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Sambucus ebulus extracts exhibit modulatory activity on inflammation and phagocytosis as revealed by changed gene expression in a model of LPS-treated J774A.1 macrophages

M. N. Todorova*, O. B. Tasinov, M. G. Pasheva, B. K. Galunska, Y. D. Kiselova-Kaneva

Department of Biochemistry, Molecular Medicine and Nutrigenomics, Faculty of Pharmacy, Medical University – Varna, Tzar Osvoboditel 84B, Varna 9002, Bulgaria

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Dwarf elder, also known as elderberry (*Sambucus ebulus* L., SE) is a popular herb in Bulgarian folk medicine, known for its antiseptic, anti-inflammatory and diuretic action. Despite its wide application as anti-inflammatory and immune stimulatory remedy, there is no data on the possible protective effect of SE total extract (TE) or anthocyanin fraction (AF) under conditions of induced inflammation. The health benefits of consuming *S. ebulus* fruits that are rich in polyphenols, especially anthocyanins, have been the focus of an *in vitro* investigation. The aim of the present study was to evaluate the effect of SE fruit total extract and anthocyanin fraction on the expression of genes associated with the inflammatory response and phagocytosis in a model of LPS-stimulated J774A.1 macrophages. TE and the purified AF from *S. ebulus* fruits exhibited anti-inflammatory activity by reducing LPS-induced expression of TLR-4, TNFα, IL-6, IL-1β, IL-1RN, CRP, COX-2 and iNOS. Stimulation of phagocytic enzymes NOX and MPO expression was also reduced by pretreatment with TE and AF. The results of the *in vitro* studies suggest a potential anti-inflammatory effect of the obtained fractions, which appear to modulate the expression of proteins directly related to control inflammatory processes: TLR-4, TNFα, IL-6, IL-1β, IL-1RN, CRP, COX-2, iNOS, as well as enzymes associated with phagocytosis NOX and MPO.

Keywords: *Sambucus ebulus* fruits, LPS, herbal extracts, antioxidants, anthocyanins

INTRODUCTION

Traditions of folk medicine in many countries around the world have preserved the millennial knowledge of the beneficial effects of herbs on health. Recent studies of the healing action of plants traditionally used in countries such as China, India, the countries of South America, the Mediterranean, etc., are numerous and usually the rich experience of folk medicine has served as a starting point for discovering new healing remedies [1-3]. Studying the molecular effects of medicinal plants and their biologically active phenolic compounds (proanthocyanidins, anthocyanins, flavonols, and phenolic acids) [4] and identifying new molecular targets for their action is essential for the development of nutrition, biotechnology and pharmacy science. Development of new foods and medicines is important for addressing public health and food safety challenges.

The immune system is a complex system of molecules, cells, and tissues that interact in concert to control and eliminate infectious agents, malignant and transformed cells, and other unwanted antigens. The immunomodulatory effects of medicinal plant extracts are most commonly associated with their ability to influence processes such as inflammation [4-8], phagocytosis [9, 10], activation of the complement system [11-13], etc. Inflammation is an immunological defense mechanism by which the body responds to damage or infection by sending *M.* cells of the immune system, which eliminate dead and/or dying cells of their own body, at the appropriate places. Cellular signaling pathways are responsible for appropriate tuning of the inflammatory process. However, in some pathological situations, chronic inflammation can lead to diseases such as rheumatoid arthritis, high fever, atherosclerosis, glomerulonephritis, gastroenteritis, etc., sometimes even promoting cancer progression [1, 14-16]. Inflammatory mediators, such as pro-inflammatory cytokines, can significantly stimulate progression of inflammation [17]. That is why counteracting inflammation is usually on the level of inflammatory mediators production and secretion [18, 19].

The expected inhibitory effects of plant extracts on the expression of inflammatory factors are described in detail in the literature [8, 20, 21]. However, there are few reports of a stimulatory effect on the expression and such studies are related to studies designed to demonstrate immune-stimulatory activity [5, 7, 22-28].

S. ebulus fruits contain organic acids and flavonols, glycosides, anthocyanins, phytosterols, phenols, triterpenes, tannins, iridoid glycosides, cardiac glycosides, derivatives of caffeic acid,

* To whom all correspondence should be sent:
E-mail: gidema@abv.bg

chlorogenic acid, ursolic acid, lectins and many other compounds with biological activity [29, 30]. The anthocyanins present in elderberries are important for the beneficial health effects associated with their antioxidant properties. In ethnopharmacology *S. ebulus* is used in gastrointestinal inflammatory disorders [1] and in autumn-winter period for immune-stimulation against acute respiratory infections [1]. These applications are a prerequisite for the expectations for perspective pharmacological effects of *S. ebulus* extracts as an anti-inflammatory and immune modulatory remedy.

The aim of the present study was to evaluate the effect of SE fruit total extract and anthocyanin fraction on the expression of genes associated with the inflammatory response and phagocytosis in a model of LPS-stimulated J774A.1 macrophages.

MATERIALS AND METHODS

Plant material

Ripe *Sambucus ebulus* fruits were collected during maturity period (September-October 2017) from Shkorpilovtsi (Varna region, Bulgaria). After appropriate transportation in light-protected containers and careful removal of peduncles without disturbing the integrity of the fruits the collected fruits were frozen at -20ºC.

Extraction procedure

Three different extracts were obtained: total acetone extract (TE) (70% acetone); hydrophilic fraction (HF) obtained from TE by chloroform fractionation, and anthocyanin fraction (AF) obtained from HF by solid phase extraction. To remove sugars and organic acids from the total extract and to obtain purified anthocyanin fraction, solid phase extraction was used. Immature (C18) solid phase extraction columns, Hypersep C18 / 500 mg (Thermo Scentific, USA), were selected to retain the hydrophobic organic compounds. At each step dry residues were obtained by vacuum evaporation below ≤40ºC with a rotary vacuum evaporator (Genevac SP Scientific, United Kingdom). The resulting dry material from each step was dissolved either in dH_2O for chemical analyses or in cell culture media DMEM for cell culture experiments.

The results of the three spectrophotometric methods revealed the content of total polyphenols in the TE (522.8 ± 4.83 mg/L), the content of total flavonoids in the extracts and the obtained fractions (630.2 ± 7.25 mg /L), and the content of total monomeric anthocyanin pigments (161.97 ± 0.80 mg/L), which decreased by 25% in the purification process. With the UPLC-UV-MS method developed, we found 0.84 mg/100 g fresh weight (FW) of epicatechin, 0.15 mg/100 g FW of quercetin and 0.05 mg/100 g FW of campherol in the total fruit extract of *S. ebulus*.

Cells culture

J774A.1 mouse macrophage cell line was obtained from the American Type Culture Collection (ATCC). Cells were cultured in 75 cm^3 flasks at 37°C in a humidified chamber containing 5% of CO_2 in DMEM (Sigma-Aldrich) with 4.5 g/L of glucose, L-glutamine and supplemented with fetal bovine serum (FBS, Sigma-Aldrich) to a final concentration of 10% and penicillin/streptomycin mixture to a final concentration of 100U/ml of each. Cells were sub-cultivated until 80% confluence was achieved.

Experimental procedure

The experimental procedure is based on a model of pretreatment with SE extracts, followed by LPS stimulation. Cells (J774A.1 macrophages) were seeded in 6-well flasks with a density of 2×10^5 cells/well. After overnight incubation, the cells were pretreated with a medium containing TE and AF. The pre-treatment media contained established final concentrations of 5, 15 and 135 μg/mL. Prior to treatment, the extracts were filtered through a 0.2 μm filter to eliminate bacterial contamination. After 24 h of incubation with extracts containing the medium the latter was removed and replaced with a medium with 100 ng/mL of LPS (*Escherichia coli*, 026: B6). Using this model, the following experimental groups were obtained: untreated control; TE pretreatment (5, 15 and 135 μg/mL) with LPS treatment; AF pretreatment (5, 15 and 135 μg/mL) with LPS treatment.

Determination of levels of gene expression

To determine the level of gene expression of selected genes in the cell cultures, two-step quantitative Real-Time PCR was used. Gene expression values were calculated by the $2^{-\Delta\Delta Ct}$ method and were presented in relative units as compared to the untreated control at which the expression level of the analyzed gene was considered to be 1. The results were presented as the mean (n=3) of the relative units ± standard error of mean (SEM). Expression of the following genes was studied: COX2 - F: TGAGCAACTATTCCAAACCAGC; R: GCACGTAGTCTTCGATCACTATC; MCP-1 – F: GGCTCAGCCAGATGCAGTTAA; R:CCTACTCATTGGGATCATCTTG; IL – 6 – F: CTGCAAGAGACTTCC; R:

GAAGTAGGGAAGGCC; TNFα - F: CCCTCACACTCAGAT CATCTTCT; R: GCTACGACGTGGGCTACAG; CRP - F: GTCTGCTACGGGGATTGTAGA R: GCACCTTGGGTTTCC CATCAA; IL - 1β – F: TTCAGGCAGGCACTA; R: CCACGGGAAAGACAC; IL – 1RN – F: GCTCATTGCTGGGTACTTACAA; R: CCAGACTTGGCACAAGACAGG; TLR4 - F: AGGCACATGCTCTAGCACTAA; R: AGGCTCCCCAGTTTAACTCTG; iNOS – F: GGCAGCCTGTGAGACCTTTG; R: GCATTGGAAGTGAAGCGTTTC; NOX – F: AGAGGAGAGCCCTTATCCCAACC; R: TGTCCAGAATTTCTTGAGCCTTG; MPO – F: GACATGCCCACCGAATGACAA; R: CAGGCAACCAGCGTACAAAG. β-actin (F: CAAGAAGGAAGGCTGGAAAAG; R: ACGGCCAGGTGATCACTATTG) served as an endogenous control.

Statistical processing and graphical presentation of the results

The values obtained were represented as the average of a minimum of three measurements ± SEM. The data were statistically treated with one-way ANOVA, at a confidence level $p<0.05$ and compared with Student's *t*-test. Data processing was performed using the statistical software product Graph Pad Prism (Ver. 5.0 Graph Pad Software, Inc.).

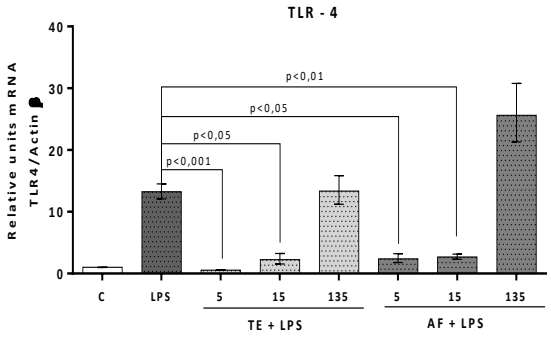
RESULTS AND DISCUSSION

Expression of genes associated with inflammatory response

Inflammation is characterized with increased plasma levels of some inflammatory factors, such as TNFα, IL-6, MCP-1 and others. Macrophages play an important role in innate and adaptive immune responses by the release of various factors, such as proinflammatory cytokines, reactive oxygen forms, nitrogen species.

LPS treatment induces gene expression by increasing both the cytosolic protein levels of cytokines (IL-1β and IL-6) and pro-inflammatory enzymes (iNOS) by activating the NF-*κ*B transcription factor [31]. TLR4 activates the translocation of NF-*κ*B and AP-1 to the nucleus, followed by binding to inflammatory regulators of cytokines and molecules, including TNFα, IL-6, NO and COX-2, responsible for targeted eicosanoids synthesis during inflammation. LPS can activate several extracellular signaling pathways, including NF-*κ*B and MAPKs [32, 33]. NF-*κ*B is a key transcriptional regulator of the inflammatory response and plays an important role in the development of inflammatory process and cellular damage. It is activated in response to various extracellular stimuli, including oxidative stress, LPS and cytokines [33-35]. TLR4 pathway in macrophages is a key target in studies of the potential properties of multiple phytochemicals exhibiting anti-inflammatory properties [36].

In support of this was the result obtained by us, under LPS treatment, in which the expression of all of the studied inflammation-related genes (TLR4, TNFα, IL-6, IL-1β, MCP-1, iNOS, COX-2 and CRP) was expectedly higher in comparison to the untreated control group of cells (Fig. 1) [37]. In order to investigate possible protective activity of *S. ebulus* fruit preparations in LPS-induced inflammatory response models we performed a 24 h pretreatment of the cells with 5, 15 and 135 μg/mL extract/fraction in culture media (Fig. 1).



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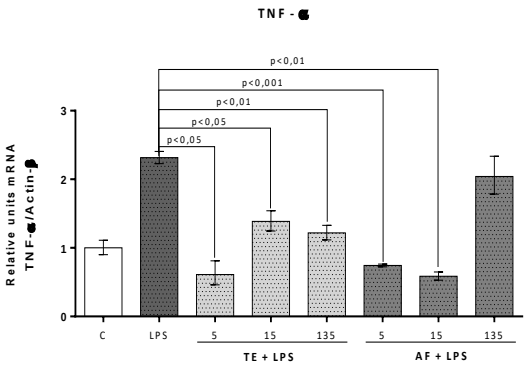

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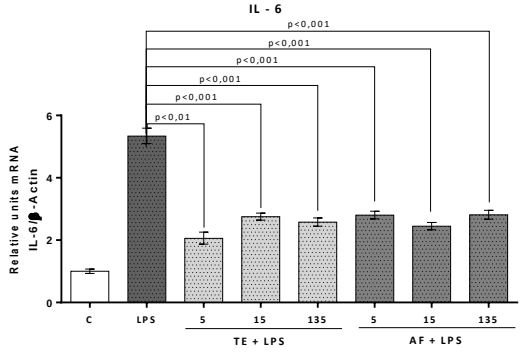

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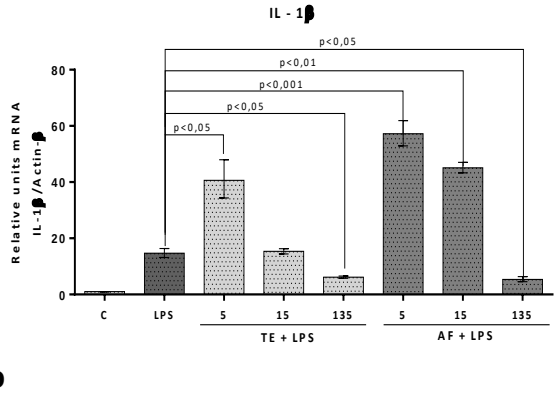

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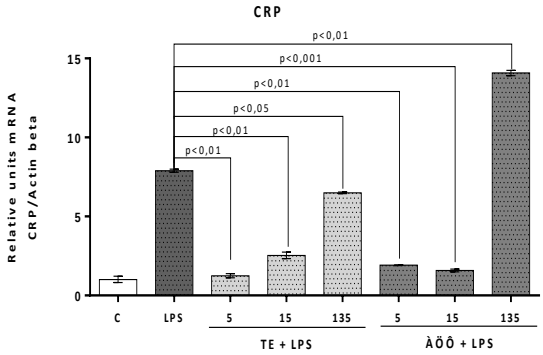

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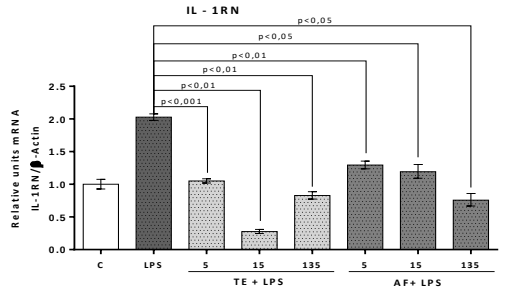

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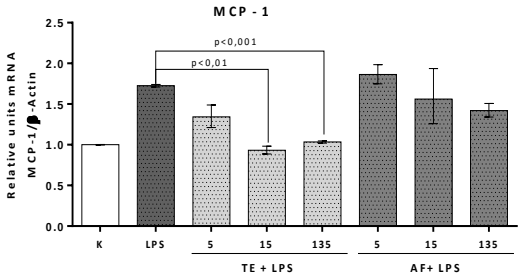

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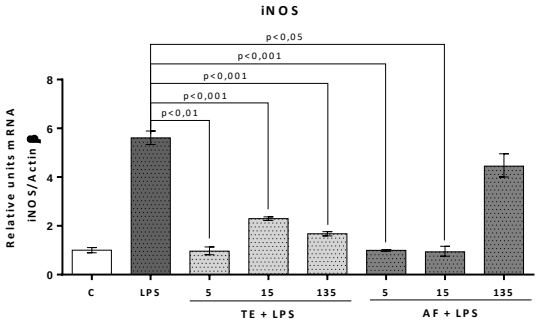

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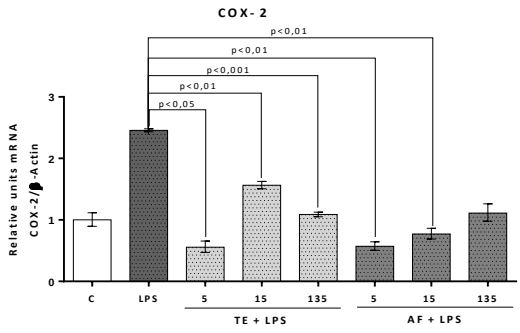

H

Figure 1. Inflammation-related genes expression levels in J774A.1 macrophages treated with 5, 15 and 135 μg/mL *S.ebulus* total extract (TE) and anthocyanin fraction (AF) in presence of 100 ng/mL LPS. (A) TLR-4, (B) TNF-α, (C) IL-6, (D) IL-1β, (E) IL-1RN, (F) MCP-1, (G) iNOS, (H) COX-2, (J) CRP.

Pretreatment of macrophages with TE and AF significantly reduced the upregulating effect of LPS on the expression of IL-6 and IL1-RN in all applied concentrations of TE and AF. Similar was the effect on TLR-4, iNOS, TNFα, COX-2 and CRP, with the exception of the highest AF concentration applied (135 μg/mL).

For the experiment we have selected 5, 15 and 135 μg/mL concentrations of polyphenols in the TE and AF extracts. These concentrations were selected on the basis of previous non-published data about TE and AF cytotoxicity using a MTT test. Results of the test represented slight proliferative and strong proliferative activity for 5 μg/mL and 15 μg/mL, respectively and cytotoxic effect of 135 μg/mL for both TE and AF. The expected inhibitory effect of 135 μg/mL on the gene expression of these gens in LPS stimulated macrophages might be due to its cytotoxic activity, which interferes and predominates over AF anti-inflammatory potential. AF had no significant lowering effect on LPS-induced MCP-1 expression.

The overall assumption is that herbal extracts and natural products can stimulate the immune system in conditions of tumor and infectious diseases [5, 20, 38] and to positively suppress it in conditions of autoimmune diseases [39, 40]. There are a lot of data demonstrating a possible immune stimulatory activity of plant preparations and they refer predominantly to unstimulated cells, e.g., for various preparations containing *Echinacea purpurea* on RAW264.7 macrophage cell culture [19]. *Uncaria tomentosa* extract has the same effect in experiments with primary rat macrophages [20], increasing IL-6 expression under the influence of the extract, both in non-stimulated and LPS-treated cells. Aqueous

extract of *Platycodon grandiflorum* stimulates the expression of IL-6 in murine peritoneal macrophages [21], and aqueous extract of *Prunella vulgaris* - the expression of IL-6 in RAW264.7 macrophages [5]. Our studies have also found induction expression of this cytokine as a result of the treatment of macrophages (J774A.1) with a plant extract from *Agrimonia eupatoria* [7]. Concerning the *in vivo* effects, the data are contradictory - from inhibitory [22, 23] to activatory [24, 25].

The potential of phytochemicals and/or plant extracts to decrease the effect of various stimuli on inflammation has been established by a number of studies. For example, some anthocyanins show an inhibitory effect on COX-2 by inhibiting C/EBP, AP-1, and NF-κB [29], as well as by suppressing iNOS enzyme and mRNA expression in LPS-stimulated RAW 264 cells [20, 30]. The anthocyanin-rich red raspberry fraction exhibits anti-inflammatory properties by suppression of the expression of iNOS and COX-2, as well as the activity of IKK in LPS/IFN-γ stimulated RAW264.7 macrophages [8]. Anthocyanins in raspberry extracts suppress LPS-induced NF-κB activation and COX-2 production. Subsequently, suppression of COX-2 gene expression may reduce the production of pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNFα [31, 32].

Namely, suppression of above mentioned pathways may be a possible mechanism for the anti-inflammatory effects of *S. ebulus* extracts. Aqueous and ethanol extracts from *S. ebulus* have been previously established to inhibit NF-κB transcription in cells [36].

Expression of phagocytosis-associated genes

In pro-inflammatory activation, phagocytic cells, such as macrophages and monocytes, produce a large amount of ROS, mainly in the form of superoxide anion and subsequent formation of radicals that accompanies the "respiratory burst" [39]. The process of high oxygen consumption and production of the superoxide anion that accompanies the respiratory burst is controlled by the phagocytic NADPH oxidase (NOX enzyme) [40]. Activated phagocytic oxidase releases a superoxide anion within the phagosome, which then undergoes disruption in H_2O_2 by superoxide dismutase (SOD). Myeloperoxidase catalyzes the next reaction of formation of hypochlorous acid. In our study we analyzed the expression levels of two phagocytosis related genes: NOX and MPO.

In LPS-stimulated macrophages, the NOX and MPO expression levels were significantly increased over the untreated control ($p < 0.001$) (Fig. 2). In this study, we show that TE and AF of *S. ebulus* fruit inhibit the MPO and NOX expression in LPS-stimulated macrophages.

TE and AF from *S. ebulus* fruits inhibited in a concentration-dependent manner the expression of both MPO and NOX, which explains their anti-inflammatory effect in LPS-stimulated macrophages. MPO stimulates macrophages to produce active oxygen forms (AOF), which in turn enhances the expression of TNFα and other proinflammatory cytokines [41, 42]. Our findings are in line with reports by other authors [43]. The result obtained in our study is confirmed by other authors in studies done on cells treated with strawberry extracts before incubation with LPS [44]. These results suggest that TE and AF of *S. ebulus* fruits can reduce LPS-induced inflammation indirectly by inhibiting MPO-related release and inflammation. Pre-treatment of J774A.1 macrophages with TE and AF from fruits of *S. ebulus* decreases intracellular AOF levels, attenuating apoptosis in LPS-treated cells. This makes it possible to claim that berry extracts counteract LPS-mediated inflammation by modulating signaling pathways and reducing AOF.

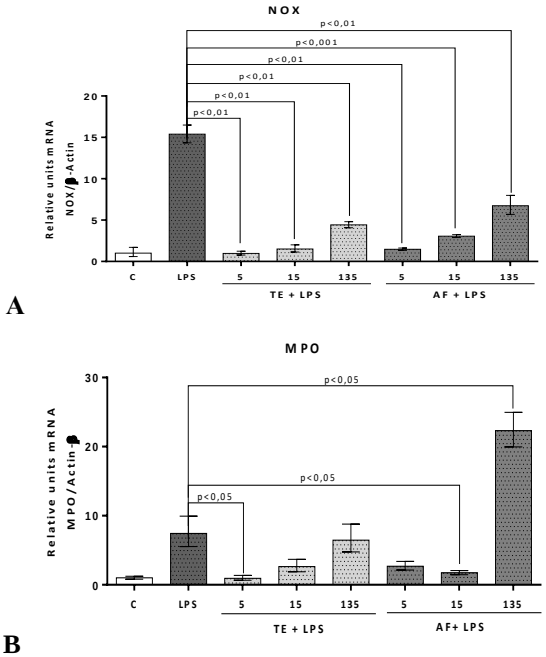

Figure 2. Phagocytosis-related genes expression levels in J774A.1 macrophages treated with 5, 15 and 135 μg/mL *S. ebulus* total extract (TE) and anthocyanin fraction (AF) in presence of 100 ng/mL LPS (A) NOX, (B) MPO.

Fruits of *S. ebulus are* rich in various polyphenol compounds, and it is difficult to identify a single component responsible for the observed effect. In future studies, it is necessary to perform additional

studies of the structure-activity relationship or to perform biocontrolled isolation of phenolic fractions.

CONCLUSIONS

The investigated TE and ACF have an effect on processes related to immune defense - inflammation and phagocytosis. Under conditions of induced inflammatory response, the test extracts administered as pretreatment mitigate the stimulatory action of LPS by lowering the expression levels of all the genes involved in inflammation (TLR-4, TNFα, IL-6, IL-1β, IL-1RN, CRP, COX-2; iNOS) and phagocytosis (NOX, MPO). The TE and the AF exhibit a pronounced modulatory effect on the expression of inflammation- and phagocytosis-related proteins. This finding supports the indications of the folk medicine about the use of *S. ebulus* fruits for improvement of immune response against infections.

Additional *in vivo* and analytical studies are needed to further disclose the mechanisms of action of the fruit extracts of *S. ebulus* in order to obtain more data on its feasibility as a feedstock in functional foods and curative remedies development and application as supportive and complementary therapy.

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REFERENCES

1. S. Kultur, *J Ethnopharmacol*., **111**, 341 (2007).
2. B. Saric-Kundalic, C. Dobes, V. Klatte-Asselmeyer, J. Saukel, *J Ethnopharmacol,*. **131(**1), 33 (2010).
3. M. El Beyrouthy, N. Arnold, A. Delelis-Dusollier, F. Dupont, *J Ethnopharmacol.*, **120**, 315 (2008).
4. M. Kähkönen, A. Hopia, H. Vuorela, J. Rauha, K. Pihlaja, T. Kujala, M. Heinonen, *J. Agric. Food Chem.*, **47**, 3954 (1999).
5. E. Han, J. Choi, Y. Hwang, H. Park, C. Choi, Y. Chung, *Food Chem. Toxicol.*,**47**, 62 (2009).
6. S. W. Min, S. N. Ryu, D. H. Kim, *Int. Immunopharmacol.*, **10**, 959 (2010).
7. D. Ivanova, O. Tasinov, D. Vankova, Y. Kiselova, *Bulg. J. Agric. Sci*., **19**, 171 (2012).
8. L. Li, L. Wang, Z. Wu1, L. Yao1, Y. Wu, L. Huang, K. Liu, X. Zhou, D. Gou1, *Scientific Reports*, **4**, 6234 (2014).
9. X. Zhang, H. Wang, H. Song, *Bioorg. Med. Chem. Lett.*, **16**, 949 (2006).
10. K. Patil, S. Jalalpure, S. Wadekar, *Indian J. Pharm. Sci.*, **71**, 68 (2009).
11. N. Cumashi, M. Ushakova, M. Preobrazhenskaya, *Glycobiology*, **17**, 541 (2007).
12. H. Kim, J. Lim, W. Kim, *The Proceedings of the Nutritional Society*, **71**, 166 (2012).
13. M. Nassiri Asl, H. Hosseinzadeh, *Phytother. Res.*, **22**, 709 (2008).
14. A. Hiermann, Hagers Enzyklopädie der Arzneistoffe und Drogen, 14th edn., Stuttgart, Wissenschaftliche Verlagsgesellschaft mbH, 2007.
15. A. Nelly, D. Annick, D. Frederic, *J. Ethnopharmacol*., **120**, 315 (2008).
16. J. Benitez, J. M. Ferreras, R. Munoz, Y. Arias, R. Iglesias, M. Cordoba-Diaz, R. del Villar, T, *Med. Chem*., **1**, 65 (2005).
17. Y. Hwang, J. Choi, H. Yun, E. Han, H. Kim, J. Kim, *Food Chem. Toxicol.*, **49**, 93 (2011).
18. W. Shusong, H. Xi, W. Xiaosong, Q. Si, H. Jianhua, Z. Shirui, H. De-Xing, *J. Funct. Foods*, **17**, 514 (2015).
19. J. Rininger, S. Kickner, P. Chigurupati, *J. Leucoc. Biol.*, **68,** 503 (2000).
20. I. Lemaire, V. Assinewe, P. Cano, *J. Ethnopharmacol.*, **64**, 109 (1999).
21. C. Choi, J. Kim, Y. Kim, *Cancer Letters*, **166**, 17 (2001).
22. D. Arçari, W. Bartchewsky, T. W. dos Santos, *Obesity*, **17**, 2127 (2009).
23. C. Chuang, A. Bumrungpert, A. Kennedy, A. Overman, T. West, B. Dawson, M. McIntosh, *J. Nutr. Biochem.*, **22**, 89 (2011).
24. V. Barak, T. Halperin, I. Kalickman, *Eur. Cytokinet. Netw.*, **12(**2), 290 (2001).
25. A. Sullivan, J. Laba, J. Moore, *Immunopharmacol. Immunotoxicol.*, **30**, 553 (2008).
26. D. Hou, K. Kai, J. Li, S. Lin, N. Terahara, M. Wakamatsu, *Carcinogenesis*, **25**, 29 (2004).
27. M. Hamalainen, R. Nieminen, P. Vuorela, M. Heinonen, E. Moilanen, *Mediators Inflamm*., 45673 (2007).
28. A. Munoz-Espada, B. Watkins, *J. Nutr. Biochem*., **17**, 589 (2006).
29. J. Wang, G. Mazza, *J. Agric. Food Chem*., **50**, 850 (2002).
30. M. Jabbari, B. Daneshfard, M. Emtiazy, A. Khiveh, M. Hashempur, *J. Evid.-Based Integr. Med*., **22**, 996 (2017).
31. S. Kim, J. Park, J. Lee, W. Yang, G. Park, H. Kim, *Mediators Inflamm.*, 1 (2015).
32. Y. Lu, S. Suh, C. Kwak, K. Kwon, C. Seo, Y. Li, Y. Jin, X. Li, S. Hwang, O. Kwon, Y. Chang, Y. Park, S. Park, J. Son, C. Kim, H. Chang, *Int. Immunopharmacol.*, **12**, 175 (2012).
33. T. Lawrence, *Cold Spring Harb. Perspect. Biol*, **1**, a001651 (2009).
34. J. Park, H. Cho, J. Kim, K. Noh, J. Yang, J. Ahn, M. Lee, Y. Song, *Clin. Chim. Acta*., **351**, 185 (2005).
35. C. Park, J. Park., K. Noh, J. Shin, Y. Song, *J. Ethnopharmacol*, **133**, 834 (2011).
36. A. Jacobs, L. Ignarro, *J. Biol. Chem*., **276**, 47950 (2001).
37. O. Tasinov, PhD Thesis, MU, Varna, 2015.
38. D. Kelley, R. Rasooly, R. Jacob, A. Kader, B. Mackey, *J. Nutr.*, **136**, 981 (2006).

39. M. Valko, D. Leibfritz, J. Moncol, M. Cronin, M. Mazur, J. Telser, *IJBCB*, **39**, 44 (2007).
40. W. Nauseef, *J. Biol. Chem*., **283**, 16961 (2008).
41. S. Nelson, W. Summer, *Infect. Dis. Clin. North Am*., **12**, 555 (1998).
42. M. Gelderman, D. Lefkowitz, S. Lefkowitz, A. Bollen, N. Moguilevsky, *Proc. Soc. Exp. Biol. Med*., **217**, 81 (1998).
43. Y. Fu, E. Zhou, Z. Wei, W. Wang, T. Wang, Z. Yang, N. Zhang. *Biochem. Pharmacol*., **90**, 126 (2014).
44. J. Mo, P. Panichayupakaranant, N. Kaewnopparat, A. Nitiruangjaras, W. Reanmongkol, *J Ethnopharmacol.*, **148**, 901 (2013).