, editors. *Mucosal Health in Aquaculture*. San Diego: Academic Press; 2015, p. 211-72.
3. Bricknell I, Dalmo RA. The use of immunostimulants in fish larval aquaculture. *Fish and Shellfish Immunology*. 2005 19:457-72.
4. Kousha A, Ringø E. Antibacterial effect of aquatic extract of *Heracleum* Spp. hogweed plants from Europe on thirteen different bacteria. *Pharmaceutical Chemistry Journal*. 2015 48:675-8.
5. Van Hai N. The use of medicinal plants as immunostimulants in aquaculture: A review. *Aquaculture*. 2015 446:88-96.
6. Asgarpanah J, Mehrabani GD, Ahmadi M, Ranjbar R, Ardebily MSA. Chemistry, pharmacology and medicinal properties of *Heracleum persicum* Desf. Ex Fischer: A review. *Journal of Medicinal Plants Research*. 2012 6:1813-20.

- 386 7. Hemati A, Azarnia M, Angaji SA. Medicinal effects of *Heracleum persicum*
387 (Golpar) Middle-East Journal of Scientific Research. 2010 5:174-6.
- 388 8. Souri E, Farsam H, Sarkheil P, Ebadi F. Antioxidant activity of some
389 furanocoumarins isolated from *Heracleum persicum* Pharmaceutical Biology. 2004 42:396-
390 9.
- 391 9. Ødegård J, Olesen I, Dixon P, Jeney Z, Nielsen H-M, Way K, et al. Genetic
392 analysis of common carp (*Cyprinus carpio*) strains. II: Resistance to koi herpesvirus and
393 *Aeromonas hydrophila* and their relationship with pond survival. Aquaculture. 2010 304:7-
394 13.
- 395 10. Hoseinifar SH, Soleimani N, Ringø E. Effects of dietary fructo-
396 oligosaccharide supplementation on the growth performance, haemato-immunological
397 parameters, gut microbiota and stress resistance of common carp (*Cyprinus carpio*) fry.
398 British Journal of Nutrition. 2014 112:1296-302.
- 399 11. Hoseinifar SH, Khalili M, Rufchaei R, Raeisi M, Attar M, Cordero H, et al.
400 Effects of date palm fruit extracts on skin mucosal immunity, immune related genes
401 expression and growth performance of common carp (*Cyprinus carpio*) fry. Fish & Shellfish
402 Immunology. 2015 47:706-11.
- 403 12. Ross NW, Firth KJF, Wang A, Burka JF, Johnson SC. Changes in hydrolytic
404 enzyme activities of naive Atlantic salmon *Salmo salar* skin mucus due to infection with
405 the salmon louse *Lepeophtheirus salmonis* and cortisol implantation. Diseases of Aquatic
406 Organisms. 2000 41:43-51.
- 407 13. Lazado CC, Lund I, Pedersen PB, Nguyen HQ. Humoral and mucosal defense
408 molecules rhythmically oscillate during a light–dark cycle in permit, *Trachinotus falcatus*.
409 Fish & Shellfish Immunology. 2015 47:902-12.
- 410 14. Siwicki A, Anderson D. Nonspecific Defense Mechanisms Assay in Fish. II.
411 Potential Killing Activity of Neutrophils and Macrophages, Lysozyme Activity in Serum and
412 Organs and Total Immunoglobulin Level in Serum. Poland, Olsztyn; 1993.
- 413 15. Bradford MM. A rapid and sensitive method for the quantitation of
414 microgram quantities of protein utilizing the principle of protein-dye binding. Analytical
415 biochemistry. 1979 72:249-54.
- 416 16. Demers NE, Bayne CJ. The immediate effects of stress on hormones and
417 plasma lysozyme in rainbow trout. Developmental & Comparative Immunology. 1997
418 21:363-73.

- 419 17. Yano T. Assay of hemolytic complement activity. In: Stolen J, Fletcher T,
420 Anderson D, Hattari S, Rowley A, editors. Techniques in Fish Immunology. New Jersey SOS
421 Publications; 1992.
- 422 18. Caipang CMA, Lazado CC, Brinchmann MF, Rombout JHWM, Kiron V.
423 Differential expression of immune and stress genes in the skin of Atlantic cod (*Gadus*
424 *morhua*). Comparative Biochemistry and Physiology Part D: Genomics and Proteomics.
425 2011 6:158-62.
- 426 19. Rajan B, Lokesh J, Kiron V, Brinchmann MF. Differentially expressed
427 proteins in the skin mucus of Atlantic cod (*Gadus morhua*) upon natural infection with
428 *Vibrio anguillarum*. BMC Veterinary Research. 2013 9:1-11.
- 429 20. Angeles Esteban M. An overview of the immunological defenses in fish skin.
430 ISRN Immunology. 2012 2012:29.
- 431 21. Subramanian S, MacKinnon SL, Ross NW. A comparative study on innate
432 immune parameters in the epidermal mucus of various fish species. Comparative
433 Biochemistry and Physiology - B Biochemistry and Molecular Biology. 2007 148:256-63.
- 434 22. Aranishi F, Mano N, Hirose H. Fluorescence localization of epidermal
435 cathepsins L and B in the Japanese eel. Fish Physiology and Biochemistry. 1998 19:205-9.
- 436 23. Kennedy J, Baker P, Piper C, Cotter PD, Walsh M, Mooij MJ, et al. Isolation
437 and analysis of bacteria with antimicrobial activities from the marine sponge *Haliclona*
438 *simulans* collected from Irish waters. Marine Biotechnology. 2009 11:384-96.
- 439 24. Fernandes JMO, Smith VJ. A novel antimicrobial function for a ribosomal
440 peptide from rainbow trout skin. Biochemical and Biophysical Research Communications.
441 2002 296:167-71.
- 442 25. Hjelmeland K, Christie M, Raa J. Skin mucus protease from rainbow trout,
443 *Salmo gairdneri* Richardson, and its biological significance. J Fish Biol. 1983 23:13-22.
- 444 26. Boshra H, Li J, Sunyer JO. Recent advances on the complement system of
445 teleost fish. Fish & Shellfish Immunology. 2006 20:239-62.
- 446 27. Hajhashemi V, Sajjadi SE, Heshmati M. Anti-inflammatory and analgesic
447 properties of *Heracleum persicum* essential oil and hydroalcoholic extract in animal
448 models. Journal of Ethnopharmacology. 2009 124:475-80.
- 449 28. Jian J, Wu Z. Influences of traditional Chinese medicine on non-specific
450 immunity of Jian Carp (*Cyprinus carpio* var. Jian). Fish and Shellfish Immunology. 2004
451 16:185-91.

29. Radhakrishnan S, Saravana Bhavan P, Seenivasan C, Shanthi R, Poongodi R. Influence of medicinal herbs (*Alteranthera sessilis*, *Eclipta alba* and *Cissus quadrangularis*) on growth and biochemical parameters of the freshwater prawn *Macrobrachium rosenbergii*. *Aquaculture International*. 2014 22:551-72.

30. Adel M, Lazado CC, Safari R, Yeganehe S, Zorriehzahra MJ. Aqualase®, a yeast-based in-feed probiotic, modulates intestinal microbiota, immunity and growth of rainbow trout *Oncorhynchus mykiss*. *Aquaculture Research*. 2016;*In Press*. 10.1111/are.13019.

Table 1. Dietary formulation and proximate composition of the basal diet (%)

Ingredients	
Fish meal	40.0
Wheat flour	21.0
Soybean meal	13.5
Gluten	5.5
Soybean oil	6.0
Fish oil	6.0
Mineral premix [*]	3.0
Vitamin premix [*]	2.0
Binder [†]	2.0
Anti fungi [‡]	0.5
Antioxidant [§]	0.5
Proximate composition (% dry matter basis)	
Dry matter	91.12
Crude protein	36.81
Crude lipid	11.33
Ash	3.50
Fiber	11.58

^{*} Details of the premix [10].

[†] Amet binder [™], Mehr Taban-e- Yazd, Iran

[‡] ToxiBan antifungal (Vet-A-Mix, Shenandoah, IA)

[§] Butylated hydroxytoluene (BHT) (Merck, Germany)

Table 2. Changes in the performance indicators of common carp (*Cyprinus carpio*) following feeding with diets supplemented with *H. persicum* powder at different inclusion levels. Values are presented as the mean $\pm$ SE.

	Persian hogweed (g Kg ⁻¹)			
	0	2.5	5	10
Final weight (g)	50.24 $\pm$ 3.17 ^a	53.23 $\pm$ 1.02 ^{ab}	55.41 $\pm$ 1.06 ^b	55.93 $\pm$ 2.01 ^b
Weight gain (g)	26.86 $\pm$ 3.31 ^a	29.73 $\pm$ 1.28 ^{ab}	31.83 $\pm$ 1.07 ^b	32.55 $\pm$ 1.90 ^b
SGR	1.55 $\pm$ 0.13 ^a	1.66 $\pm$ 0.06 ^{ab}	1.74 $\pm$ 0.04 ^b	1.78 $\pm$ 0.06 ^b
FCR	2.37 $\pm$ 0.11 ^a	2.15 $\pm$ 0.10 ^{ab}	1.96 $\pm$ 0.05 ^b	1.90 $\pm$ 0.08 ^b
Survival rate (%)	100	100	100	100

Values in a row with different letter denote significant difference ($P < 0.05$)

Title: Dietary phytoimmunostimulant Persian hogweed (*Heracleum persicum*) has more remarkable impacts on skin mucus than on serum in common carp (*Cyprinus carpio*)

Authors: Seyed Hossein Hoseinifar, Fazel Zoheiri, Carlo C. Lazado

HIGHLIGHTS

- Persian hogweed is a flower-bearing herb rich in bioactive compounds.
- Carp diet was supplemented with the powder form of the herb at 3 inclusion levels.
- Immunological defences were altered following an 8-week feeding trial.
- Changes in immune factors were more pronounced in skin mucus than in serum.
- *H. persicum* is a potential phytoimmunostimulant in carp aquaculture.