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Bactericidal Capacity of Serum and Enhancement of Specific Cell-Mediated Immunity Subsequent to *Hipopphae Rhamnoides* Treatment in Pigs

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RESEARCH ARTICLE

Abstract

Low-input farming subject's swine to increased immune stress and subsequent infectious risk, which could be prevented by phytotherapy. *Hypopphae rhamnoides* is a widespread shrub in both Europe and Asia. The research aimed at investigating the influence of a whole fruit extract on antibacterial effect of the serum in extensively raised, two batches, sea buckthorn *in vivo* treated/untreated pigs. Scavenging effect over DPPH was used to estimate the antioxidant potential of the sea buckthorn commercial syrup (Steaua Divina©). Minimal inhibiting (MIC) and minimal bactericidal (MBC) capacity of the serum were tested and spectrophotometrically measured against *Shigella* spp., *Kitococcus sedentarius*, *Staphylococcus werneri* and *Staphylococcus sciuri*. The antioxidant activity of the syrup was intermediate (54.65 %). Sera from treated pigs had bactericidal and bacteriostatic effect on *Shigella* spp. and *S. sciuri*, respectively. *Shigella* spp. growth was inhibited at serum dilutions of ½-1/32 while much lower ½-1/4 dilutions were active against *S. sciuri*. In untreated pigs, the sera were inefficient in either stopping or totally inhibiting bacterial growth. The MBC and MIC values obtained for the sera of pigs treated with *Hippophae rhamnoides* syrup supported the positive effect of the *in vivo* treatment inducive of potential protection against bacterial diseases.

Keywords: pigs, *Hippophae rhamnoides*, MBC, MIC, pathogenic bacteria

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INTRODUCTION

Low input, small-scale pig farms were expanding due to the preference for a more natural habitat provided to animals and the improvement in meat quality. Nevertheless, environment conditions on these farms suffer disruptions, causing daily inequality in raising conditions, as well as an increased infectious pressure. Such continuous changes could induce stress and immunosuppression (Gentry et al., 2002; Barnett and Hemsworth, 1986). Considering the free-range system as an open housing system, various factors can affect swine health: improper batching; close contact between animals, poor hygiene; improper pest control etc. Some of those, such as overcrowding, favour an easy spread of potentially pathogenic microbes and their persistence in the group. All the above-mentioned factors can lead to disease, when the immune status is either underdeveloped or functioning inappropriately and such examples could include opportunistic pathogens that express enhanced virulence in immunosuppressed pigs (Gebreyes

et al., 2008; Mannelli et al., 1997). Any highly transmissible disease, that affects a large number of animals, has major economic consequences through animal loss, costs for the therapy and potential zoonotic impact.

To lower the number of affected animals and, implicitly, the disease occurrence, the importance of immune strength is utmost. To improve the antimicrobial potence and to reduce antibiotic intake, which could lead to antimicrobial resistance or residues in the meat, it is important to seek for alternative immune stimulating products with increased bioavailability such as vegetal components or extracts (e.g. sea buckthorn or other phytoterapeutics) (Li et al., 2015; Hermenean and Pribac, 2003).

Sea buckthorn, is a deciduous shrub, widespread in Europe and Asia belonging to the kingdom of *Plantae*, class *Magnoliopsida*, order *Rosales*, family *Elaeagnaceae*, genus *Hippophae*. The plant is well-known for its high content in vitamins (A, B1, B12, C, E and K) and flavonoids, and also minerals (Ca, Mg, K, Se, Na, Fe, Zn, P, Co, Cu, Cr, Ni, Mn, Li etc.), essential fatty acids, free aminoacids etc. It also possesses a strong antioxidant effect, thus having major therapeutic importance by inhibiting the free radical production and potentiates the production of intracellular antioxidants, such as GSH and GPx (Li and Schroeder, 1996; Patel et al., 2012). Sea buckthorn has multiple uses in traditional medicine (Wani et al., 2016). By stimulating the cellular and humoral immune response, by restoring integrity and number of macrophages (enhanced phagocytosis process) (Suryakumar and Gupta, 2011), preserving the structure and functionality of lymphocytes and activating the majority of the immune cells (Geetha et al., 2002; Prakash et al., 2005) sea buckthorn has also been considered as an immune modulator. Sea buckthorn has antibacterial properties, due its high content of phenolic compounds, which inhibit the growth of Gram negative bacteria (e.g. *Yersinia enterocolitica*), but also has an inhibitory effect on Gram positive bacteria such as *Bacillus* spp. (*B. subtilis*, *B. coagulans*, *B. cereus*), *Listeria monocytogenes* and *Staphylococcus aureus*, as quantified by the MIC (minimum inhibitory concentration) test (Negi et al., 2005; Yue et al, 2017).

The research aimed at studying the effect of sea buckthorn in fattening pigs, raised in a conventional, free range system as a way to improve their immune potency and resistance to disease.

MATERIALS AND METHODS

Two batches of mix breed pigs (n=9) were: *a)* orally treated with 5 ml of commercial syrup obtained (store bought, Steaua Divina©) from fruits /day/individual for 5 days (n=5), *b)* and left untreated (n=4). Pigs were identified by numbers (S2 to S10) and were clinically healthy. All animals subjected to testing were raised on a traditional, extensive farm. The immuno-active capacity and the ability to neutralize bacterial populations of the *in vivo* administered sea buckthorn syrup were investigated.

To establish the antioxidant activity of sea buckthorn syrup, DPPH assay was performed, by assessing free radical scavenging effect over 1,1-diphenyl l-2-picrylhydrazyl (DPPH) radical (Odriozola-Serrano et al., 2008). An amount of 100 µl of the syrup was mixed with 3.9 ml of DPPH (0.025g /l). The mixture was stirred and maintained in dark environment for 30 minutes. The absorbance of the samples was measured at 515 nm (Shimadzu 1700 UV-VIS) against a methanol blank. Results were expressed as percent over standard DPPH absorbance, calculated by the following formula:

$$\text{Radical scavenging activity \%} = 100x\ [(A_{DPPH}-A_{sample})/A_{DPPH}]$$

where A_{DPPH} is the absorbance of the DPPH solution and A_{sample} is the absorbance of the sample after incubation (Sharma and Bhat, 2009).

Similarly, the total polyphenol content of the sea buckthorn syrup was evaluated according to the Folin-Ciocalteu method (Blainski et al., 2013). Absorbance was read at 750 nm with a Shimadzu UV-VIS 1700 spectrophotometer. The standard curve was carried out using concentrations of 0, 0.25, 0.50, 0.75, 1 mg/ml of gallic acid. Total polyphenol content of the syrup was expressed in gallic acid equivalents, mg of GAE/100 ml sample. This assay was done triplicate.

To assay the specific cell-mediated immunity before and after treatment in pigs, lymphocyte blast transformation test was performed. The used protocol evaluates the *in vitro* proliferative response of the lymphocytes to mitogens, which nonspecifically stimulate this cells, by measuring glucose consumption and thus the cellular reactivity (Rigby et al., 1984).

In this case, the response to stimulation with PHA (phytohemagglutinin obtained from *Phaseolus vulgaris*), which induces the transformation of T lymphocytes, was tested. To perform the test, blood sample were collected on tubes containing lithium heparin. The blood samples were diluted with RPMI 1640 medium, in 1:4 ratio, and subsequently distributed in 96-well plates, in duplicate. Twelve experimental variants of each sample were tested (untreated control, PHA - standard mitogen, 5 µg/ml, 70% alcohol control 7.5 µg/ml, alcoholic plant extracts (Plantextract, Germany) of: *Echinacea angustifolia*, *Echinacea purpurea*, *Hippophae rhamnoides*, *Thymus vulgaris*, *Vaccinium myrtillus*, *Sylibum marianum*, *Arnica montana*, and 2 hydro-alcoholic extracts – *Hippophae rhamnoides* and *Betula* spp. – 7.5 µg/ml/well of each extract). All extracts were commercial ones, for human use, obtained according to the procedures described in the German Pharmacopoeia.

The plates were then incubated for 48 h at 37°C in 5% CO2 atmosphere and residual glucose tested (ortho-

toluidine method) at the end of the incubation period. The colour of the complex formed between glucose and ortho-toluidine was tested spectrophotometrically using a SUMAL PE2 spectrophotometer at a wavelength of 610 nm. Stimulation indices (SI %) expressed as percentage of the initial culture medium were calculated by the following formula:

$$SI\% = (A_{culture\ medium} - A_{sample} / A_{culture\ medium}) \times 100$$

where $A_{culture\ medium}$ represents the glucose concentration of initial RPMI and A_{sample} is the sample final glucose concentration.

Minimal inhibiting (MIC) and minimal bactericidal (MBC) concentrations of the serum from both sea buckthorn syrup treated and control animals were tested against *Shigella* spp., *K. sedentarius*, *S. werneri* and *S. sciuri*, identified in the samples collected from the tested pigs' skin. For that, the sera were diluted in simple broth from ½ to 1/256 in 96-well plates. Bacterial colonies of each strain were harvested after 24 h of incubation at 37°C and diluted with sterile saline to 0.5 on McFarland scale. Each well was inseminated with the same amount of this suspension, for each bacterial strain. All plates were incubated in similar conditions to the bacterial strains and the optical densities of the cultures were spectrophotometrically measured (535 nm, d=0.5, SUMAL PE2, Karl Zeiss, Jena) (Wiegand et al., 2008). The last well with no bacterial growth was considered to be the MIC of the serum, while the reinsemination from the same well, accompanied with growth or lack of growth, indicated either the MBC or the bacteriostatic effect of the sera.

Statistical processing of the data. The Excel program was used to calculate the averages and the significance of the differences of SI % before and after the therapy, versus placebo treated group by means of the t- Student test.

RESULTS AND DISCUSSIONS

Sea buckthorn was used in traditional medicine, especially in areas where the shrub is natively found, for centuries. Researches concerning its medicinal properties connected with its composition are relatively widely extended. It has been demonstrated that in very small dietary concentrations (400 to 800 ppm), sea buckthorn enhanced the function of macrophages, avoided toxic effects of T2 fusario-toxin, increased the total Ig levels and also the Newcastle disease antibody levels in chickens (Ramsamy et al., 2010).

The antioxidant activity of the used commercial sea buckthorn syrup was intermediate, with an average percent of 54.65% and a total polyphenol content of 215,45 mg/100 ml (Table 1).

Table 1. The antioxidant activity and polyphenol content of *Hippophae rhamnoides* commercial syrup

Variant	RSA % DPPH	mg GAE/100 ml
V1	54,57	212,81
V2	54,57	220,74
V3	54,81	212,81

Notes: RSA-radical scavenging activity, GAE-gallic acid equivalents, V1, V2, V3- the triplicate samples

Following the obtained results (Table 2 and Table 3) a positive influence of the sea buckthorn syrup on the specific cell-mediated immunity could be noticed. An increase in the SI calculated for the treated batch was observed, which suggested an increase in the adaptability of the specific cellular system under potential microbial aggression.

Similarly, the response to classical mitogens has increased and the plant extracts used *in vitro* showed stimulating effects, especially that of *Echinacea purpurea,* with a higher average SI% than other tested compounds, both pre and post therapy. Various extracts induced a higher increase in the post-therapeutic SI% (*Arnica montana* with 33.18%, *Echinacea angustifolia* with 30.94%) as compared to *E. purpurea* (20.49%), which showed a high SI% even before the *in vivo* sea buckthorn therapy. Such behavior is encouraging for the possible association of extracts that have stimulated the *in vitro* blastogenesis with sea buckthorn syrup administered orally to pigs, which could increase the non-specific immune response but could also facilitate the post-vaccine response in these pigs as well as enhanced healing in certain situations (e.g. surgery, traumatic wounds) (Heinrich et al., 2018).

Table 2. SI % - treated pigs before application of the therapeutic protocol

Sample Mitogen	S2	S5	S7	S8	S9	Average
M	44,85	55,76	36,97	18,18	50,91	41,33
PHA	67,88	83,03	70,30	49,09	52,73	64,61
Alc	41,21	76,36	72,73	46,67	73,94	62,18
Ea	78,79	72,12	41,21	40,61	64,85	59,52
Ep	68,49	58,18	58,79	87,88	78,79	70,42
Cat1	74,55	66,67	39,40	54,55	70,30	61,09
Thy	57,58	60,61	53,94	43,64	66,67	56,49
Vm	74,55	56,97	73,94	62,43	57,58	65,09
Sy	79,39	62,43	63,64	35,15	58,79	59,88
Ar	76,97	56,36	64,85	20,61	51,52	54,06
Cat2	79,39	58,79	49,70	72,12	53,33	62,67
Mest	78,18	62,43	45,46	60,61	57,58	60,85

Notes: M- control, PHA - phytohemagglutinin, Alc- control alcohol, Ea - culture treated with alcoholic extract *E. angustifolia*, Ep - culture treated with alcoholic extract *E. purpureea*, Cat1 - culture treated with alcoholic extract *Hippophae rhamnoides* , Thy - culture treated with alcoholic extract *Thymus vulgaris*, Vm - culture treated with alcoholic extract *Vaccinum myrtillus*, Sy - culture treated with alcoholic extract *Sylibum marianum*, Ar - culture treated with alcoholic extract *Arnica montana*, Cat2 - culture treated with hydroalcoholic extract *Hippophae rhamnoides*, Mest - culture treated with hydro-alcoholic extract *Betula pendula*, S 2...10 –individual pig number

Table 3. SI % in treated pigs after application of the therapeutic protocol

Sample Mitogen	S2	S5	S7	S8	S9	Average
M	52,12	68,49	55,15	58,18	64,24	59,64
PHA	85,45	86,06	80,61	79,39	67,27	79,76
Alc	52,73	76,97	76,97	69,70	97,37	71,15
Ea	82,42	83,03	83,03	73,94	70,91	77,94
Ep	84,85	82,42	82,42	89,09	82,42	84,85
Cat1	76,97	83,03	83,03	85,45	75,76	81,09
Thy	66,06	73,94	73,94	57,58	70,91	68,73
Vm	76,36	86,06	86,06	72,12	72,12	76,97
Sy	81,21	66,67	66,67	61,21	66,67	69,09
Ar	80,61	85,45	85,45	58,18	67,27	72,00
Cat2	83,64	85,45	85,45	86,67	72,73	85,58
Mest	84,24	60,61	60,61	71,52	64,24	72,49

Notes: M- control, PHA - phytohemagglutinin, Alc- control alcohol, Ea - culture treated with alcoholic extract *E. angustifolia*, Ep - culture treated with alcoholic extract *E. purpureea*, Cat1 - culture treated with alcoholic extract *Hippophae rhamnoides* , Thy - culture treated with alcoholic extract *Thymus vulgaris*, Vm - culture treated with alcoholic extract *Vaccinum myrtillus*, Sy - culture treated with alcoholic extract *Sylibum marianum*, Ar - culture treated with alcoholic extract *Arnica montana*, Cat2 - culture treated with hydroalcoholic extract *Hippophae rhamnoides*, Mest - culture treated with hydro-alcoholic extract *Betula pendula,* **S 2...10 –individual pig number**

The untreated batch showed no increasing of the SI percent (Table 4 and 5).

Table 4. SI % in untreated pigs before experiment

Sample Mitogen	S3	S4	S6	S10	Average
M	49,09	52,73	20,00	46,06	41,97
PHA	78,18	64,24	58,79	60,00	65,30
Alc	39,40	63,03	40,00	67,88	52,58
Ea	67,27	77,58	29,09	60,61	58,64
Ep	71,52	72,12	64,24	81,82	72,42
Cat1	50,91	70,91	63,64	64,24	62,43
Thy	59,39	82,42	47,88	51,52	60,30
Vm	50,30	71,52	62,43	52,12	59,09
Sy	72,12	66,06	43,03	70,91	63,03
Ar	58,79	58,18	83,64	67,88	67,12
Cat2	59,39	75,15	40,61	61,21	59,09
Mest	66,06	76,97	51,52	67,88	65,61

Notes: M- control, PHA - phytohemagglutinin, Alc- control alcohol, Ea - culture treated with alcoholic extract *E. angustifolia*, Ep - culture treated with alcoholic extract *E. purpureea*, Cat1 - culture treated with alcoholic extract *Hippophae rhamnoides* , Thy - culture treated with alcoholic extract *Thymus vulgaris*, Vm - culture treated with alcoholic extract *Vaccinum myrtillus*, Sy - culture treated with alcoholic extract *Sylibum marianum*, Ar - culture treated with alcoholic extract *Arnica montana*, Cat2 - culture treated with hydroalcoholic extract *Hippophae rhamnoides*, Mest - culture treated with hydro-alcoholic extract *Betula pendula,* **S 2...10 –individual pig number**

Table 5. SI % in untreated pigs after experiment

Sample Mitogen	S3	S4	S6	S10	Average
M	45,45	24,24	26,66	47,87	36,06
PHA	61,21	49,69	60,00	69,09	60,00
Alc	16,36	47,27	45,45	41,81	37,72
Ea	84,24	29,69	29,09	58,18	50,30
Ep	61,81	46,66	64,84	52,12	56,36
Cat1	56,34	73,33	64,84	60,00	63,63
Thy	38,78	48,48	49,09	67,87	51,06
Vm	49,69	56,36	60,60	58,18	56,21
Sy	72,72	73,93	43,03	60,00	62,42
Ar	68,48	76,36	84,84	60,00	72,42
Cat2	58,18	50,90	41,81	61,21	53,03
Mest	61,21	66,06	50,30	56,36	58,48

Legend: M- control, PHA - phytohemagglutinin, Alc- control alcohol, Ea - culture treated with alcoholic extract *E. angustifolia*, Ep - culture treated with alcoholic extract *E. purpureea*, Cat1 - culture treated with alcoholic extract *Hippophae rhamnoides* , Thy - culture treated with alcoholic extract *Thymus vulgaris*, Vm - culture treated with alcoholic extract *Vaccinum myrtillus*, Sy - culture treated with alcoholic extract *Sylibum marianum*, Ar - culture treated with alcoholic extract *Arnica montana*, Cat2 - culture treated with hydroalcoholic extract *Hippophae rhamnoides*, Mest - culture treated with hydro-alcoholic extract *Betula pendula,* **S 2...10 –individual pig number**

Monitoring the t-test values (Table 6), it became obvious that the syrup treatment led to a significant increase of the SI%, at different levels for different cultural variants, while the stimulation of the lymphocytes in the untreated

group was statistically non-significant.

T**able 6.** The statistical significance of the SI increase following sea buckthorn syrup therapy, versus controls

	Variant	M	PHA	Alc	Ea	Ep	Cat1	Thy	Vm	Sy	Ar	Cat2	Mest
Treated	**t value**	0.02	0.03	0.13	0.03	0.02	0.01	0.02	0.01	0.15	0.04	0.01	0.16
	Significance	sign	sign	non-sign	sign	sign	sign	sign	sign	non-sign	sign	sign	non-sign
Untreated	**t value**	0.28	0.2	0.1	0.32	0.01	0.42	0.19	0.31	0.48	0.27	0.25	0.15
	Significance	non-sign	non-sign	non-sign	non-sign	sign	non-sign	non-sign	non-sign	non-sign	non-sign	non-sign	non-sign

Notes: sign – significant, non-sign – non significant, M- control, PHA - phytohemagglutinin, Alc- control alcohol, Ea - culture treated with alcoholic extract *E. angustifolia*, Ep - culture treated with alcoholic extract *E. purpureea*, Cat1 - culture treated with alcoholic extract *Hippophae rhamnoides* , Thy - culture treated with alcoholic extract *Thymus vulgaris*, Vm - culture treated with alcoholic extract *Vaccinum myrtillus*, Sy - culture treated with alcoholic extract *Sylibum marianum*, Ar - culture treated with alcoholic extract *Arnica montana*, Cat2 - culture treated with hydroalcoholic extract *Hippophae rhamnoides*, Mest - culture treated with hydro-alcoholic extract *Betula pendula*

These results are supported by other researchers, who mention that sea buckthorn has immunomodulatory activity, by stimulating lymphocyte's production of IL-2 and γ-IFN – activating cell-mediated immune response (Geetha et al., 2005). Also, has a potent antioxidant effect by reducing intracellular reactive oxygen species levels, thus protecting cells from injuries caused by oxidative stress (Kim et al., 2015).

Subsequent to incubation, the sera from treated pigs had a bactericidal effect on *Shigella* spp. and a bacteriostatic one on *S. sciuri* (Table 7). *Shigella* spp. growth was inhibited after repeated cultivation at serum dilutions of ½-1/32 while for *S. sciuri* the active dilutions were much lower ½-1/4. On other strains, the sera didn't had bacteriostatic or bactericidal effect. Also, the sera obtained from untreated pigs were inefficient in stopping bacterial growth or killing any of the tested bacteria.

Table 7. Sera dilutions (from treated pigs) and its effect on different bacterium specie

Bacterium	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
Shigella spp.	-	-	-	-	-	+	+	+
Kytococcus spp.	+	+	+	+	+	+	+	+
S. sciuri	-	+	+	+	+	+	+	+
S. warneri	+	+	+	+	+	+	+	+

Similar to cell-mediated immunity, without going into detail, it can be stated that, at least against *Shigella*, the sea buckthorn syrup enhanced the humoral mediated immunity.

CONCLUSIONS

In vitro antibacterial activity of the serum from pigs treated with *Hippophae rhamnoides* syrup supported the positive effect of the *in vivo* treatment inductive of increased MIC and MBC, useful in protection against bacterial diseases, thus reducing the potential consumption of antibiotics. Sea buckthorn syrup induced an adaptive immune system stimulation, with differences depending on each animal, by increasing the specific immune response, and also enhanced the response to other plant extracts, therefore advocating for its use as an adjuvant during vaccine period. The results support the supplementation of the diet in growing pigs with sea buckthorn syrup, thus providing the animals an increased cell-mediated immunity and a better resistance against diseases which could occur due to mainly bacterial pathogens in the low-input farming system.

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Conflicts of Interest

The authors declare that they do not have any conflict of interest.

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