International Journal of Agriculture, Environment and Bioresearch

Vol. 3, No. 04; 2018

ISSN: 2456-8643

POLYPHENOLS FROM SPENT BLACK COFFEE GROUND MODULATE PLATELET ACTIVITY

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ABSTRACT

Spent coffee grounds (SCG) obtained after preparing different coffee beverages, considered as a waste, is an excellent source of polyphenols with prominent antioxidative activity. The present study is focused on the SCG obtained as a result of preparation of black - so called "Turkish" coffee. The effects of polyphenol-rich black coffee SCG extracts on platelets activation and platelet-monocytes and platelet-neutrophils aggregate formation in vitro were investigated. The antiplatelet activity of defatted black SCG extracts were measured using flow cytometry, in blood samples from healthy subjects. The effects of investigated polyphenol-rich extracts on P-selectin and GPIIbIIIa expression on platelets and their further aggregation with monocytes and neutrophils were assessed in samples activated by adenosine diphosphate as platelet agonist. All tested extracts induced the significant, dose-dependent inhibitory effect both on P-selectin and GPIIb/IIIa receptors (15%). Statistically significant and dose-dependent inhibitory effect was recorded on platelet aggregation with monocytes (20%) and neutrophils (22%). These results suggest that black coffee SCG can be recommended as a valuable and low-cost source of polyphenols with observed antiplatelet activity that could be used as an ingredient in functional food.

Keywords: Black coffee, flow cytometry, spent coffee grounds, platelets, polyphenols

1. INTRODUCTION

Coffee is one of the most popular and the most traded beverage today, consumed many times a day by a large number of people all over the world. Different types of coffee brew, the Turkish coffee, Espresso, Cappuccino, Vienna coffee, Mazagran coffee or Ireland coffee, refer to different ways of preparation rather than the land the coffee is grown in [1]. Coffee culture is highly developed in the region of Balkan countries and the black coffee prepared in a traditional way is still largely consumed in domestic environment and taverns, so called "Turkish" (or "Greek" or "Serbian") coffee. This coffee cooking method is also known as the "mud coffee brewing method" in which a substantial amount of solid residue, the spent coffee grounds (SCG), is obtained as a waste product. SCG obtained from different types of coffee, although consider as a waste, could be a valuable source of polyphenols, secondary plant metabolites, in both free and bound form [2-3], with exceptional antioxidative activity shown in vitro [4] – [7].

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It has been shown that moderate consumption of food rich in polyphenols have the potential to decrease the risk of developing cardiovascular disease (CVD) [8]. Numerous in vitro, in vivo and ex vivo studies have reported that polyphenol-rich food, such as dark chocolate, garlic, ginger, purple grape juice, onion, tomato, tea and wine, positively affect platelet function [9] – [10]. It was shown that direct inhibition of platelet activation and their further aggregation is a potential mechanism through which polyphenols could play a role in CVD risk reduction [11] – [12].

Healthy adults at very low risk of CVD have much lower percentage of activated platelets in circulation, compared to subjects at very high risk [13]. Activated platelets have increased numbers of expressed surface antigens, P-selectin and GPIIb/IIIa, described as platelets hyperreactivity state [14]. This state is reflected in increasing aggregation of platelets with monocytes, neutrophils and endothelial cells that can lead to acute thrombotic events and/or atherosclerosis. Thus, platelets can be considered as a rational target for the prophylactic effects of bioactive compounds obtained from food. Despite the large amount of evidence implicating SCG as an important source of polyphenols, the exploitation of SCG in the production of functional food ingredients stays scarcely reported.

We herein postulate that due to present polyphenols, black coffee SCG has the potential to modulate platelet activity. To test this assumption, we examined the in vitro the effects of polyphenol-rich extracts both on platelets activation and aggregation with monocytes and neutrophiles using flow cytometry method. To the best of our knowledge, the direct role of polyphenols from black coffee SCG on platelet function, more precisely on the expression of platelet antigens and interactions with other circulatory cells, has not be studied previously.

1. MATERIJAL AND METHODS

2.1 Black coffee

Roasted coffee “Doncafe Moment”, a blend of Coffea arabica and robusta was provided by a local company Strauss-Adriatic d.o.o., Šimanovci, Belgrade, Serbia. According to the information provided by the producer, “Doncafe Moment” is a mix of roasted coffee obtained from Rio minas (Brazil) - C. arabica and two types of Vietnamese C. robusta, blended in the confidential proportion that may vary only slightly depending on the market offer.

1.2 Preparation of the black coffee

We prepared black coffee beverage by following the traditional ‘home made’ procedure commonly used in households in Serbia [15]. Collected SCG was then spread as a thin layer, dried for 24 h in oven at 37 oC (Memmert) and stored at 3-4 °C until experiment.

1.3 Spent black coffee grounds polyphenol rich extracts

Polyphenol rich extracts were obtained by employing the response surface method (RSM), using the optimal parameters of the microwave assisted extraction, based on our previous study [7], [16]. The optimal parameters were obtained by verifying the effects of microwave power, liquid-

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to-solid ratio and extraction time on total phenolics content and antioxidative activity. The highest concentration of phenolic compounds from SCG was obtained at the following extraction conditions: microwave power of 240 W, liquid-to-solid ratio of 6 ml/g and 40 s extraction time. 20% ethanol solution was applied based on Pavlovic et al. study [17]. In this study, microwave assisted extraction was employed to examine effect of ethanol concentration and time of extraction on total polyphenol content, DPPH activity and FRAP ability. The highest concentration of extracted phenolic compounds was achieved under 40s of microwave radiation by applying 20% aqueous ethanol solution.

Obtained extracts were defatted by washing with diethyl ether.

1.4 Chemical reagents and antibodies

Total polyphenol content was measured using Folin-Ciocalteau phenol reagent (MOL Belgrade, Serbia). The gallic acid (GA) was used as a standard. Na2CO3 and MeOH were purchased from Lach-Ner (Neratovice, Czech Republic). The analytical reagent grade chemical ethanol (96%) (Zorka Pharma Šabac Hemija d.o.o., Serbia) was used. Monoclonal antibodies: (PAC1-FITC antibody), anti-CD62P-PE, anti-CD11b-PE, anti-CD14-PerCP, and isotype controls (IgG1-FITC, IgM-FITC, IgG1-PE, IgG2-PE, IgG1-PerCP and IgG2-PerCP), cell lysis buffer and CellFIX solution were purchased from BD Biosciences, USA. HEPES–Tyrode's buffer (HTB) and dimethyl sulfoxide (DMSO) and all standards were purchased from Sigma-Aldrich (Deisenhofen, Germany).

1.5 Total polyphenol content (TPC)

According to the modified method of Đorđević et al. [18], Folin-Ciocalteu method was used to determine the concentration of total polyphenols in SCG samples. For the assessment of antiplatelet activity, we dissolved obtained extract in DMSO to the concentration 50 mg GAE/ml, based on the TPC values, and the obtained solution was kept on 2-8 °C not longer than 7 days.

1.6 Antiplatelet activity

The expression of P-selectin and GPIIb/IIIa markers on platelet surface and platelet–monocyte/neutrophil aggregates formation were measured by complete blood flow cytometry [19], [20], with some modifications for in vitro testing, according to a previously published method [21]. P-selectin and GPIIb/IIIa markers were evaluated in the whole blood of five healthy donors after in vitro stimulation using suboptimal concentration of adenosine diphosphate (ADP, final conc. 0.25 µM) as agonist. The complete blood samples were obtained from volunteers following the guidelines for blood collection for the analysis of platelet activation and aggregation [21], [22]. Prior to the experiment the stock solution of SCG extract in DMSO was diluted in HEPES–Tyrode's buffer (HTB). Immediately after the venipuncture, the SCG extract solution was added to the aliquots of the whole blood (360 µL) to the final concentration of 25, 50 and 100 µg GAE/ml and the obtained samples were pre-incubated for 10

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min at 37 oC. The final concentration of DMSO did not exceed 0. 2%. Samples of the whole blood with only DMSO in the final concentration of 0.2% were used as negative controls. For the assessment of the effects on the expression of P-selectin and GPIIbIIIa, after incubation both treated and control blood samples were diluted in HTP (1:10) The aliquots of diluted blood (45 µL), were treated with ADP (0.25 µM), and incubated with monoclonal antibodies CD61-PerCP, CD62-PE and PAC1-FITC or isotope control (5 µL each) at following conditions: 20 min at room temperature in the dark. Finally, all samples were fixed in CellFix solution (350 µL) for 15 min and further analysed on flow cytometer. The number of formed platelet-monocyte and neutrophil aggregates were determined in the undiluted whole blood samples. Aliquots of the whole blood pre-incubated with SCG extracts, were treated with ADP (0.25 µM), and incubated with monoclonal antibodies (or isotope control) CD61-FITC, CD11b-PE and CD14-PerCP for 15 min in the dark at room temperature. After staining, samples were treated for 10 min with CellLysing solution washed twice in HTB and finally treated with the CellFix solution for 15 min. Samples were analysed using Becton Dickinson FACSCalibur flow cytometer and CellQuest software (Becton Dickinson). The platelets were identified by the expression of CD6 as a pan-platelet marker. Appropriate non-specific controls were used for setting the positive regions for P-selectin and GPIIb/IIIa analysis. The light scatter properties and the differential expression of CD14 and CD11b were used for monocytes and neutrophils identification. As a negative control samples stained with isotype-matched monoclonal antibodies were used. The number of platelet–monocyte and platelet-neutrophil aggregates was measured by the change of expression of platelet specific CD61 in the whole population of monocytes and neutrophils. All procedures were approved by the Clinical Zemun Hospital Ethical Committee and before entering the study all volunteers signed the informed consent form. The blood samples were taken in the morning, after overnight fasting.

1.7 Statistics

We simultaneously analysed bioactive content of SCG, separated from three replicate samples of prepared coffee beverage. The Wilcoxon signed-rank test was used to analyse the differences in number of activated platelets and number of formed aggregates with monocytes and neutrophils in samples pre-treated with polyphenol rich extracts compared to DMSO-treated samples. All data are shown as mean values with their standard deviation. Statistical analyses were performed using SPSS software (SPSS, Chicago, IL, USA). Differences were considered statistically significant at $p<0.05$.

2. RESULTS AND DISCUSSION

The results are presented as percent change (%) of P-selectin or GPIIbIIIa positive platelets (analysed in a pool of 20,000 platelets) after the treatment with different concentrations of defatted SCG extracts (25, 50 and 100 µgGAE/ml), and subsequent in vitro activation with ADP and compared to a vehicle-treated activated control samples. The obtained results are presented in Figure 1.

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Figure 1. The effects of black coffee SCG on the percentage of P-selectin (a) and a GPIIb-IIIa (b) positive platelets of healthy subjects (n = 5) induced by the agonistic action of ADP (0.25 µM) in vitro. Data is expressed as percent change (%) to positive control (solvent pre-treated, ADP induced cells) and shown as mean ± SEM. Significantly different from the control: * $p < 0.05$; (Wilcoxon signed rank test).

The effects of the treatment with different concentrations of black coffee SCG extracts on the percentage of platelet aggregates with monocytes and neutrophils, in samples activated with ADP in vitro, were compared to a negative (vehicle-only) control, and expressed as percent change (%). The number of platelet aggregates with monocytes and neutrophils, were measured in a pool of 1000 monocytes and 10000 neutrophils, respectively. The results are shown in Figure 2.

Figure 2. The effects of black coffee SCG on percentage of platelets aggregates with monocytes (PMA), (a) and platelets aggregates with neutrophils (PNA), (b) of healthy subject, after in vitro exposure to ADP as agonists (0.25 µM). Data is expressed as percent change (%) to positive control (solvent pre-treated, ADP induced cells) and shown as mean ± SEM. Significantly different from the control: * $p < 0.05$; (Wilcoxon signed rank test).

The significant inhibitory effect on P-selectin expression was observed for all tested extracts, with the inhibition of 14.33 ± 0.50% observed with the highest used concentration (100 µgGAE/ml). A dose-dependent effects on P-selectin was shown with different concentration of polyphenols ($r2 = 0.9956$, $p = 0.040$). The significant inhibitory effect on GPIIb/IIIa was recorded at the higher concentrations used (50 and 100 µgGAE/ml) up to 15.66 ± 0.83%. Marked statistically significant and dose dependent ($r2 = 0.9987$, $p = 0.032$) inhibitory effect of SCG extracts on platelet aggregation with monocytes was observed with all three tested concentrations. The highest concentration tested (100 µgGAE/ml), induced the decrease in platelet-monocyte aggregates of 21.90 ± 1.90%. The statistically significant effect on platelet aggregation with neutrophils was observed only at the concentration of 50 µgGAE/ml. The highest inhibitory effect, 20.00 ± 11.43% is observed at the highest concentration of polyphenols used (100 µgGAE/ml), with high value of SD due to the marked interindividual variation.

The presented results confirm previous published results showing that espresso coffee SCG polyphenol reach extracts induce dose dependent inhibition of platelet activation with decrease in expression of P-selectin (8.7%) and GPIIb/IIIa (5.6%) and also decrease platelet aggregation with monocytes and neutrophils up to 20% [7]. Also, it was shown both in human and animal studies, in vitro, in vivo and ex vivo that coffee beverage has a potential to decrease platelet aggregation induced by ADP and other agonists (arachidonic acid or collagen) [23-25]. The inhibitory effects of total polyphenols present in SCG are significantly higher than the effects of total polyphenols present in extracts obtained from the correspondent beverage (unpublished data). The obtained inhibitory effects on P-selectin and on GPIIb/IIIa up to 6% with the highest concentrations tested (100 µgGAE/ml) for beverage compared to around 15% for correspondent SCG, may indicate a more favourable profile of bioactive compounds in SCG. The physiological

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significance of our results is confirmed by earlier study of Huo and Ley [26], performed in animals, where injections of 7% of activated platelets in mice, for 4 weeks, 3 times a day, intensify the formation of atherosclerotic lesions by 30% by increasing the recruitment of monocytes. This effect is shown to be induced by the expression of surface antigen, P-selectin and GPIIb/IIIa, resulting in augmented platelets interaction with other cells. The activated form of GPIIb/IIIa, a receptor for fibrinogen, mediates platelet-platelet aggregation and is essential for the platelet plug formation. P-selectin binding to the monocytes and neutrophils counter-receptor, P-selectin glycoprotein ligand (PSGL)-1, contributes to the pathogenesis of atherosclerosis. Comparing to results obtained by Huo and Ley (2004), our data showing the significant inhibitory effect of all tested concentrations of black coffee SCG on platelet aggregation with monocytes up to 21.90 ± 1.90% point to the substantial potential of SCG in the prevention of atherosclerotic lesions development. The modulation of platelet function is considered as an underlying mechanism for their prevention role in developing CVD, by reducing the risk of atherosclerosis development [12]. Observed dose-dependent activity of investigated extracts supports conclusions on the specific antiplatelet activity of polyphenols present in black coffee SCG. However, it was shown that increase in dose does not guarantee an increase in the effect, either due to system saturation or bioavailability of the agents [27]. In that sense, the direct relationship to a biological response should be confirmed in an in vivo setting with the larger cohort.

The limitation of the study is that it does not take into account an extensive metabolism of polyphenols after absorption [28], and the activity of their human metabolites in physiologically relevant concentrations [29]. Nonetheless, our in vitro findings provide proof-of-concept for the antiplatelet effects of black coffee SCG polyphenols. Further research should determine the bioavailability of polyphenols present in SCG, identify bioactive metabolites and dose-response relationship in optimally designed proof-of-principle clinical trial. Observed antiplatelet effect rationalize the use of black coffee SCG as a source of polyphenols and a low-cost food ingredient aiming to preserve and promote optimal CVH. In addition, based on the European Food Safety Authority (EFSA) “Guidance on the scientific requirements for health claims related to antioxidants, oxidative damage and cardiovascular health”, the reduction in platelet aggregation is accepted as a beneficial effect of food and dietary constituents, in the context of maintaining CVH enabling claims related to foods or substances affecting disturbed platelet function [30]. From the domain of functional food and nutraceutical industry, the possibility of SCG utilization based on current available technologies represents an exciting opportunity to attain new functional ingredients with high nutritional value. Given that the SCG remained after preparing black coffee beverage is usually thrown directly into the water supply, collection and re-use of these residues could also contribute to the reduction of coffee consummation environmental footprint [31].

3. CONCLUSION

The SCG extract in all concentrations of polyphenols tested (25, 50 and 100 µgGAE/ml) has shown the significant inhibitory effect on both on P-selectin and GPIIb/IIIa receptors induced by

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ADP in vitro, and the effect was dose dependent. Statistically significant and dose dependent inhibitory effect was recorded on platelet aggregation with monocytes, in all three concentrations tested. Based on our findings, black coffee SCG is recommended as a valuable and low-cost source of polyphenols with observed antiplatelet activity that could be used as a functional food ingredient. Additional research is needed to identify the responsible components for the observed antiplatelet effects, the amounts needed to reach effective results in vivo and the clinical significance of these findings for the development CVD and maintenance of CVH. This study provides good knowledge base for SMSs business opportunity for exploitation of black coffee SCG in Balkan and Middle East countries where this type of coffee is in widespread use.

Acknowledgements: This work was supported by the Project III41030 and TR31035 financed by the Serbian Ministry of Education, Science and Technological Development.

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