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Original Scientific Paper

The effect of the *Satureja montana* ethanol extract on the morphological changes of erythrocytes

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ABSTRACT:
The present study investigated the antioxidant activity of ethanol extracts of the leaves of *Satureja montana* and their influence on the membrane stability of erythrocytes *ex vivo*. The ethanol extracts showed a very potent antioxidant activity of EC_{50} = 0.055mg/ml. Rat blood samples were treated with 96% ethanol extracts in different concentrations of 100 μg/ml, 200 μg/ml, and 300 μg/ml, and morphological analyses were carried out. The results showed significant differences in the shape of the erythrocytes incubated with higher extract concentrations. Considerable morphological changes were observed at a concentration of 200 μg/ml which was characterised by the highest percentage of stomatocytes, while the highest percentage of echinocyte formation was observed at a concentration of 300 μg/ml. The results of this investigation indicated that the ethanol extracts of *S. montana* exhibited a possible protective effect on the membrane stability of erythrocytes.

Keywords:
winter savory, plant extract, antioxidant activity, blood cells

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INTRODUCTION

Recent literature data suggest that phytotherapy has been promoted as an alternative to synthetic drug treatment. The antioxidant activity of different plant extracts have been shown in numerous studies (Bors *et al.* 2012; Bonarska-Kujawa *et al.* 2014). Natural antioxidants are assumed to protect cells against oxidative stress, which might otherwise lead to cell damage (Cyboran *et al.* 2011). Oxygen reactive species (ROS) are often formed as the by-product of oxygen metabolism. When the production of ROS increases, they tend to negatively affect essential cellular components such as proteins, lipids, and nucleic acids (Wu *et al.* 2013). Polyphenols are plant metabolites involved in defence against different types of abiotic stressors such as high temperatures, solar radiation, drought, flood, salt, and heavy metals. The leaves and fruits of *Ribes nigrum* L. contain polyphenols with antioxidant and anti-inflammatory activities (Tabart *et al.* 2012). Some plant species such as *Chelidonium majus* L. (Biswas *et al.* 2008) and *Hydrastis canadensis* L. (Karmakar *et al.* 2010) have been tested for their possible anticancer potential. In that sense, polyphenols exert anticancer activity, inhibiting the multiplication and growth of cancer cells (Bishayee *et al.* 2011). Quercetin, a plant flavonol from the flavonoid group of polyphenols, exhibits significant antioxidative effects in non-cellular systems (red blood cell membranes or plasma) (Zbikowska *et al.* 2014). The interactions of polyphenols at the surface of membranes through hydrogen bonding between the polar head groups of lipids and hydrophilic flavonoids may act to reduce the access of harmful molecules such as oxidants. This interaction should be considered among the factors which contribute to their antioxidant effects, thus protecting the structure and function of the membranes (Erlejman *et al.* 2004; Oteiza *et al.* 2005; Hendrich 2006).

Species of the family Lamiaceae are widely used as fragrances and flavouring agents, and due to the biological activity of the secondary metabolites are well known in traditional herbal medicine. *Satureja montana* L. (La-

miaceae), winter savory, is an aromatic species used both as a spice and as a traditional medicinal plant.

Previous histochemistry research of the secretion compounds of the peltate and capitate glandular trichomes of some *Satureja* species (Marin *et al*. 2010, 2012a) showed similarities in the content of secretory products, which were characterised by phenols, terpenes, tannins, and lipids. Due to the presence of phenols in the essential oil of *S. montana* leaves, it is proven to have very strong pharmacological activities and antioxidative properties (Marin *et al*. 2012b).

The volatile components of *S. montana* essential oil and methanol or ethanol extracts have been reported to exhibit potential antimicrobial and antioxidative activity (Mihajilov-Krstev *et al*. 2014; El-Hagrassi *et al*. 2018; Hudz *et al*. 2020). Winter savory extract has shown antioxidative, anti-inflammatory, and hepatoprotective effects (Milijašević *et al*. 2022), as well as antiviral properties against a variety of viruses, including the cucumber mosaic virus (CMV) and human immunodeficiency virus type 1 (HIV-1) (Tepe & Cilkiz 2015).

Since there is no data on the influence of ethanol extracts of the leaves of *S. montana* on the membrane stability of erythrocytes, this study aimed to determine the possible antioxidant activity of the ethanol extracts and the effect of the various extract concentrations on the morphological changes to erythrocytes.

MATERIALS AND METHODS

Plant material. The plant material (aerial parts) was collected near the town of Benkovac (Croatia) (N 44°02′, E 15°36′). A voucher specimen was deposited in the herbarium of the Institute of Botany and Botanical Garden Jevremovac, Faculty of Biology, University of Belgrade (BEOU, No. 16496), Serbia.

The leaves of *S. montana* were very finely milled to obtain the corresponding powder. Five grams of dry powder were extracted with 96% ethanol at 37°C for 24 h. The extract was then filtered through a filter paper and was evaporated under reduced pressure at 60°C using a rotavapor apparatus until it became dry. About 100 mg/g of 96% ethanol extract was obtained.

Antioxidant activity. The antioxidant activity of the ethanol extract was measured in terms of hydrogen donating or radical scavenging ability by using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay. The decrease in absorbance at 517 nm after a 30 min reaction in darkness was determined by a UV-VIS spectrophotometer.

The percentage of inhibition of the DPPH radical by the samples was calculated according to the following equation:

$$\text{DPPH radical scavenging (\%)} = A_c - A_u / A_c \times 100$$

where A_c is the absorbance of the control and A_u is the absorbance of the remaining DPPH radical after reaction with the antioxidant for 30 min.

Blood smears. Wistar strain rats were used for the experiment. The animals were housed in a temperature-controlled room at 22 ± 1°C. They were maintained under intermittent 12-h periods of light and dark and were given commercial rat food and tap water *ad libitum*. The blood from the tail vein was collected in tubes containing heparin. The blood was centrifuged (2500 rpm/min at 4°C, for 5 min), the plasma was discarded and the erythrocytes were washed three times with phosphate-buffer saline (pH 7.4). The erythrocytes were treated with *S. montana* ethanol extract in varying concentrations of 100 µg/ml, 200 µg/ml, and 300 µg/ml and incubated at 37°C for 1 h (Bors *et al*. 2012). The erythrocytes which were incubated with phosphate-buffered saline (PBS) were used as the controls. After 1 h of incubation, ten blood smears for the control and ten smears for each different type of extract concentration were routinely prepared, left to dry for 1–2 h, and then fixed with methanol. The blood smears were stained with May-Grunwald-Giemsa, rinsed with buffer, and dried. The morphology of the erythrocytes was observed using a light microscope (Leica, Microsystem, Germany) at 100× magnification, and the images were obtained with a digital camera (Leica, Microsystem, Germany).

Statistical analysis. The obtained data were analysed using GraphPad Prism, Version 5.03 software. The one-way analysis of variance (ANOVA) with post hoc multiple comparison procedure was used to assess any statistical differences. The data are expressed as arithmetic means ± SE, where differences were considered to be statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Erythrocytes are very sensitive to various influences and serve as a simplified model (Halliwell & Gutteridge 2017) for assessing the anti-inflammatory potential of investigated drugs or compounds. Anti-inflammatory activity has been observed in many plants including *Syzygium aromaticum* L., *Curcuma longa* L. (Mueller *et al*. 2010), *Zingiber officinale* Rosc. (Terry *et al*. 2011), and *Piper betle* L. (Alam *et al*. 2013). The ethanolic root-extract of *Piper chaba* Trel. & Yunck. showed considerable anti-inflammatory activity, it might serve to stabilise the membrane of red blood cells by preventing the release of lytic enzymes and other active inflammatory mediators, thus effectively inhibiting hemolysis (Yesmin *et al*. 2020).

Erythrocytes are commonly employed in the evaluation of oxidative stress since they are prone to oxidative reactions because of relatively high oxygen tension and

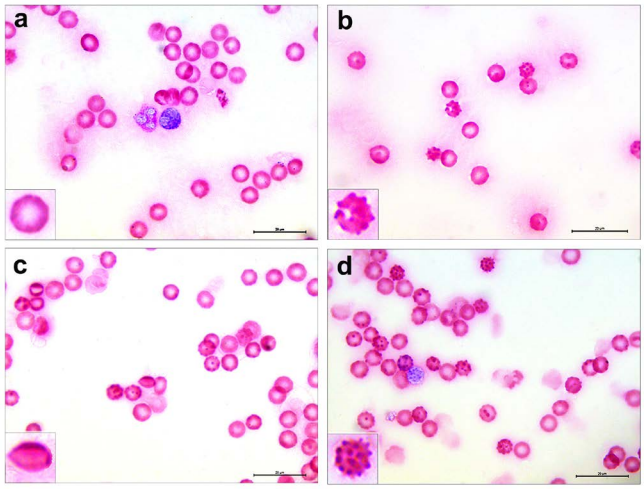

Fig. 1. a) Control erythrocytes incubated with PBS; erythrocytes incubated with ethanol extract: b) 100 µg/ml; c) 200 µg/ml; and d) 300 µg/ml. Scale bars = 20 *µm*.

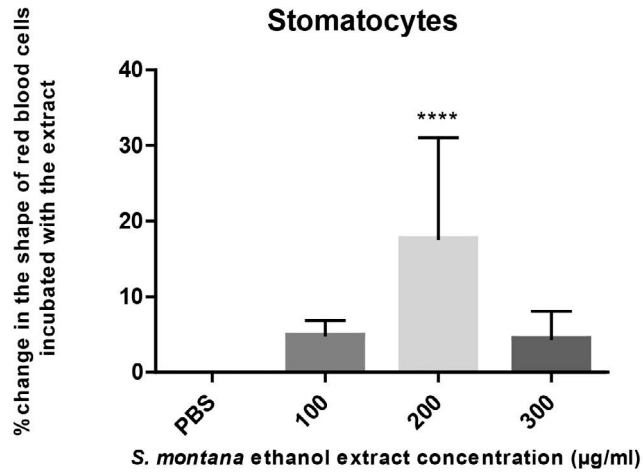

Fig. 3. Percentage of stomatocytes of the control (PBS) and incubated with the ethanol extract of *S. montana* in varying concentrations (100 µg/ml, 200 µg/ml, and 300 µg/ml).

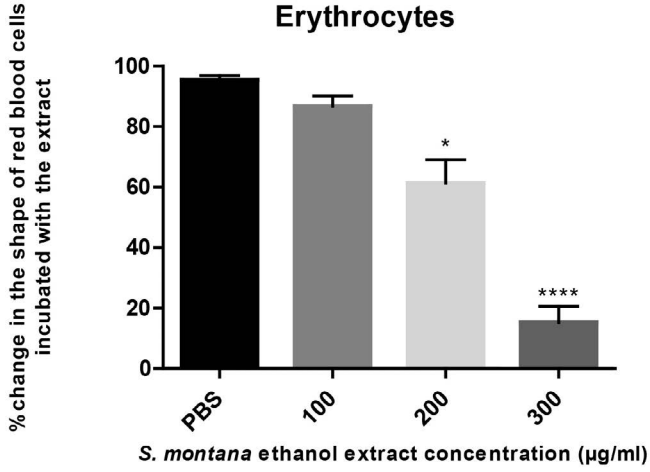

Fig. 2. Percentage of erythrocytes of the control (PBS) and incubated with the ethanol extract of *S. montana* in varying concentrations (100 µg/ml, 200 µg/ml, and 300 µg/ml).

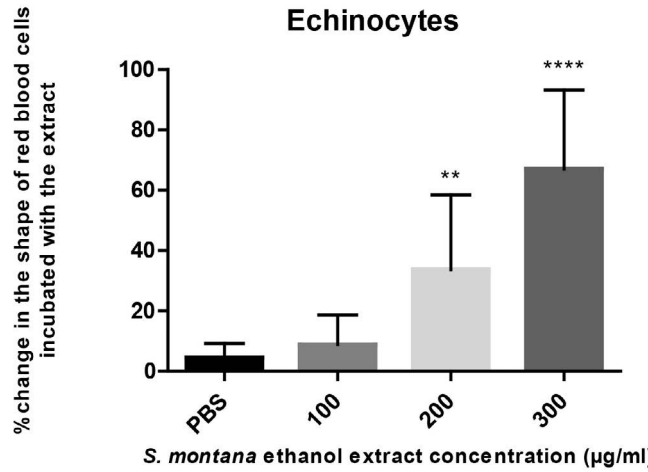

Fig. 4. Percentage of echinocytes of the control (PBS) and incubated with the ethanol extract of *S. montana* in varying concentrations (100 µg/ml, 200 µg/ml and 300 µg/ml).

the presence of polyunsaturated lipid-rich membranes (Dluya *et al.* 2013). The compounds such as quercetin contained in the leaf extracts of *Psidium guajava* L. (Vijayakumar *et al.* 2019) and *Moringa oleifera* L. (Laksmiani *et al.* 2022) have a wide range of biological activities. Due to its high antioxidant activity quercetin prevents the formation of reactive oxygen species (ROS), it has the ability to inactivate already produced ROS, to increase the activity of antioxidant enzymes, and to inhibit lipid peroxidation (Russo *et al.* 2012).

Previous research (Bonarska-Kujawa *et al.* 2011) showed the significant antioxidant properties of quercetin-3-O-glucoside in relation to biological membranes. A study on the effect of quercetin on erythrocytes reported that changes in the shape of the erythrocytes may result from the interaction of quercetin with cytoskeleton proteins (Pawlikowska-Pawlega *et al.* 2003).

It is known that significant changes to the shape of erythrocytes can lead to their elimination from circulation. The translocation of phosphatidylserine (PS) from the internal to the external surface of the plasma membrane is the mechanism whereby other apoptotic cells are recognised and eliminated by phagocytic macrophages (Marczak *et al.* 2007).

Antioxidant activity of the ethanol extract. In the present study, in terms of the EC_{50} values (the concentration of antioxidants required to achieve an absorbance equal to 50% of that of the control containing no antioxidants) the ethanol extract of the leaves of *S. montana* exhibited very potent antioxidant activity EC_{50} = 0.055mg/ml. Our previous studies using GC–MS analysis (Marin *et al*. 2012b) showed the presence of the phenolic monoterpene carvacrol, followed by p-cymene, g-terpinene, borneol, and thymol in *S. montana*. Carvacrol is one of the main components which exerts significant biological activity, and it may be suggested that it was responsible for the morphological changes in the incubated cells. Similar results were obtained in other studies (Sousa *et al*. 2012; Sirooli *et al*. 2015). In the studies carried out by Slavkovska *et al*. (2001) and Kremer *et al*. (2015) different phenolic compounds were identified in *S. montana*.

The results gained by Suwalsky *et al*. (2008) indicated that polyphenol-rich medicinal plant extracts cause the formation of echinocytes. Using various methods other research showed that polyphenols bind to the erythrocyte surface and protect red blood cells from potential oxidative stress and damage (Oteiza *et al*. 2005). According to the literature data, flavonoids cause a reduction in cellular membrane fluidity by their entry into the hydrophobic core. They may also interact with both the proteins and lipids of cellular membranes and the depth of their localisation in a membrane depends on their chemical structure (Hendrich 2006). Recent findings concluded that although sage, savory, raspberry, and yarrow extracts all showed antioxidant activity, savory was the most promising antioxidant extract and was the most effective in preventing hemolysis (Giao *et al*. 2010).

Morphological changes to the erythrocytes. The results in our investigation showed significant differences in the shape of the erythrocytes incubated with higher ethanol extract concentrations compared to the control. Instead of the biconcave disc shape, the erythrocytes revealed morphological changes in the cell membrane, which were characterised by the formation of stomatocytes and echinocytes. Micrographs of the control erythrocytes (Fig. 1a) showed the biconcave disc shape and normal structure of the erythrocytes' surface membrane.

It was found that those erythrocytes incubated for 1 h with *S. montana* extract at concentrations of 100 μg/ml, 200 μg/ml, and 300 μg/ml exhibited different morphology (Fig. 1b–d). In addition to the formation of echinocytes (Fig. 1b) and stomatocytes (Fig. 1c), the strongest effect on the surface of the cell membrane, the formation of dendrites, which are typical of echinocyte form, was observed after incubation with the highest ethanol extract concentration (Fig. 1d).

The analysis revealed that the studied extracts also induced changes in the percentages of the different formations of blood cells.

Percentage of erythrocyte formation. It was shown (Figs. 2–4) that higher concentrations of the ethanol leaf extracts (100 μg/ml, 200 μg/ml, and 300 μg/ml) caused significant changes to the percentages of different erythrocyte formation. The lowest concentration of the ethanol extract (100 μg/ml) led to the formation of echinocytes; at a concentration of 200 μg/ml, in addition to echinocytes, the appearance of stomatocytes was also observed, which were the most numerous, while the concentration of 300 μg/ml was characterised with by highest percentage of echinocyte formation. Higher ethanol extract concentrations resulted in more pronounced changes to the erythrocyte shape in comparison to the control.

The most significant changes were observed in the ethanol extract at a concentration of 200 μg/ml, which was characterised by an increase in stomatocyte formation, and at a concentration of 300 μg/ml with an increase in the shape variation of the echinocytes and a decrease in the normal shape of the erythrocytes. It is possible that the compounds present in the *S. montana* extracts were incorporated into the erythrocyte membranes as according to Bukowska *et al*. (2009), xenobiotics exert significant effects on plasma membranes because of interactions with their components, whereby echinocytes are formed by the insertion of xenobiotics in the outer monolayer, whereas stomatocytes are formed as a result of xenobiotic accumulation in the inner monolayer of the cell membrane. In our research, echinocytes and stomatocytes were formed after red blood cell treatment with all of the extracts studied. Similar results were observed in the investigation of the effect of *Uncaria tomentosa* (Willd. ex Schult.) DC. extracts on erythrocyte morphology (Bors *et al*. 2012).

CONCLUSIONS

Although the present research did not establish the mechanism by which the secondary constituents of the ethanol extracts of *S. montana* leaves affect the change in shape of erythrocytes, we were able to conclude that the highly potent antioxidant property of carvacrol was possibly responsible for interacting directly with the membranes of the erythrocytes, thus causing the changes in their shape. These results indicate that the different concentrations of the ethanol extracts did not disturb the function of the biological membrane, thus potential anti-inflammatory properties could be expected, as well as beneficial effects on the organism by protecting the cell membrane against oxidation. Free radicals which damage cells lead to inflammation, so natural compounds may provide a very suitable base for the creation of new anti-inflammatory medications.

In our future research, it would be interesting to determine whether these morphological changes were accompanied by any other alterations to the erythrocyte

membranes, and how they reflect on the functional capacity of the cells.

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REZIME

Botanica SERBICA

Uticaj etanolnog ekstrakta *Satureja montana* na morfološke promene eritrocita

Marija Marin i Snežana Branković

U istraživanju je ispitana antioksidativna aktivnost etanolnog ekstrakta listova *Satureja montana* i njegov uticaj na stabilnost membrana eritrocita *ex vivo*. Ekstrakt etanolnog ekstrakata je pokazao veoma značajnu antioksidativnu aktivnost EC50 = 0,055 mg/ml. Uzorci krvi pacova tretirani su 96% etanolnim ekstraktom *S. montana* koncentracije 100 µg/ml, 200 µg/ml i 300 µg/ml i potom je izvršena morfološka analiza. Rezultati su pokazali da postoje značajne razlike u obliku eritrocita inkubiranih sa rastućim koncentracijama ekstrakata. Značajne morfološke promene uočene su pri koncentraciji od 200 µg/ml koje su se karakterisale najvišim prisustvom stomatocita, a najviša zastupljenost ehinocita zapažena je pri koncentraciji od 300 µg/ml. Rezultati ovog istraživanja su pokazali da etanolni ekstrakt *S. montana* ima potencijalni zaštitni efekat na stabilnost membrana eritrocita.

Ključne reči: rtanjski čaj, biljni ekstrakt, antioksidativna aktivnost, ćelije krvi