

## The effects of methanol extract of *Galium verum* L on cardiac redox state in hypertensive rats

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The aim of this study was to assess the effects of methanol extract of *G. verum* on redox status of isolated heart of spontaneously hypertensive rats after ischemia. Twenty-four *Wistar albino* rats were divided into three groups: untreated control rats and rats that received 125 and 250 mg/kg *G. verum* extract for 4 weeks per os. Index of lipid peroxidation (measured as TBARS) and parameters of antioxidative defence system such as level of reduced glutathione (GSH) and activities of catalase (CAT) and superoxide dismutase (SOD) were spectrophotometrically determined in heart homogenate. The index of lipid peroxidation in heart tissue was lower in both treated groups compared to the control group. On the other hand, the activity of SOD was significantly higher after consumption of both doses, while the activity of CAT was significantly higher only after treatment with a higher dose of extract. Based on our results we might conclude that 4-week treatment with methanol extracts of *G. verum* has the potential to modulate myocardial redox signaling after ischemia, thus significantly alleviating cardiac oxidative stress and exerting dose-dependent antioxidant properties. Future studies are certainly necessary to fully clarify the role of this plant species in myocardial I-R injury.

**Keywords:** *Galium verum*. Ischemia-reperfusion injury. Isolated rat heart. Oxidative stress.

### INTRODUCTION

Myocardial infarction is one of the most common causes of mortality due to ischemia-reperfusion (I-R) injury (Kalogeris *et al.*, 2016). Timely restoration of blood flow to an ischemic heart is essential for limiting the infarct size, but it can paradoxically amplify tissue damage which is known as I-R injury. It has been considered that factors that mostly contribute to myocardial dysfunction are increased generation of prooxidants, pronounced inflammatory response, and intracellular and mitochondrial Ca<sup>2+</sup> overload (Frank *et al.*, 2012). There is evidence that the

use of plant extracts rich in polyphenols may reduce the harmful effects of I-R injury thanks to their antioxidant and anti-inflammatory potential (Akhlaghi, Bandy, 2009).

*Galium verum* L. (*G. verum*) is perennial herbaceous plant, belonging to the *Rubiaceae* family (Bradic, Petkovic, Tomovic, 2018). It is also known by its name Lady's bedstraw and yellow bedstraw (Farcas *et al.*, 2018). The plant is widely distributed in Europe, North Africa and Asia (Bradic, Petkovic, Tomovic, 2018). *G. verum* has been used in traditional medicine for centuries as a diuretic, choleric, spasmolytic, diaphoretic, against diarrhea, sedative, anticancer agent for exogenous treatment of psoriasis and for skin injury (Lakić *et al.*, 2010; Schmidt *et al.*, 2014; Demirezer *et al.*, 2006). Also, it is believed that *G. verum* has beneficial effects against liver disorders and cardiovascular diseases (Schmidt *et al.*, 2014). Phytochemical investigation shows presence of

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iridoid glycosides, phenolic compounds, anthraquinones and triterpenes and small amounts of tannins, saponins, essential oils, waxes, pigments and vitamin C (Bradic, Petkovic, Tomovic, 2018; Lakić *et al.*, 2010).

Although many traditional indications of *G. verum* have been confirmed by scientific research, its role in myocardial I-R injury as well as its potential to attenuate I-R-induced oxidative damage has still not been fully clarified. Therefore, the aim of this study was to investigate the effects of methanol extract of *G. verum* on the redox status of the isolated heart of Wistar albino rats after ischemia.

## MATERIAL AND METHODS

This study was performed at the Faculty of Medical Sciences, University of Kragujevac, Serbia. The protocol was approved by the Ethical Committee for the welfare of experimental animals of the Faculty of Medical Sciences, University of Kragujevac, Serbia. All experiments used in this study were performed in accordance with the EU directive on the protection of animals used for experimental and other scientific purposes (86/609/EEC) and the principles of Good Laboratory Practice (GLP).

### Plant material and extract preparation

Methanol extract of *G. verum* was prepared using heat reflux extraction and chemical characterization was performed as described in our previous research (Bradic *et al.*, 2018). Once a day, just before administration, the extract was dissolved in tap water and then administered to experimental animals.

### Animals and experimental design

Twenty-four Wistar albino rats (male, eight weeks old, body weight  $150 \pm 30$  g) were included in this study. They were fed with commercial rat food (20% protein rat food, Veterinary Institute Subotica, Serbia) *ad libitum*. The rats were housed at a temperature of  $22 \pm 2^\circ\text{C}$  and were illuminated daily for 12 hours by automatic illumination. The animals were divided into three groups:

1. **Control group** – the group that drank only tap water
2. ***G. verum* group 1** – the group that drank tap water with the 125 mg/kg methanol extract of *G. verum*
3. ***G. verum* group 2** – the group that drank tap water with the 250 mg/kg methanol extract of *G. verum*

After completing the 28-day protocol of *G. Verum* administration, the rats were induced to a state of short-time narcosis using a mixture of ketamine (10 mg/kg) and xylazine (5 mg/kg) that was applied intraperitoneally, with heparin used as an anticoagulant prior to that. The animals were then sacrificed by decapitation. The chest of the rats was opened using midline thoracotomy. The hearts were removed immediately following thoracotomy and immersed in a cold saline solution. The hearts were then cannulated to the Langendorff perfusion apparatus which provided retrograde perfusion under constant coronary perfusion pressure (CPP) of 70 cmH<sub>2</sub>O. The buffer used for retrograde perfusion was the Krebs-Henseleit buffer, with the following components (in mmol/l): NaCl 118, KCl 4.7, CaCl  $\times$  2H<sub>2</sub>O 2.5, MgSO<sub>4</sub>  $\times$  7H<sub>2</sub>O 1.7, NaHCO<sub>3</sub> 25, KH<sub>2</sub>PO<sub>4</sub>, 1.2, glucose 11, pyruvate 2. The buffer was balanced with 95% O<sub>2</sub> and 5% CO<sub>2</sub> and was heated to 37°C. Buffer pH was 7.4.

During stabilization and reperfusion, a sensor (transducer BS473-0184, Experimetria Ltd., Budapest, Hungary) was placed inside the left ventricle to measure the parameters of heart function. To achieve a stable rhythm, the hearts had to undergo 30-minute perfusion at CPP of 70 cm H<sub>2</sub>O. The hearts were then subjected to a 20-minute long total ischemia period followed by 30 minutes of reperfusion.

### Biochemical analysis

After completing the experiments, all rat hearts were frozen at  $-80^\circ\text{C}$ , and then a 0.5 section of each tissue was homogenised in a 5ml phosphate buffer with pH 7.4 on ice using an electrical homogeniser. Afterwards, the homogenates were centrifuged at  $1200 \times g$  for 20 min at  $4^\circ\text{C}$ . The supernatants obtained in this way were then isolated and stored at  $-80^\circ\text{C}$  until further use in biochemical analyses. Index of lipid peroxidation (measured as thiobarbituric acid reactive substances (TBARS)) and the

parameters of the antioxidant defence system, which include activity of superoxide dismutase (SOD), catalase (CAT) and level of reduced glutathione (GSH) were determined spectrophotometrically in the heart tissue.

#### *Determination of the Index of Lipid Peroxidation measured as TBARS*

The degree of lipid peroxidation in the heart tissue was estimated by measuring TBARS, using 1% thiobarbituric acid in 0.05 NaOH, which was incubated with the heart tissue at 100°C for 15 min and measured at 530 nm. Krebs–Henseleit solution was used as a blank probe (Ohkawa, Ohishi, Yagi, 1979).

#### *Determination of CAT and SOD*

Heart tissue homogenates were used in cardiac SOD and CAT activity determination. SOD activity was assessed using the epinephrine method by Beutler. A heart tissue homogenate was mixed with carbonate buffer, and after the mixing was completed, epinephrine was added. Detection was performed at 470 nm. The amount of SOD in heart tissue was expressed as U/g of tissue (Beutler, 1984). CAT buffer, heart tissue sample and 10 mM H<sub>2</sub>O<sub>2</sub> were used in CAT determination. Detection was performed at 360 nm. The amount of CAT in the heart tissue was expressed as U/g of tissue (Aebi, 1984).

#### *Determination of reduced glutathione (GSH)*

The level of reduced glutathione was determined based on GSH oxidation with 5,5-dithiobis-6,2-

nitrobenzoic acid using the method reported by Beutler et al. (1963). Detection was performed at 420 nm. The amount of GSH was expressed as nmol/g tissue (Beutler, Duron, Kelly, 1963).

#### **Statistical analysis**

IBM SPSS Statistics 20.0 for Windows was used for statistical analysis of data within the control and both *G. Verum* groups. Values were expressed as mean ± standard deviation (SD). Distribution of data was checked using the Shapiro-Wilk test. Data were analyzed using a one-way analysis of variance (ANOVA). Statistically significant values that were considered were those of  $p < 0.05$ .

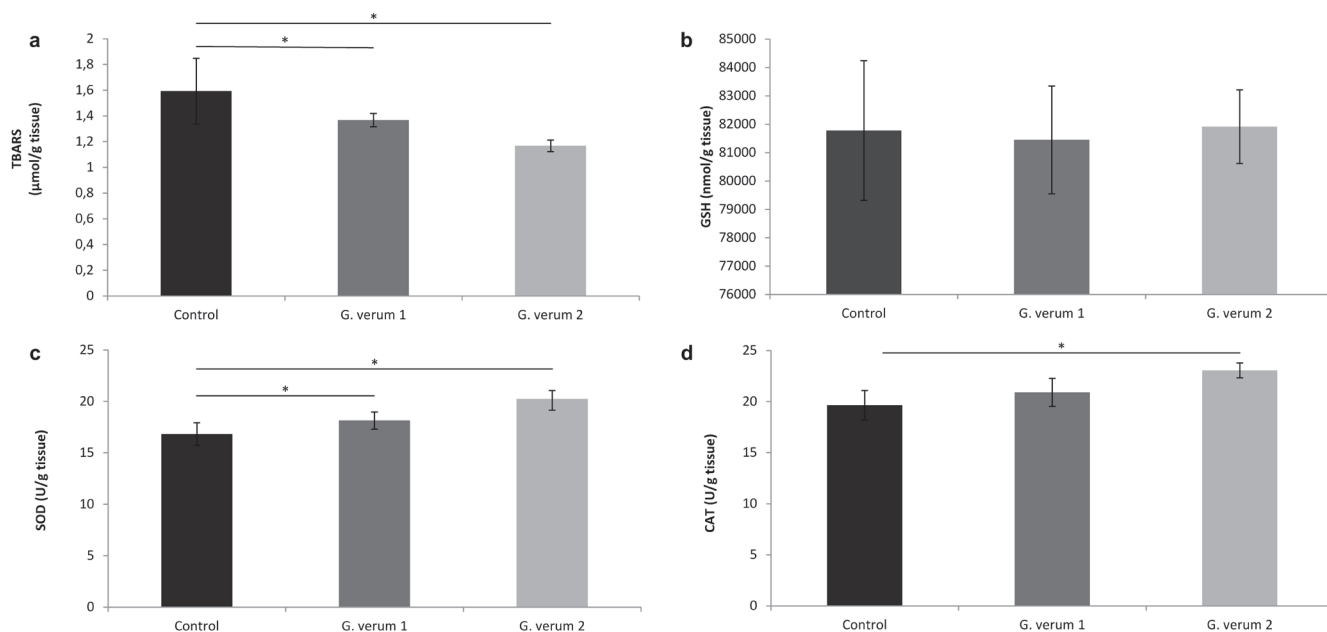
## **RESULTS**

### **Index of lipid peroxidation (measured as TBARS)**

The index of lipid peroxidation in heart tissue was lower in both groups treated with *G. verum* compared to the control group (Figure 1 (a)).

#### *Parameters of antioxidant defence system*

The activity of SOD was significantly higher in both groups treated with *G. verum* in comparison to the control group. The activity of CAT was significantly higher in the *G. Verum group 2* compared to the control group, while the activity of CAT was similar in the *G. Verum group 1* and control group. The level of GSH was similar in the observed groups (Figure 1 (b, c d)).



**FIGURE 1** - Parameters of oxidative stress and antioxidant defence system in heart tissue in healthy, *G. verum 1* and *G. verum 2* treated rats. (a) TBARS value comparison between groups, (b) SOD value comparison between groups, (c) CAT value comparison between groups, (d) GSH value comparison between groups.

\*Statistical significance at the level of  $p < 0.05$  within control group; data are presented as means  $\pm$  SD. S: stabilisation; I: ischemia

## DISCUSSION

Numerous research data suggest that oxidative stress is one of the most important factors contributing to myocardial damage after ischemia (Kalogeris *et al.*, 2012). In that sense, one of the possible therapeutic approaches in salvaging the heart might be the application of agents that act on cellular and enzymatic sources of ROS overproduction (Panche, Diwan, Chandra, 2016).

Great scientific effort has been invested in assessing the role of plant extracts rich in polyphenols and flavonoids against reperfusion-induced heart damage. It has been well documented that polyphenols have antioxidant and anti-inflammatory potential which might be efficient in both prevention and treatment of cardiac diseases (Akhlaghi, Bandy, 2009). *G. verum* is a plant rich in polyphenols and flavonoids such as quercitrin, rutin, hyperoside, chlorogenic and caffeic acids and stands out as a potential candidate with antioxidant properties

in ischemia-induced heart damage (Bradic, Petkovic, Tomovic, 2018; Mocan *et al.*, 2016).

According to our previous investigation, 4-week treatment with methanol extract of *G. verum* in a dose of 500 mg/kg significantly improved cardiac function after ischemia and alleviated production of most of measured pro-oxidants (Bradic *et al.*, 2018). Therefore we aimed to investigate if lower doses of the same extract might exert similar benefits on I-R injury and whether the effects are dose-dependent.

The results of our study have shown that I/R injury was related to increased oxidative stress, as evidenced by markedly higher TBARS. Under normal physiological conditions, there is a balance between continuously produced pro-oxidants and antioxidant system that counterbalances the effects of oxidants (Birben *et al.*, 2012; Gupta *et al.*, 2014). Prominent lipid peroxidation in our study might be explained by the fact that re-introduction of oxygen during the early phase of reperfusion is a stimulus for the generation of

pro-oxidants which strongly destruct cell components (Kalogeris *et al.*, 2012). Regarding the components of antioxidant defence system, myocardial SOD activity, as one of the first lines in antioxidant protection, was higher in groups treated with *G. verum*. On the other hand, only a higher dose of extract was capable of enhancing myocardial CAT activity compared to the control group. The level of GSH remained constant within the observed follow-up period, thus suggesting that decrease in TBARS was independent of GSH modulation. Our previous study also investigated the effects of methanol extract of *G. verum* on the redox status of the isolated heart of Wistar albino rats after ischemia. The results are similar, but the groups treated with lower doses tend to have better antioxidative protection as shown by lower TBARS (Bradic *et al.*, 2018).

Observed antioxidant potential of *G. verum* extract in the current research is probably a consequence of the additive effect of all present constituents. It has been known that polyphenols are a large group of molecules found in many plant species that mainly contribute to defence against UV radiation and various pathogens. These molecules consist of many hydroxyl groups connected to aromatic rings, and are classified according to their chemical structure into phenolic acids, flavonoids, lignans, stilbenes (Manach *et al.*, 2004). Flavonoids are polyphenol compounds based on the flavan nucleus and they are classified by chemical structure, which strongly influences their biochemical activity and metabolism (Cook, Samman, 1996). These bioactive compounds exhibit a myriad of biological effects, such as anti-inflammatory, antiviral, antibacterial, anti-ischemic, antioxidant and even some pro-oxidant effects. For our research, it is important to emphasize that pro-oxidant effect depends on flavonoid concentration and certain reaction conditions, but it is suggested that mild oxidative stress may induce cellular antioxidative defence system and may be beneficial in preventing further oxidative damage (Procházková, Boušová, Wilhelmová, 2011). The antioxidant activity of flavonoids depends on configuration, substitution, and total number of hydroxyl groups, but also on the occurrence, position, structure, and total number of sugar moieties in flavonoids glycosides. Several mechanisms have been described

to participate in the antioxidant action of flavonoids, while the most significant is their tendency to scavenge ROS. However, they have potential to suppress ROS formation by inhibiting enzymes such as microsomal monooxygenase, glutathione S-transferase, mitochondrial succinoxidase, NADH oxidase, or by chelating trace elements involved in free radical generation (Kumar, Pandey, 2013). Flavonoids also show indirect antioxidant capacity by modulating gene expression and by inducing the endogenous antioxidant enzymatic defence system. Polyphenol and flavonoid compounds scavenge suddenly generated ROS during restoration of flow and that might be one of the explanations for our finding. Endogenous antioxidant defence system components, particularly SOD and CAT, are also activated by these natural biomolecules, resulting in attenuation of oxidative stress-induced tissue damage. Impact on transcription-mediated signaling is responsible for the long-lasting antioxidative effect of these natural molecules (Mattera *et al.*, 2017). Observed effects of *G. verum* extract on cardiac redox status are probably a consequence of additive and synergistic antioxidative activities of all present bioactive compounds.

## CONCLUSION

Based on our findings we might conclude that 4-week treatment with methanol extract of *G. verum* has dose-dependent potential to alleviate cardiac oxidative stress that contributed to harmful effects of I/R injury. A higher dose was associated with greater antioxidative activity and stronger capability of modulating enzymes involved in antioxidative protection. However future studies are necessary to fully clarify the role of *G. verum* as an additional strategy in preventing myocardial damage in the presence of hypertension or other chronic diseases.

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Received for publication on 26<sup>th</sup> February 2020

Accepted for publication on 03<sup>rd</sup> January 2021