

## Original article

**Immune-enhancing capacity of peppermint (*Mentha piperita* L.)  
in the endangered Danube salmon (*Hucho hucho* L.)**Cerbu Constantin Gheorghe, Marina Spinu, Sandru Carmen Dana, VasIU Aurel, Giupana Radu Mihai,  
Niculae Mihaela, Muresan Elena Andruta\* and Brudasca Gheorghe FlorinelDepartment of Clinical Sciences-Infectious Diseases, University of Agricultural Sciences and  
Veterinary Medicine, Str. Manastur No.3-5, Cluj-Napoca, Romania\*Food Engineering Department, University of Agricultural Sciences and Veterinary Medicine,  
Str. Manastur No.3-5, Cluj-Napoca, Romania

Received August 1, 2018; Revised September 15, 2018; Accepted September 20, 2018; Published online December 30, 2018

**Abstract**

The Danube salmon (*Hucho hucho* L.) represents the flagship of the Salmonidae family. The species is severely divided within the Danube River drainage, where most residents exclusively depend on (re) stocking since natural reproduction is very limited due to reasons like major habitat changes and flow regime alterations. Appropriate immunity is essential in preserving the health of fish, especially when restocking forces the fish to adapt to a new environment. Consequently, medicinal plants could be used as an immune-enhancing resource. This study aimed to evaluate the effects of peppermint (*M. piperita* L.) extract on cell-mediated immunity in Danube salmon (*H. hucho*), depending on sex and compared to classical mitogens. Blood was sampled from a number of 30 adult huchens (males: n = 15; females: n = 15). The leukocyte blast transformation test for measuring the *in vitro* reactivity of mononuclear cells was performed. Blast transformation indices were calculated versus untreated controls by glucose concentration measurements (Orto-toluidin method). Results showed a significant ( $p < 0.001$ ) difference between the *in vitro* response to peppermint versus control for both males and females. The stimulation index (%) averaged 23.43 for males and 18.77 for females. The overall cell-mediated response to peppermint extract was mainly influenced by sex. However, the enhancing effect can be used a tool of immune stimulation and modulation for future restocking programs.

**Key words:** *Hucho hucho* L., Danube salmon, immune system, immune modulation, plant extracts

**1. Introduction**

The endangered Danube salmon, *Hucho hucho* (Linnaeus, 1758), or huchen as well-known in Central and South-Eastern Europe, represents the flagship of the *Salmonidae* family (Weiss and Schenekar, 2016). Until recent times, the species was present in the Danube River and most of its mountain and submontane tributaries (Formicki *et al.*, 2013). Today, the species occupies only about a quarter of its original range in Europe, mainly due to river regulation, dam and reservoir construction, major water consumption by industry and agriculture, river pollution and accelerated eutrophication (Holcik *et al.*, 1988; Holcik, 1990; Formicki *et al.*, 2013). The species is endangered (EN) according to IUCN (International Union for Conservation of Nature) (Freyhoff and Kottelat, 2008) and listed under Annexes II and V of the European Habitat Directive, as well as Appendix III of the Bern Convention of European Wildlife and Natural Habitats (Weiss and Schenekar, 2016). The huchen is also reported in Annexes II (designated as Sites of Community Importance must be protected

under the Natura 2000 Network and the sites managed in accordance with the ecological requirements of the species) and V of the EU Habitat Directive (Ihut *et al.*, 2014).

The study of fish diseases and fish immune system requires extensive knowledge of the physiology in a peculiar environment and of the numerous agents that can cause various infections (Cerbu *et al.*, 2015). The immune system represents the only defence mechanism of invertebrates and a fundamental defence framework in fish (Magnadottir, 2006). The anatomical and functional integrity of the immunological defence mechanisms are extremely important and depend on the dynamics of host-aggressor interrelations. Interest for the use of new immunostimulants as an alternative to various substances and antibiotics currently used for the control of fish pathologies is growing partially because immunostimulants, in contrast to vaccines, enhance the innate (or non-specific) immune response (Galeotti, 1998; Sakai, 1999; Galina, 2009). As the Danube salmon represents the flagship of the Salmonidae family, it is very important to find new ways into preserving the species. A series of preventive measures are deemed sustainable and Food and Agriculture Organization (FAO) of the United Nations encourages the scientific community to investigate further on: (i) 'the role of good nutrition in improving aquatic animal health', (ii) 'harnessing the host's specific and non-specific defence mechanisms in controlling aquatic animal diseases', (iii) 'use of immunostimulants and non-specific immune-enhancers to reduce susceptibility to

**Author for correspondence:** Dr. Cerbu Constantin Gheorghe  
Assistant Professor, Department of Clinical Sciences-Infectious  
Diseases, University of Agricultural Sciences and Veterinary Medicine,  
Str. Manastur No.3-5, Cluj-Napoca, Romania.

**E-mail:** constantin.cerbu@usamvcluj.ro

**Tel.:** +40-264596384/173; +40-752181611

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Email: ukaaz@yahoo.com; Website: www.ukaazpublications.com

disease', iv. 'use of probiotics and bioaugmentation for the improvement of aquatic environmental quality' and v. 'reducing the use of chemicals and drugs in aquaculture' (Subasinghe *et al.*, 1997). It is well known that a boost of the innate immune system in fish can be initiated by many immunostimulants. Yet, some of the immunostimulants could not be used because of various disadvantages, such as high cost, limited effectiveness upon parenterally administration, *etc.* (Dugenci *et al.*, 2003). On the other hand, a large number of plants have been used in traditional medicine for the treatment and control of several diseases (Duke, 1987). Herbs are currently used in commercial aquaculture as growth-promoting substances, antimicrobial agents, nutrients, as well as many other applications. Their potential to prevent and control fish diseases is also under study. Boosting the immune response using medicinal plant products has become the centre of extensive scientific investigations (Galina, 2009; Manoharachary and Nagaraju, 2016; Dang, 2018; Nooreen, 2018). Regardless that peppermint is a very popular herb that has been used in humans and mammals for a long time, its effects on fish received limited attention (Adel *et al.*, 2015). The plant is present in the natural habitats of the huchen. In this framework, the aim of the study was to evaluate the effects of an alcoholic peppermint (*M. piperita*) extract on cell-mediated immunity in Danube salmon (*H. hucho*), depending on sex and compared to classical mitogens.

## 2. Materials and Methods

### 2.1 Fish

The research was carried out on a number of 30 mature, healthy Danube salmon (*H. hucho*), aged between 4 and 11 years (males: n=15, average length: 87.4 cm, average weight: 6.11 kg; females: n=15, average length: 81.8 cm, average weight: 5.27 kg) reared in a mountain lake in South-Central Romania, in 32 m<sup>2</sup> special nets, at a maximum depth of 4 m. To the best of our knowledge, this was the Europe's last population of huchens reared in a mountain lake. Sampling was done during spawning at a water temperature of 4°C. The fishes were anesthetized with tricaine methane-sulfonate (MS-222, Western Chemical Inc., USA), 80 mg/l in 500 l special containers, and sampling was done by puncturing the caudal vessels.

### 2.2 Leukocytes blast transformation test

The leukocyte blast transformation test is used to assess the *in vitro* reactivity of mononuclear cells to sensitizing (*in vivo* encountered) antigens, chemicals or other compounds. Cell growth was quantified by means of the glucose consumption technique as described by Khokhlova *et al.*, (2004). Part of the blood sample (1 ml) was diluted with four times the amount of RPMI 1640 (Sigma-Aldrich, USA) with 5% FCS and antibiotics at pH 7.4. The mixture was distributed in five wells of a 96-sterile-wellplate (100 ml per well). Twelve variants were tested once for each individual, namely: i. untreated control culture, ii. phytohaemagglutinin-M (PHA) (1 µl per well) treated culture, iii. phytohaemagglutinin-M (PHA) (2 µl per well) treated culture, iv. phytohaemagglutinin-M (PHA) (5 µl per well) treated culture, v. lipopolysaccharide (LPS) (1 µl per well) treated culture, vi. lipopolysaccharide (LPS) (2 µl per well) treated culture, vii. lipopolysaccharide (LPS) (5 µl per well) treated culture, viii. concanavalin A (Con A) (1 µl per well) treated culture, ix. concanavalin A (Con A) (2 µl per well) treated culture, x. concanavalin A (Con A) (5 µl per well) treated culture, xi. alcohol (1.5 µl per well) treated culture and xii. peppermint (1.5 µl per

well) treated culture. The quantities of mitogens were established when using the same technique during preliminary studies as being the most effective *in vitro* for salmonids. The cultures were incubated for 48 h at 4°C and 5% CO<sub>2</sub>. Glucose concentrations were measured in the initial medium and in all variants at the end of the incubation period, using a standard (100 Mg/dl) glucose solution, by means of an Orto-toluidine colorimetric test. In order to do that, 12.5 µl of the cultural supernatants were transferred to 0.5 ml of orto-toluidine reagent, boiled for 8 min, cooled suddenly in cold water and read in a spectrophotometer at 610 nm wavelength (Sumal PE2, Karl Zeiss, Germany), using the reagent as a blank. The transformation index (TI) was calculated using the following formula:

TI (%) = [(MG – SG)/MG]/100, where TI=blast transformation index, MG=glucose concentration in the initial culture medium and SG=glucose concentration in the sample after incubation. All the reagents were of analytical grade while the peppermint (*M. piperita*) extract used was a commercially available, human use product, obtained by the procedures described in the German Pharmacopoeia.

During this study, animal welfare and protection legislation valid in EU and Romania were fully obeyed.

### 2.3 Statistical analysis

Minitab 16.0 was used for statistical interpretation. Results were expressed as Mean ± standard deviation (SD).

## 3. Results and Discussion

Fish, either cartilaginous or bony, are the most primitive vertebrates with a thymus, and possess T cells similar to those in mammals. There are numerous studies in fish establishing that the thymus is the fundamental organ for development of T lymphocytes from early thymocyte progenitors to functionally competent T cells (Nakanishi, 2015).

In this study, immune responses of fishes differed among sexes (control, PHA, LPS, Con A, alcohol and mint) and showed a significant difference ( $p < 0.001$ ) between the *in vitro* response versus control, for both males and females. The stimulation index for peppermint (*M. piperita*) averaged 23.43 (± 11.68%) for males (Table 1) and 18.77 (± 8.30%) for females (Table 2).

For males, the stimulation index increased with concentration for PHA and LPS but the pattern was not followed by Con A, in which the highest concentration (5 µl) averaged the lowest value (14.82%). Instead, in females, the only mitogen for which the TI values increased with concentration was Con A. For the other mitogens, no patterns could be identified. In males, the highest value of TI was recorded for LPS (5 µl): 24.47%, while in females for alcohol (1.5 µl): 29.72%.

An interpretation of the results with regard to the functionality of the immune response mediated by T cells showed that there was a strong response to mitogens including the peppermint (*M. piperita*) even though the incubation temperature was low. Based on previous studies (Savu, 2012), immunity in salmonids is stronger at lower temperatures. This finding could be explained by the fact that the Danube salmon is found in mountain rivers where the average water temperature is low. As most of the restocking programs involving the Danube salmon failed because of maladaptation, the current study suggested an effective alternative for the stimulation of the

immune system - the peppermint (*M. piperita*). Peppermint extract has multiple advantages over the classical immunostimulants: it is easy to obtain, it is less expensive and it can be given orally, which is the most convenient method for immunostimulation. However, the effect is dose-dependent and there is always a potential for overdosing (Gallina, 2009). The use of peppermint as immunostimulant for the Danube salmon may also be of environmental value because of its presence in the huchen's habitat and because of its biodegradability. Moreover, this opens new perspectives into research regarding the use of other medicinal plants as immunostimulants for threatened fish species and in aquaculture.

#### 4. Conclusion

Immunological investigations showed that there are quantitative differences between the functionality of the immune system in *H. hucho* based on the individual. The overall cell-mediated response to peppermint extract was mainly influenced by sex, which could be explained by the changes that occurred in fish during spawning. Furthermore, the enhancing effect of peppermint (*M. piperita*), a medicinal plant which is almost ubiquitous, could be implied as a tool for immune stimulation and modulation for future restocking programs of the endangered Danube salmon (*H. hucho*).

**Table 1:** Dose dependant *in vitro* blast transformation index (%) in male huchen (Mean  $\pm$  standard deviation, SD)

	Control	PHA 1 $\mu$ l	PHA 2 $\mu$ l	PHA 5 $\mu$ l	LPS 1 $\mu$ l	LPS 2 $\mu$ l	LPS 5 $\mu$ l	ConA 1 $\mu$ l	ConA 2 $\mu$ l	ConA 5 $\mu$ l	Alcohol 1.5 $\mu$ l	<i>M. piperita</i> 1.5 $\mu$ l
Mean	14.62	16.96	19.96	21.03	15.89	20.34	24.47	15.23	22.99	14.82	21.08	23.43
$\pm$ SD	4.14	9.53	12.43	11.27	10.52	12.60	14.34	12.48	13.51	10.65	14.73	11.68

**Table 2:** Dose dependant *in vitro* blast transformation index (%) in female huchen (Mean  $\pm$  standard deviation, SD)

	Control	PHA 1 $\mu$ l	PHA 2 $\mu$ l	PHA 5 $\mu$ l	LPS 1 $\mu$ l	LPS 2 $\mu$ l	LPS 5 $\mu$ l	ConA 1 $\mu$ l	ConA 2 $\mu$ l	ConA 5 $\mu$ l	Alcohol 1.5 $\mu$ l	<i>M. piperita</i> 1.5 $\mu$ l
Mean	13.41	20.98	9.07	9.21	8.70	12.68	11.45	14.18	16.52	23.84	29.72	18.77
$\pm$ SD	13.28	17.19	6.92	8.95	9.86	6.82	7.53	7.96	14.09	18.44	17.98	8.30

#### Conflict of interest

We declare that we have no conflict of interest.

**Note:** The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest, or non-financial interest in the subject matter or materials discussed in this manuscript.

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