

## *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

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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, TNF $\alpha$ , IL-6, IL-1 $\beta$ , 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, TNF $\alpha$ , IL-6, IL-1 $\beta$ , 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].

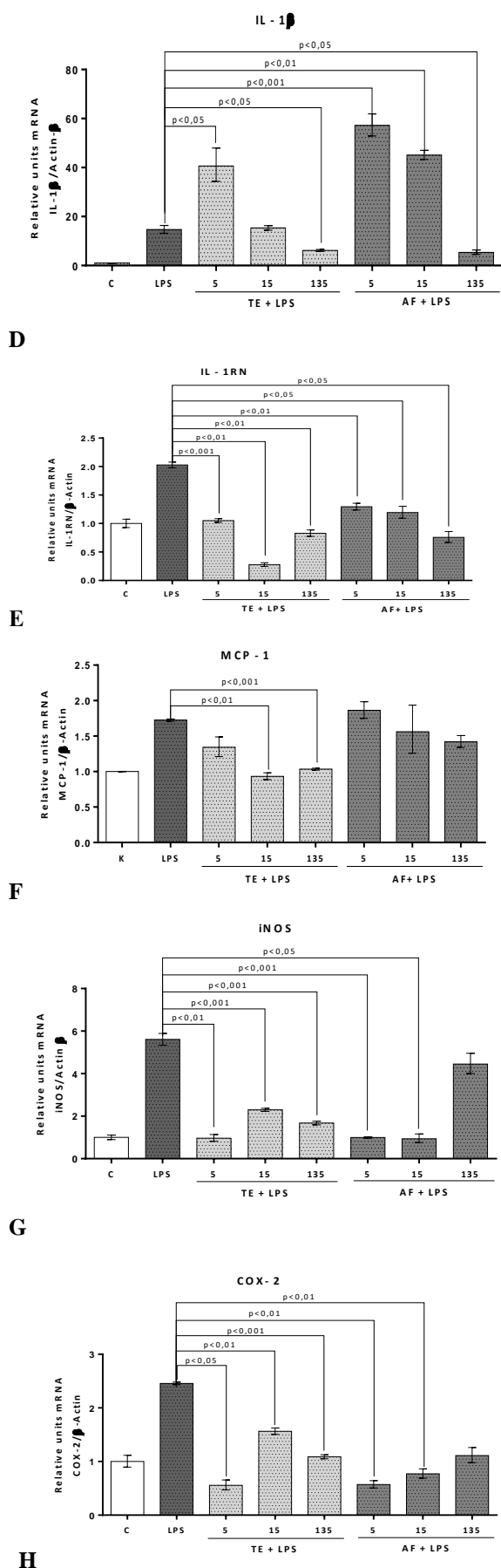
The expected inhibitory effects of plant extracts on the expression of inflammatory factors are described in detail in the literature [8, 20, 21]. However, there are few reports of a stimulatory effect on the expression and such studies are related to studies designed to demonstrate immune-stimulatory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

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**Figure 1.** Inflammation-related genes expression levels in J774A.1 macrophages treated with 5, 15 and 135  $\mu\text{g/mL}$  *S. ebulus* total extract (TE) and anthocyanin fraction (AF) in presence of 100 ng/mL LPS. (A) TLR-4, (B) TNF- $\alpha$ , (C) IL-6, (D) IL-1 $\beta$ , (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, TNF $\alpha$ , COX-2 and CRP, with the exception of the highest AF concentration applied (135  $\mu\text{g/mL}$ ).

For the experiment we have selected 5, 15 and 135  $\mu\text{g/mL}$  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  $\mu\text{g/mL}$  and 15  $\mu\text{g/mL}$ , respectively and cytotoxic effect of 135  $\mu\text{g/mL}$  for both TE and AF. The expected inhibitory effect of 135  $\mu\text{g/mL}$  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- $\kappa$ B [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- $\gamma$  stimulated RAW264.7 macrophages [8]. Anthocyanins in raspberry extracts suppress LPS-induced NF- $\kappa$ B activation and COX-2 production. Subsequently, suppression of COX-2 gene expression may reduce the production of pro-inflammatory cytokines IL-1 $\beta$ , IL-6, IL-8 and TNF $\alpha$  [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- $\kappa$ B transcription in cells [36].

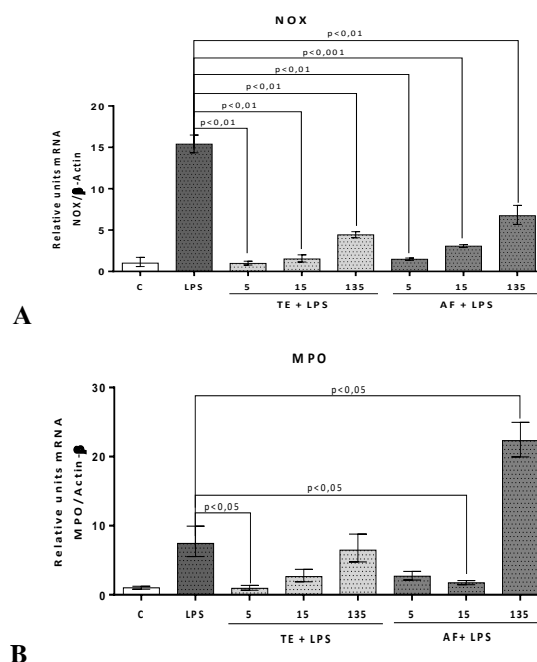
#### Expression of phagocytosis-associated genes

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<sub>2</sub>O<sub>2</sub> 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 TNF $\alpha$  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  $\mu$ g/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, TNF $\alpha$ , IL-6, IL-1 $\beta$ , 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. Klatter-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. Wu<sup>1</sup>, L. Yao<sup>1</sup>, Y. Wu, L. Huang, K. Liu, X. Zhou, D. Gou<sup>1</sup>, *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).