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ORIGINAL ARTICLE

Effects of *Sambucus nigra* and *Aronia melanocarpa* extracts on immune system disorders within diabetes mellitusMagda Badescu¹, Oana Badulescu¹, Laurentiu Badescu², and Manuela Ciocoiu¹¹Department of Pathophysiology and ²Department of Cellular and Molecular Biology, University of Medicine and Pharmacy “Grigore T. Popa” Iasi, Iasi, Romania

Abstract

Context: The fruits of *Aronia melanocarpa* Elliot (Rosaceae), (black chokeberry), and *Sambucus nigra* L. (Caprifoliaceae), elderberries are rich in anthocyanins. Many studies have reported that anthocyanins are beneficial in diabetes due to their capacity to stimulate insulin secretion and reduce oxidative stress.

Objective: The purpose of this study is to prove the biologically active properties of polyphenols extracted from *S. nigra* and *A. melanocarpa* fruit. The study also details the influence of plant polyphenols on immune system imbalances within diabetes mellitus.

Materials and methods: Polyphenolic extract was administered to Wistar rats 0.040 g/kg body every 2 d for 16 weeks. The absorbencies of all the solutions were determined using a V-550 Able Jasco UV-VIS spectrophotometer. The immunomodulatory capacity of vegetal extracts was assessed by studying cytokines TNF- α and IFN- γ through the ELISA method and fibrinogen values.

Results and discussion: At 48 h, the anti-inflammatory effects of *S. nigra* and *A. melanocarpa* substances have been revealed by an increase of the TNF- α and IFN- γ levels in the diabetic group protected by these extracts. Seventy-two hours post-administration of both substances in the diabetic groups, the TNF- α level returns to the values read 24 h after substance administration. The vegetal extracts limit the production of fibrinogen in the diabetic rats under polyphenolic protection, the values being highly significant compared with the diabetic group.

Conclusions: Natural polyphenols extracted from *S. nigra* and *A. melanocarpa* modulate specific and non-specific immune defenses in insulin-deficiency diabetes and reduce the inflammatory status and self-sustained pancreatic insulinitis.

Keywords

Fibrinogen, IFN- γ , polyphenols, TNF- α

History

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Introduction

Patients with diabetes have increased serum expression of key inflammatory cytokines. One hypothesis is that hyperglycemia and the associated oxidative stress damages endothelial cells which in turn release cytokines and chemokines (Groot & Rauwen, 1998). Studies have shown that hyperglycemia-induced glycation end-products stimulate secretion of monocyte chemoattractant protein from human mesangial cells *in vitro* (Foss-Freitas et al., 2006; Yamagishi et al., 2002). The kidneys in streptozotocin-induced diabetic rat models also express elevated levels of inflammatory cytokines (TNF- α and IFN- γ) which are produced by cells in the early and late phases of the disease (Brownlee, 2001; Mensah-Brown et al., 2005).

The study of the effects of some natural extracts on the cellular and molecular mechanisms involved in immune

system disorders within diabetes mellitus is one of the main directions of the current research. Polyphenols may affect glycemia through different mechanisms, including the inhibition of glucose absorption in the gut or inhibition of its uptake by peripheral tissues (Cheynier, 2005; Manach et al., 2004; Yao et al., 2004). The metabolites of polyphenols are rapidly eliminated from plasma, which indicates that consumption of plant products on a daily basis is necessary to maintain high concentrations of metabolites in the blood (Yeh et al., 2003).

Polyphenols might stimulate TNF- α and IFN- γ secretion, thus limiting inflammatory status and development of microvascular diabetic complications. The purpose of our study is to prove the biologically active properties of polyphenols extracted from *Sambucus nigra* L. (Caprifoliaceae) (elderberries) and *Aronia melanocarpa* Elliot (Rosaceae), (black chokeberry) fruit, and to find out the way plant polyphenols influence immune system imbalances within induced diabetes mellitus. The immunomodulatory capacity of substances *S. nigra* and *A. melanocarpa* was assessed by studying their influence on the secretion of cytokines TNF- α and IFN- γ , as well as the fibrinogen value.

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Material and methods

Ripe berries of *A. melanocarpa* and *S. nigra* were collected in Botanical Garden, Iasi, Romania, in October 2009. The plant material was identified by Dr. Violeta Tanasescu. A herbarium voucher sample (AMF.09) is deposited in the Department of Pharmacognosy, School of Pharmacy, University of Medicine and Pharmacy “Gr. T. Popa”, Iasi, Romania.

Preparation of extract and chemical determinations

Sambucus nigra and *Aronia melanocarpa* fruits were washed and shade-dried. Dried and powdered fruits (50 g) were extracted with 2×250 mL acidulated methanol (0.5% HCl) using a magnetic stirrer, each time for 1 h. The extract was concentrated to dryness under reduced pressure (Büchi R-210 rotavapor, Büchi V-850 pressure controller, Büchi V-700 vacuum pump; BÜCHI Labortechnik AG, Flawil, Switzerland). The extract was completely dried before administration to animals. The amount of dry vegetable material extracted was 42.31 g/100 g from *S. nigra* fruit and 69.34 g/100 g from *A. melanocarpa* fruit. The total phenolic content in elder fruit extract was determined by the Singleton and Rossi method (Singleton & Rossi, 1965). The amount of total phenolic content was expressed as gallic acid equivalents (GAE)/100 g extract. The absorbencies of all the solutions were determined by using a V-550 Able Jasco UV–VIS spectrophotometer (JASCO, Easton, MD). The result is the mean of triplicates $\pm$ standard deviation. The total content of polyphenols was 7.63 ± 0.12 mg GAE/100 g from *S. nigra* extract and 3.67 ± 0.31 mg GAE/100 g from *A. melanocarpa* extract.

The dry polyphenol extract was diluted in dimethylsulphoxide (DMSO), 100 mL polyphenolic solution containing 840 mg natural polyphenols, 95 mL distilled water, and 5 mL DMSO. After repeated testing, it was found that the dose of polyphenols extracted from the fruits of *A. melanocarpa* and *S. nigra* to be administered as an enteral solution (by tube feeding) is 0.040 g/kg body every 2 d. The experiment used active therapeutic doses, well-determined fractions of DL50 on an experimental model of diabetes mellitus. Values of DL50 were determined using the Probit analysis method (Finney, 1971). Fractions of DL50 are doses representing 1/5, 1/10, 1/20, and 1/40 of DL50. The dose representing 1/20 of DL50 was chosen, as it is the smallest dose that determined the pharmacodynamic effect that is being researched, without producing significant toxic effects.

Experimental model

The research was carried out on Wistar white male rats, aged 28 weeks at the beginning of the study, weighing 250–280 g on the average, which were divided into four 12-rat groups, namely: *W* group – control, normal animals, that were not given natural polyphenols; *DM* group – rats with diabetes caused by the rapid intraperitoneal injection of a single dose of streptozotocin (STZ); *A* group – rats who were administered *A. melanocarpa* polyphenolic extract for a period of 16 weeks; *S* group – rats who were administered *S. nigra* polyphenolic extract for a period of 16 weeks; *DM+A* group – rats who were administered an *A. melanocarpa* polyphenolic extract for 16 weeks after diabetes mellitus induction; *DM+S* group – rats who were administered a *S. nigra*

polyphenolic extract for 16 weeks after diabetes mellitus induction.

Diabetes was induced by STZ [2-deoxy-2(3-methyl-5-nitro-ureido)-*p*-glucopyranose] (cytotoxic antibiotic synthesized of *Streptomyces achromogenes*) obtained from Sigma S-0130 (Sigma, St. Louis, MO), Batch 31K1379, and delivered in a single dose of 60 mg/kg body mass, as 1% intraperitoneal (i.p.) solution, after 18 h of fasting. Streptozotocin delivery causes insulin-deficiency diabetes through selective endocrino-chemical pancreatectomy, destroying only the cells of Langerhans' islets (Saltiel & Pessin, 2002).

At the end of the experiment, the 44-week-old animals were killed by cardiac puncture under ketamine anesthesia (100 mg/kg body weight). Blood samples were collected using sodium citrate as an anticoagulant buffer, at a 9:1 blood/citrate ratio, or without anticoagulant. Aliquots of plasma and serum were frozen and kept at -80°C for later analysis.

Biochemical methodology

TNF- α and *IFN- γ* determination

The immunomodulatory capacity of substances *A. melanocarpa* and *S. nigra* was assessed by studying their influence on the secretion of cytokines *TNF- α* (Pfeffer et al., 1993) and *IFN- γ* (Dalton et al., 1993). For this we used mononuclear cells (i.e. peripheral blood mononuclear cells (PBMC)) from the venous peripheral blood collected from 10 diabetic donors and 10 normal donors. PBMC separation was done by the density gradient centrifugation of 10 mL of heparinized blood collected from each patient, using 5 mL of Ficoll–Hypaque 1077 (Sigma, St. Louis, MO). The PBMC ring formed above the Ficoll layer was collected, washed using 15 mL of simple RPMI (Sigma, St. Louis, MO), and centrifuged at 300 g, at room temperature, for 15 min. After settling, a PBMC count was done in a Bürker–Türk counting chamber. The PBMC were then resuspended in a volume of RPMI + 5% fetal serum designed to allow their culturing in 96-well culture plates at a concentration of 1×10^6 cells/mL/well. PBMC were incubated with substance *S. nigra/A. melanocarpa* in different concentrations (50, 100, and 150 $\mu\text{g/mL}$ /well). The culture plates were incubated in an incubator at 37°C , 5% CO_2 . The cytokines secreted in the PBMC supernatant were measured after 24, 48, and 72 h of incubation, when the culture plates were centrifuged at 300 g, at room temperature, for 5 min. The supernatant in each well was collected and used to determine the *TNF- α* and *IFN- γ* values by the ELISA method, using Endogen[®] Human *TNF- α* and *IFN- γ* ELISA kits, respectively.

The value of the fibrinogen was coagulometrically determined, using the Clauss (1957) method. The experimental study fulfils all the requirements of the guide regarding the use of laboratory animals and biological preparations issued by the International Society of Pain Study (IASP) and the European Council Committee (86/609/EEC). This study was approved by the Laboratory Animal Care Committee of “Grigore T. Popa” University of Medicine and Pharmacy and the rats were kept in accordance with the general guidelines for the care and use of laboratory animals recommended by the Council of European Communities (approval no. 9803/12.09.2006).

Statistical data interpretation

Data were expressed as means $\pm$ standard deviation (SD). Mann–Whitney paired test is basically the same in that they compare between two medians to suggest whether both samples come from the same population or not. A test was performed to determine whether there were significant ($p < 0.05$) differences between groups, using Statistical Software Package SPSS®, version 13 (SPSS Incorporation, Chicago, IL).

The statistical interpretation of the data considered the corresponding differences for a significance threshold: $p > 0.05$ statistically insignificant; $p < 0.01$ strong statistical significance; $p < 0.001$ very strong statistical significance.

The Pearson coefficient (r) is a measure of the linear correlation (dependence) between two variables, giving a value between +1 and -1 inclusive, where 1 is the total positive correlation, 0 is no correlation, and -1 is the total negative correlation. It is widely used as a measure of the degree of linear dependence between two variables.

Results

The TNF- α serum level was checked in both control and diabetic rats groups 24, 48, and 72 h, after substance A. *melanocarpa* or *S. nigra* was administered. About 24 h after substance administration, the control rats that were given

substance A. *melanocarpa* had significantly higher TNF- α values than those that were given the same concentration of substance *S. nigra*, but only when the concentration was 150 $\mu\text{g/mL}$ ($p < 0.001$) (Figure 1). About 24 h after substance administration, the diabetic rats included in the DM + A and DM + S groups that were given the same concentration of substance, i.e., 150 $\mu\text{g/mL}$, had a significantly higher TNF- α secretion value than the DM group ($p < 0.01$).

Forty-eight hours after substance administration, the control rats that were given substance *S. nigra* had significantly different mean TNF- α values, starting with 50 $\mu\text{g/mL}$, as compared with the control rats that were given substance A. *melanocarpa* ($p < 0.01$) (Figure 2). The control rats that were administered the same concentration of substance A. *melanocarpa* had significantly higher values of TNF- α than those that were administered substance *S. nigra*, starting with a concentration of 100 $\mu\text{g/mL}$ ($p < 0.01$). The diabetic rats that were given 150 $\mu\text{g/mL}$ of substance A. *melanocarpa* exhibited significantly lower mean TNF- α secretion values than the control rats ($p < 0.01$); statistically speaking, the mean TNF- α values were significantly lower in the diabetic rats than in the DM + S group, regardless of the concentration of substance *S. nigra* that was administered ($p < 0.01$ at a concentration of 50 $\mu\text{g/mL}$, and $p < 0.001$ at over 100 $\mu\text{g/mL}$ of substance *S. nigra*).

Figure 1. Influence of substances S and A on TNF- α secretion 24 h after administration: in the diabetic groups compared with controls were found significantly lower average TNF- α values in groups receiving substance A (except for the dose of 50 mg/mL ($p < 0.05$), or substance S regardless of the concentration ($p < 0.01$ at a concentration of 50 mg/mL and $p < 0.001$ at a concentration 100 mg/mL). Diabetic rats receiving in 24 h substance A or S at a concentration of 150 mg/mL caused a significant increase in TNF- α as compared with the diabetic rats who had not received either A or S ($p < 0.01$). In diabetic rats, the same concentrations of solutions A and S did not result in significant differences ($p < 0.05$).

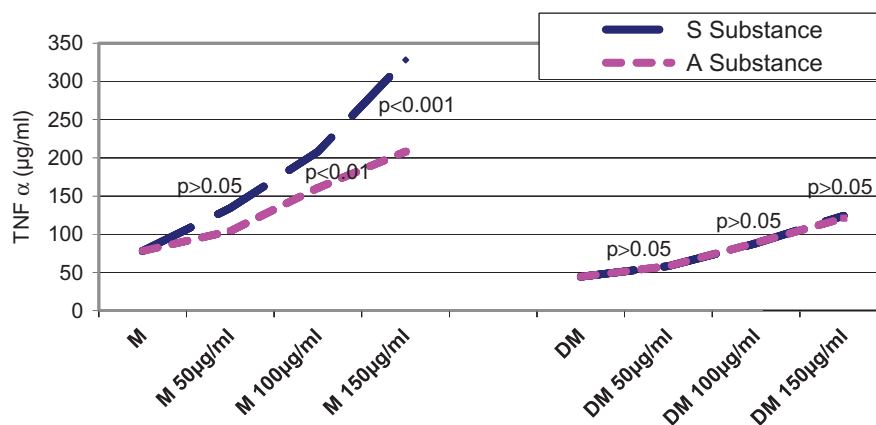
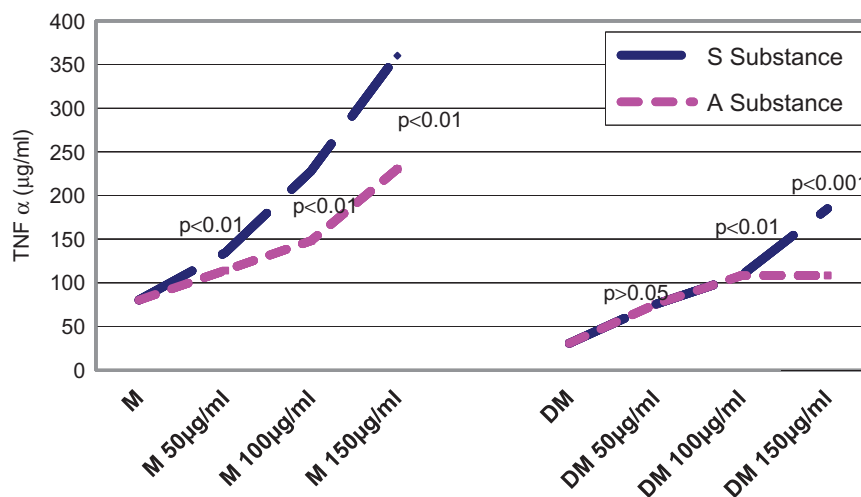


Figure 2. Influence of substances S and A on TNF- α secretion 48 h after administration: 48 h after administration, both control and diabetic rats have shown a significant increase in a dose-dependent secretion of TNF- α for both substances. Average values of TNF- α were significantly lower in diabetics compared with controls, regardless of the concentration or substance administered ($p < 0.01$ at a concentration of 50 mg/mL and $p < 0.001$ over 100 mg/mL).



Seventy-two hours after substance administration, the diabetic groups that were given the same concentrations of substance *A. melanocarpa* or *S. nigra* did not exhibit statistically significant differences ($p > 0.05$) (Figure 3).

The influence of substances *A. melanocarpa* and *S. nigra* on IFN- γ secretion in control and diabetic rats groups was assessed at different points in time, i.e., 24, 48, and 72 h after administration. About 24 h after substance administration in concentrations of at least 100 $\mu\text{g/mL}$, IFN- γ secretion was significantly higher in the DM + S or DM + A group rats than in the DM group rats ($p < 0.05$) (Figure 4).

In the case of substance *A. melanocarpa*, an increase in IFN- γ secretion was noticed after 48 h of administration, regardless of the group. The increase was significant in groups which received substance *A. melanocarpa*, compared with those who received substance *S. nigra*. As far as

the diabetic group that was administered substance *A. melanocarpa* is concerned, IFN- γ increases significantly in time with the increase of the administered dose of substance *A. melanocarpa*. The IFN- γ serum level was much higher when the substance *S. nigra* was administered than when substance *A. melanocarpa* was given, regardless of the dose administered to the diabetic group.

Forty-eight hours after the administration of substance *S. nigra* or *A. melanocarpa*, the control rats that were given the same concentration of substance *A. melanocarpa*, i.e. 100 $\mu\text{g/mL}$, exhibited significantly higher values than the ones that were administered substance S ($p < 0.01$) (Figure 5). Forty-eight hours after the administration of at least 50 $\mu\text{g/mL}$ of substance *A. melanocarpa*, IFN- γ secretion increased very significantly in the diabetic rats that were given the substance as compared with the regular diabetic rats ($p < 0.001$).

Figure 3. Influence of substances S and A on TNF- α secretion 72 h after administration: the level of TNF- α after 72 h from the administration of both substances, both in control groups and in diabetic groups, it is about the same values and the same characteristics as those recorded at 24 h after administration.

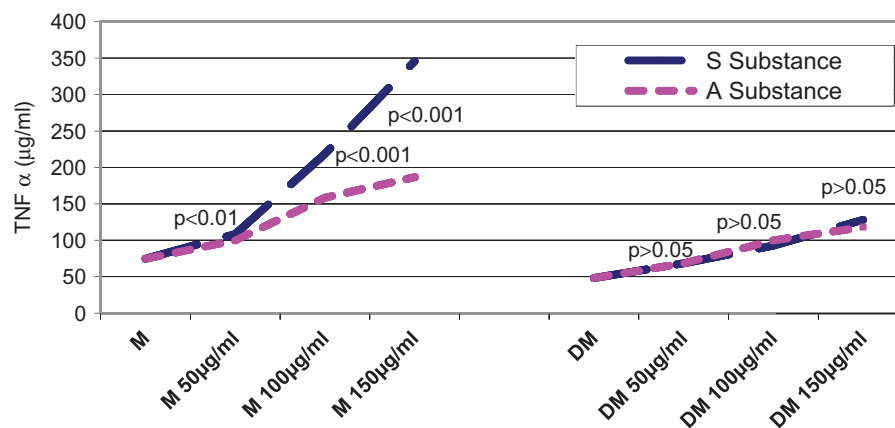


Figure 4. Influence of substances S and A on IFN- γ secretion 24 h after administration: diabetic group receiving at 24 h, either substance S or substance A, in a concentration of at least 100 mg/mL had a significant increase in secretion of IFN- γ compared with the diabetic group to which these substances have not been administered ($p < 0.05$). At the same concentration, A and S have shown significant differences ($S > A$) if administered with a concentration of at least 100 mg/mL ($p < 0.05$).

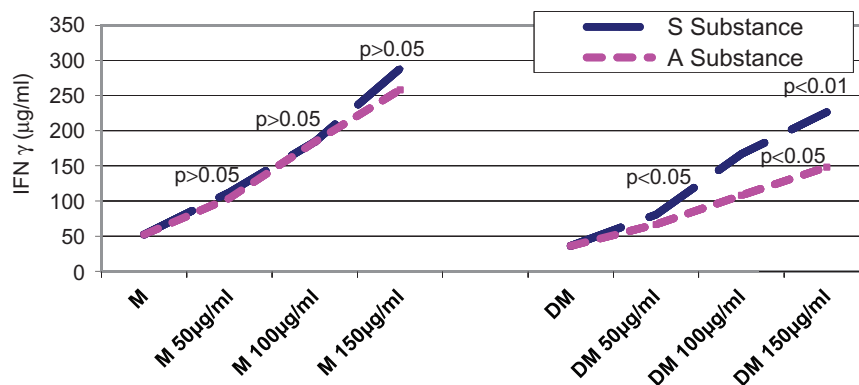


Figure 5. Influence of substances S and A on IFN- γ secretion 48 h after administration: in diabetic rats after 48 h of taking at least 50 mg/mL of substance S, the secretion of IFN- γ has increased significantly in comparison with the untreated diabetic rats ($p < 0.01$); the same phenomenon is observed with the administration of A substance as well, but the serum level of IFN- γ was lower than in the case of the administration of substance S regardless of the dose.

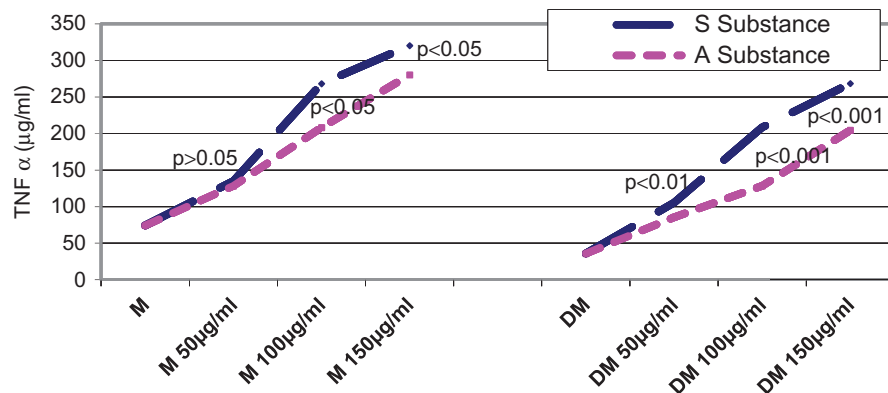


Figure 6. Influence of substances S and A on IFN- γ secretion 72 h after administration: the diabetic group who received at least 00mL of either S or A substance, compared with the untreated diabetic group, was evidenced a significant increase in IFN- γ ($p < 0.001$); diabetic groups who received the same concentration of solution A or S did not differ statistically significant ($p > 0.05$).

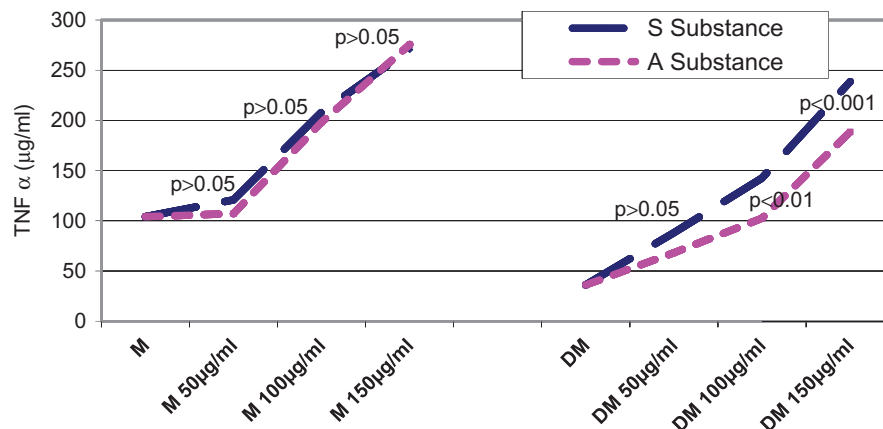


Table 1. Influence of substance *A. melanocarpa* and *S. nigra* on fibrinogen (g/L).

The studied group	M	M + A	M + S	DM	DM + A	DM + S
Mean $\pm$ SD	2.40 $\pm$ 0.22	2.56 $\pm$ 0.35	2.50 $\pm$ 0.24	12.05 $\pm$ 0.66	6.0126 $\pm$ 0.47	5.0346 $\pm$ 0.58
Statistical comparisons with Mann–Whitney paired test						
M/M + A	M/M + S	M/DM	M/DM + A	M/DM + S	M + A/M + S	M + A/DM
$p > 0.05$	$p > 0.05$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p > 0.05$	$p < 0.001$
Pearson's correlation						
$r = -0.380$	–	–	$r = 0.565$		$r = 0.577$	
$p < 0.05$			$p < 0.01$		$p < 0.001$	
M + A/DM + A	M + A/DM + S	M + S/DM	M + S/DM + A	M + S/DM + S	DM/DM + A	DM/DM + S
$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$

Seventy-two hours after substance administration, the control rats that were given substance *S. nigra* or *A. melanocarpa* exhibited very strong significant differences of the mean TNF- α values as compared with the rats that were not given such substances, starting with a concentration of 100 μ g/mL ($p < 0.001$) (Figure 6).

Seventy-two hours after the administration of both substances in the diabetic groups, the TNF- α level returns to approximately the same values read 24 h after substance administration.

The fibrinogen serum concentration is about five times higher in the DM group than in the M group, and it drops very significantly ($p < 0.001$) in the polyphenol-protected groups M + A and M + S. Although it does not reach the reference values, the DM + A and DM + S groups exhibit a fibrinogen serum concentration that is more than 50% lower than in the DM group (Table 1).

Discussion

The direct destruction of the β cells through STZ can expose the “cryptic” antigens of the immune system. The infiltration of the pancreatic islands of Langerhans with mononuclear cells (macrophages and T lymphocytes) is one of the characteristics of non-insulin-dependent diabetes mellitus. The lesion of the β -insular cells occurs following direct contact with the infiltrated cells and/or with the products secreted by these cells, called cytokines.

TNF- α plays a major role in the inflammatory process; it stimulates the macrophages in the synthesis of other proinflammatory cytokines: IL-1, IL-6, and IL-12.

These interleukins, especially IL-1 and IL-6, contribute greatly to inflammation intensification (Heinrich et al., 1990; Pickup et al., 2000). On a local level, TNF- α enhances TCD₈ lymphocytes adhesion to the vascular endothelium and stimulates their cytotoxicity. It is a proinflammatory cytokine. TNF- α is directly involved in the destruction of insular B-cells, in insulin-dependent diabetes mellitus (McNerlan et al., 2002). In contrast, inflammation, which is a non-specific defense mechanism, should be stimulated whenever microbial infections related to diabetes mellitus are involved. Any TNF- α deficit increases the risk of infections related to diabetes mellitus. The immune response is controlled both by direct interactions between different types of co-operating cells and by the synthesis products they secrete (Ozer et al., 2003).

The anti-inflammatory effects of *S. nigra* and *A. melanocarpa* substances have been revealed by an increase of the TNF- α and IFN- γ levels in the diabetic group protected by these two extracts compared with the unprotected diabetic group. Under these circumstances, a statistical check of these differences is necessary to be able to set the best concentration and the most efficient substance for inducing an increase in TNF- α secretion.

The existence in *A. melanocarpa* and *S. nigra* of a high concentration of anthocyanins and proper flavones is a premise for the positive therapeutic effects of these agents on immune system disorders in the evolution of diabetes mellitus (Pascual-Teresa & Sanchez-Ballesta, 2008; Scalbert et al., 2005). Dietary polyphenols may offer an indirect protection by activating endogenous defense systems and by modulating cellular signaling processes such as NF- κ B

activation, glutathione biosynthesis, MAPK proteins, and PI3-kinase/Akt pathway (Gray et al., 2000; Nijveldt et al., 2001; Rahman et al., 2006).

The results of our research are in agreement with those quoted in previous studies, according to which the chronic inflammatory reaction related to diabetes mellitus is improved under the action of vegetal polyphenols (Bell & Gochenaur, 2006; Trompezinski et al., 2003; Ursini et al., 1999).

Interferon γ is secreted by activated lymphocytes under the action of specific antigens or of mitogenic substances and it is a type II interferon. Since, *in vivo*, it is synthesized by activated T lymphocytes, by B series lymphoblasts and by large granular lymphocytes (NK cells), interferon γ is considered to be the immune type (Chen & Liu, 2009).

The inflammatory immune response of the islets of Langerhans is known to represent the pathogenetic stage of type 1 diabetes mellitus and to materialize in the insulinitis process. The monocytes/macrophages and activated T lymphocytes are the cells infiltrating the islets. It is now that the immunological markers, including anti-islet cytoplasmic antibodies (detected by immunofluorescence), anti-insulin antibodies, and many others, may be detected in the serum. Once the destructive autoimmune process is triggered, this will involve the lymphocytes, natural killer cells (NK), macrophages, and mast cells. The cytokines produced by these cells seem to be responsible for the damage suffered by the pancreatic β -cells. The presence in the diabetic patients' serum of one or several immunological markers, in significant concentrations, is higher (reaching up to 90%) during the "immunologically active" period preceding the clinical disease onset and then it drops progressively (Mavridis et al., 2008).

IFN- γ is expressed in the pancreas of diabetic mice and transgenic overexpression of IFN- γ by beta cells in normal mice leads to the development of autoimmune diabetes (Campbell et al., 1991). Furthermore, development of autoimmune diabetes in the non-obese diabetic mice can be prevented by treatment with neutralizing IFN- γ antibodies (Debray-Sachs et al., 1991).

Forty-eight hours after substance administration, substance *S. nigra* determines a significant IFN- γ secretion increase both in the control groups and in the diabetic rats groups. The *in vivo* studies conducted revealed that the *S. nigra* and *A. melanocarpa* polyphenol extract has immunomodulatory properties as it stimulates TNF- α and especially IFN- γ secretion by the peripheral blood mononuclear cells.

The fibrinogen was determined as an acute-phase protein. The extremely high synthesis of the fibrinogen in the diabetic group confirms the self-preserved inflammatory status in the evolution of diabetes mellitus. The polyphenols limit the production of fibrinogen in group DM + A/DM + S, the values being highly significant when compared with the group DM.

Inflammation, and more specifically proinflammatory cytokines and other molecules with a relevant role within the inflammatory process, may be a critical factor in the development of microvascular diabetic complications. This new pathogenic perspective may lead to important new

therapeutic considerations and new therapeutic goals for the treatment of diabetes mellitus.

Conclusions

Natural polyphenols extracted from *S. nigra* and *A. melanocarpa* modulate specific and non-specific immune defense in insulin-deficiency diabetes and reduce the inflammatory status and self-sustained pancreatic insulinitis.

Declaration of interest

The authors declare that they have no competing interests.

Authors' contributions

M. B. participated in study design, has revisited critically the manuscript for important intellectual content, and given final approval of the version to be published. O. B. carried out the sample analyses and collection of data. L. B. performed the statistical analysis and interpretation of data. M. C. carried out the studies and data analyses and drafted the manuscript. All authors read and approved the final version of the manuscript.

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