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Original Article

Micellar propolis nanoformulation of high antioxidant and hepatoprotective activity

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ABSTRACT

The present study reports a promising antioxidant protection by a recently developed micellar propolis formulation, against oxidative stress in *in vitro* and *in vivo* models of toxicity. The formulation, based on poplar propolis encapsulated in poly(ethylene oxide)–β-poly(propylene oxide)–β-poly(ethylene oxide) triblock copolymer (PEO_{26}–PPO_{40}–PEO_{26}) micelles is characterized by small size (D_h = 20 nm), enhances aqueous solubility and good colloidal stability. *In vitro*, propolis-loaded PEO_{26}–PPO_{40}–PEO_{26} micelles (20–100 μg/ml) significantly increased the cell viability of human hepatoma HepG2 cells, subjected to H_2O_2-induced cell injury (0.1 mM, 1 h). Antioxidant activity and protection of the micellar propolis were evaluated in a model of carbon tetrachloride-induced hepatotoxicity in rats (10% CCl_4 solution, 1.25 ml/kg, *p.o.*) by measurement of non-enzyme (malondialdehyde and glutathione) and enzyme (catalase and superoxide dismutase) biomarkers of oxidative stress. Clinic observations, hematological, biochemical parameters and histological analysis were also performed. *In vivo*, micellar propolis (20 mg/kg b.w., *p.o.*, 14 days) ameliorated CCl_4-induced acute liver injury in rats. The oral administration of micellar propolis significantly prevented serum transaminase increases, as well as brought the levels of malondialdehyde, glutathione, and antioxidant enzymes catalase and superoxide dismutase toward the controls levels. Therefore, PEO_{26}–PPO_{40}–PEO_{26} micelles could be considered as a promising oral delivery system of propolis against oxidative stress injury in liver cells.

Introduction

Oxidative stress plays a crucial role in the pathology of different hepatic disorders such as inflammatory injury, liver cirrhosis, ischemia reperfusion impairment (Sumida et al., 2013; Nita and Grzybowski, 2016; Torok, 2016). Therefore, the interest in protective effects of natural antioxidants against oxidative stress in liver is considerable. Recent studies showed a promising antioxidant activity of propolis (Pr) by the mechanisms of scavenging, neutralizing and removing the reactive species after oxidative stress induction (ROS), and by preventing lipid peroxidation (Mavri et al., 2012; da Silva Frozza et al., 2014; Hofer et al., 2014; Jug et al., 2014; El-Guendouz et al., 2018).

Nevertheless, the poor solubility of the active lipophilic secondary plant metabolites in biological media reduces their bioavailability, which is an obstacle for effective *in vivo* application of propolis. Therefore, an increasing interest in the development of new delivery systems for weakly soluble active substances from natural origin exists, aiming to increase the solubility in biological fluids and to improve their bioavailability. Polymeric micelles are widely studied as potential drug delivery carriers (Bastakoti and Liu, 2017). Among them, polymeric micelles from amphiphilic block copolymers are explored as a platform for delivery of low soluble drugs in anticancer therapy and imaging (Bastakoti et al., 2013). Their unique properties allow the sustained release of the encapsulated hydrophobic drug and stabilization of the particle in aqueous solution. Recently, different drug-delivery nanoformulations obtained by self-assembly (quantosomes, cubosomes, spongosomes, vesicles, *etc.*) have been proposed for delivery of weakly soluble compounds, including antioxidants (Ferrer-Tasies

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et al., 2013; Angelova et al., 2011, 2018; Guerzoni et al., 2017; Zou et al., 2017). In addition to the improved delivery characteristics, the carrier of the system should allow optimal loading and preservation of antioxidant activity of the active molecule. In previous studies, we found that the encapsulation of flavonoid quercetin in chitosan-alginate nanoparticles led to achievement of higher antioxidant activity at low concentrations (Tzankova et al., 2017), and the ability of curcumin loaded in cationic polymeric micelles to scavenge ABTS radical and hypochlorite ions was higher than that of the free curcumin (Yoncheva et al., 2015). Overall, micellar formulations based on poly (ethylene oxide)–β-poly(propylene oxide)–β-poly(ethylene oxide) (PEO–PPO–PEO) triblock copolymers are among the most extensively studied for biomedical application (Kabanov et al., 2002). Various natural antioxidants, such as curcumin, silybin, *etc.* have been effectively loaded into PEO–PPO–PEO micelles (Li et al., 2009; Sahu et al., 2011; Gorinova et al., 2016). A significant improvement of pharmacokinetic parameters of the bioactive compounds loaded in PEO–PPO–PEO-based micellar formulations and increased time of systemic circulation have been demonstrated by *in vivo* experiments.

Recently, a novel micellar form of poplar propolis based on $PEO_{26}PPO_{40}PEO_{26}$ (Pluronic P85) triblock copolymer was prepared and reported by our team (Petrov et al., 2016). All biologically active lipophilic constituents of poplar (*Populus nigra* L., Salicaceae) propolis were solubilized with the aid of polymeric micelles in aqueous media. Furthermore, the small size of the micelles containing propolis (D_h = 20 nm) and the extremely good colloidal stability of the system offered an opportunity for further *in vitro* and *in vivo* characterization of its beneficial effects. This approach could also protect the unstable biologically active metabolites from premature oxidation and would give the opportunity to use the lower effective doses of antioxidant. However, attention should be given to preservation of favorable antioxidant properties of micellar propolis. The aim of the present study was to assess the antioxidant efficiency and hepatoprotective activity of the recently developed micellar form of poplar propolis in a model of oxidative stress injury *in vitro* (human hepatoma HepG2 cells) and after acute CCl_4-induced hepatotoxicity *in vivo*.

Material and methods

Chemicals

$PEO_{26}PPO_{40}PEO_{26}$ (Pluronic P85) was kindly donated by BASF. Poplar propolis was collected in the Balkan Mountains, near the town of Elena, Bulgaria. DMEM cell medium (4500 mg/l glucose, without L-glutamine) with 10% fetal bovine serum, 2 mM L-glutamine, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), hydrogen peroxide solution 30% (w/w) were supplied from Sigma-Aldrich (Germany). All other reagents were of analytical grade.

Propolis extraction

Poplar propolis, grated after cooling, was extracted for 24 h with ethanol/water (70/30, v/v) mixture at room temperature (1:10, w/v), filtered and dried using a rotatory evaporator. The dried extracts content was analyzed by GC–MS using the procedure, previously described by Petrov et al. (2016).

Preparation of propolis loaded polymeric micelles

The dried extract was dissolved in ethanol/water (70/30, v/v) mixture and used for further experiment. 4 g of poplar propolis, dissolved in 72.6 ml ethanol/water (70/30, v/v) mixture, were added to 178.2 ml distilled water containing 12 g $PEO_{26}PPO_{40}PEO_{26}$ triblock copolymer. Then, ethanol was removed from the system by rotary evaporator (50 °C, 70 mbar) giving a transparent yellowish colloidal aqueous solution.

Characterization of propolis loaded polymeric micelles

Dynamic light scattering (DLS) measurements were conducted with a Zetasizer NanoBrook 90Plus PALS, equipped with a 35 mW red diode laser (λ = 640 nm) at a scattering angle of 90°. Transmission electron microscopy (TEM) analysis was conducted with a JEOL 2100 electron microscope at an accelerating voltage of 200 kV, equipped with a digital camera. A drop of sample solution was deposited on a TEM copper grid coated with a Carbon film, and the solvent was allowed to evaporate. Propolis release from $PEO_{26}PPO_{40}PEO_{26}$ micelles was investigated in phosphate buffer (pH 7.4) by UV–vis spectroscopy using the dialysis method.

Cell lines and culturing

Human hepatocellular carcinoma cells HepG2 were purchased from European collection of cell cultures (ECACC). HepG2 were kept in culture and expanded at 37 °C in a humidified atmosphere of 5% CO_2 in culture medium DMEM cell medium (4500 mg/l glucose, without L-glutamine), with 10% fetal bovine serum (FBS, Gibco BRL) and 2 mM L-glutamine.

Model of oxidative stress in vitro

HepG2 cells were seeded at a density 4×10^4 cells/well. After 24 h the cells were pre-treated (1 h) with propolis-loaded polymer micelles (Pr-PM) (20, 50, 100, 250 μg/ml), propolis (Pr) (20, 50, 100, 250 μg/ml) and blank polymeric micelles (PM) (60, 150, 300, 750 μg/ml). HepG2 cells were exposed to H_2O_2 (0.1 mM, 1 h) to obtain submaximal cytotoxicity (approximately 60% *vs* untreated controls). Viability was assayed after 24 h by MTT-dye reduction assay (Mosmann, 1983).

Animal selection and care

The *in vivo* study was conducted on 42 male Wistar rats (body weight 250–280 g). Animals were purchased from the National Breeding Center, Sofia, Bulgaria. The rats were housed under standard laboratory conditions (20 °C ± 2 °C and humidity 72% ± 4%) with free access to water and standard pelleted rat food (ISO 9001:2008). All performed procedures were approved by the Institutional Animal Care Committee (KENIMUS) and the principles stated in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe, 1991; EU, 2010) were strictly followed throughout the experiment.

In vivo *model of CCl_4-induced liver toxicity*

The animals were divided into seven groups of six animals each (n = 6). The compounds were administered orally *via* gavage (0.5 ml/100 g b.w.) for 14 days, as described:

Group 1 (CNTR): saline (0.5 ml/100 g b.w., *p.o.*, 14 days).
Group 2 (PM): blank polymeric micelles (60 mg/kg b.w., 14 days).
Group 3 (Pr): non-loaded propolis (20 mg/kg b.w., 14 days).
Group 4 (Pr-PM): propolis-loaded polymer (propolis concentration corresponding to 20 mg/kg b.w., 14 days).
Group 5 (CCl_4): saline (0.5 ml/100 g b.w., *p.o.*, 7 days); at day 7 the animals were challenged with CCl_4 (10% solution, 1.25 ml/kg);

Table 1
Chemical composition of ethanol extract of propolis sample (% of total ion current).[a]

Compound	% of TIC	Compound	% of TIC
Aromatic acid	6.6	*Chalcones*	1.6
Benzoic acid	0.6	Pinocembrin chalcone	1.0
Cinnamic acid	0.1	Trihydroxymonomethoxy chalcone*m/z* = 502	0.2
p-Hydroxybenzoic acid	0.1	Pinobanksinacetate chalcone	0.4
p-Coumaric acid	0.6	*Flavanones and dihydroflavonols*	*37.3*
Dimethoxycinnamic acid	1.1	Pinobanksin	5.2
Ferulic acid (*Z*)	0.8	Pinocembrin	10.4
Ferulic acid (*E*)	0.8	Sakuranetin	0.2
Caffeic acid	0.2	Dihydroxymethoxyflavanone	2.1
Caffeic acid	2.1	3-Acetylpinobanksin	14.4
		Pinobanksin butanoate	0.7
Phenolic acid esters	*13.0*	Pinobanksin propanoate	1.6
Pentenyl coumarate	0.1	Pinobanksin pentanoate	1.8
Pentenyl coumarate (isomer)	0.2	Alpinone	0.2
3-Methyl-3-butenyl ferulate	0.1	Pinobanksin pentenoate	0.7
2-Methyl-2-butenyl ferulate	0.1	*Flavones and flavonols*	*22.6*
3-Methyl-2-butenyl ferulate	0.9	Galangin	8.2
2-Methyl-2-butenyl caffeate	0.6	Chrysin	7.6
3-Methyl-2-butenyl caffeate	1.9	Dihydroxymonomethoxy flavone	0.8
Phenylethyl *p*-coumarate	0.1	Kaempferol	0.9
Benzyl caffeate	2.3	Quercetin	1.1
Benzyl ferulate	0.2	Kaempferol methyl ether	1.3
Cinnamyl caffeate	1.3	Quercetin-methyl ether	1.2
Phenylethyl caffeate	3.7	Quercetin-methyl ether (isomere)	0.7
Others	*0.2*	Quercetin-methyl ether (isomere)	0.6
Phenylethyl alcohol	0.1	Dihydroxymonomethoxy flavone	0.2
Hexadecanoic acid	0.1	*Sugars*	*3.8*

[a] The ion current generated depends on the characteristics of the compound concerned and is not a true quantification.

from day 8–14 the animals were post-treated with saline (0.5 ml/100 g b.w., *p.o.*).
Group 6 (Pr/CCl_4): animals pre-treated with Pr (20 mg/kg, b.w. 7 days). At day 7, animals were challenged with CCl_4 (10% solution, 1.25 ml/kg); from day 8–14 animals were post-treated with Pr (20 mg/kg, b.w., *p.o.*).
Group 7 (Pr-PM/CCl_4): animals pre-treated with Pr-PM (20 mg/kg b.w.). At the day 7, they were challenged with CCl_4 (10% solution, 1.25 ml/kg); from day 8–14 the animals were post-treated with Pr-PM (20 mg/kg b.w., *p.o.*).

On the day 15th, blood samples were collected from the tail vein, the animals were sacrificed by decapitation and the livers were taken for evaluation.

Biomarkers of oxidative stress damage in liver

MDA determination and glutathione (GSH) depletion

MDA quantity was measured spectrophotometrically (λ 535 nm) by molar extinction coefficient 155 mM^{-1} cm^{-1} and was expressed as nmol/g liver as previously described (Bai et al., 2007). GSH was determined by spectrophotometric measurement at λ = 412 nm and expressed as nmol/g liver (Bai et al., 2007).

Analysis of antioxidant enzyme activity

Catalase (CAT) activity (nmol/g liver) was determined by measuring the decrease in absorbance at 240 nm (Aebi, 1974). Superoxide dismutase (SOD) activity (nmol/g liver) was measured s as previously described (Misra and Fridovich, 1972). The protein content was measured by the method of Lowry et al. (1951).

Biochemistry and hematology parameters

Blood samples (0.5 ml) were collected from the tail vein for evaluation of clinical hematology and biochemical assay, which were performed using BC-2800-Vet (Mindray) haematology analyser.

Histological analysis

Liver samples (0.5 cm^3) were fixed in buffered formaldehyde (4% final concentration) and then sectioned at 5 μm. Staining methods have been used according to the standard Hematoxylin–Eosin (HE) and Masson's trichrome (M) protocols. All preparations were analyzed under Leica DM 1000 LED microscope and documented by Leica ICC 50 HD camera on 20× magnification.

Statistical analysis

Results are expressed as mean values and standard deviation (SD) from at least three independent experiments. The cell survival data were normalized as percentage of untreated control set as 100% viability. One-way analysis of variance (ANOVA) with *post hoc* multiple comparisons procedure (Dunnett's test) was used to assess statistical differences in case of normal distribution. Values of $p < 0.05$, $p < 0.01$ and $p < 0.001$ were considered statistically significant. All statistical analyses were performed using GraphPad Prizm Software.

Results

Chemical composition of the propolis extract

The propolis extract used in this study was characterized by GC–MS analysis after silylation. The chemical composition of the extract is given in Table 1. The constituents of the extract were typical for the poplar type propolis: flavonoid aglycones pinocembrin, galangin, chrysis, esters of caffeic acids (phenethyl, benzyl, and pentenyl), and phenolic acids.

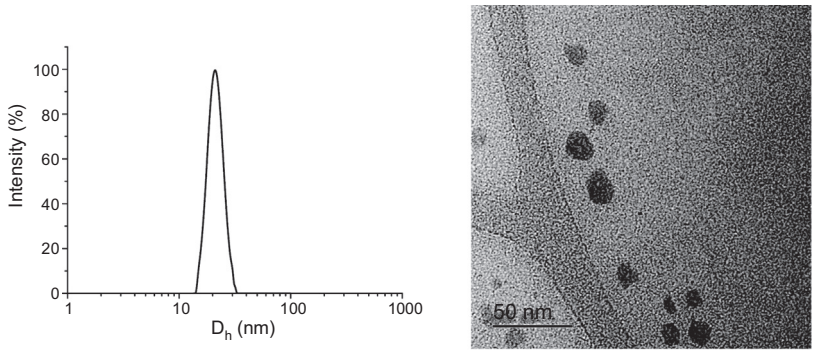

Fig. 1. Particle size distribution curve (left) and TEM micrograph (right) of poplar propolis loaded $PEO_{26}PPO_{40}PEO_{26}$ micelles. The concentrations of propolis and copolymer are 20 g/l and 60 g/l, respectively.

Preparation and physicochemical characterization of propolis-loaded PEO_{26}–PPO_{40}–PEO_{26} micelles

Micelles

Micellar formulation of poplar propolis developed recently by our team (Petrov et al., 2016) is characterized by enhanced aqueous solubility and superior colloid stability. The improved solubility of propolis in biological media is favorable for *in vivo* application and, in particular, an increase of antioxidant activity and hepatoprotective effect of micellar propolis as compared to free propolis, was considered.

Briefly, poplar propolis dissolved in ethanol/water (70/30, v/v) mixture was added to an aqueous solution of PEO_{26}–PPO_{40}–PEO_{26} and, then, the organic solvent was evaporated. The concentration of propolis and PEO_{26}–PPO_{40}–PEO_{26} copolymer in the final transparent yellowish colloidal solution were 20 g/l and 60 g/l, respectively. Dynamic light scattering and transmission electron microscopy analysis confirmed the formation of rather spherical nanoparticles with an apparent hydrodynamic diameter D_h = 21 nm and dispersity index = 0.18 (Fig. 1). The micellar formulation retained its main characteristics (transparency, stability, D_h) upon storage at temperature of 20–25 °C for several weeks. In contrast, the sample prepared without any copolymer formed a turbid unstable suspension, which tended to precipitate after 2 days of storage. Thus, the role of copolymer seems critical for fabricating a colloid solution of propolis in aqueous media. In water, the hydrophobic interactions between water-insoluble constituents of propolis and the hydrophobic PPO chains were strongly favored, and triggered the encapsulation of natural product within the cores of nano-sized PEO–PPO–PEO micelles. The hydrated PEO shell provided an excellent colloid stability of the formulation. A proof for the successful loading of micelles with propolis was provided by TEM analysis. The spherical particles possessed a high contrast unlie the blank PEO–PPO–PEO micelles. Obviously, the aromatic molecules located within the micellar core contributed, similarly to the standard staining agents, to enhance the quality of visualization of polymeric micelles. Further on, the performed *in vitro* release studies showed a slight initial weak burst effect followed by a sustained release within 56 h (Fig. 2). This fact also suggests a pronounced hydrophobic interaction between the core-forming PPO segments and the lipophilic molecules of propolis extract.

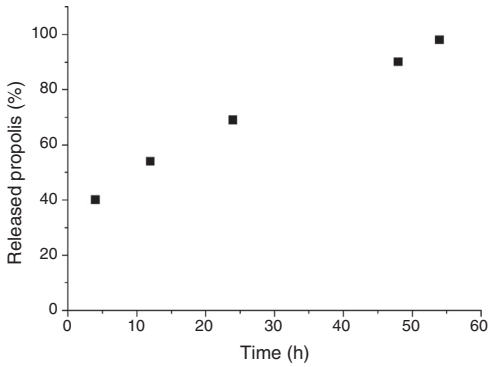

Fig. 2. Release profile of propolis from $PEO_{26}PPO_{40}PEO_{26}$ micelles in phosphate buffer (pH 7.4).

In vitro *protective effects in a model of H_2O_2-induced oxidative stress*

The safety evaluation of a new propolis-based formulation is an important step in its further characterization. Our preliminary study showed a good safety profile of blank PEO_{26}–PPO_{40}–PEO_{26} micelles (60–750 μg/ml) against HepG2 cells, since no direct cytotoxicity was observed (data not shown). Based on a good safety profile of the micellar PEO_{26}–PPO_{40}–PEO_{26} formulation on HepG2 cells, we further evaluated the effectiveness of the propolis-loaded PEO_{26}–PPO_{40}–PEO_{26} micellar formulation in H_2O_2-induced oxidative stress model *in vitro*.

The cell viability decreased by 60% ($p < 0.001$) *vs* untreated controls after H_2O_2-treatment (0.1 mmol/l, 1 h), indicating significant cell damage (Fig. 3). The pretreatment with Pr (20, 50, 100 μg/ml) and Pr-PM (propolis concentration corresponding to 20, 50 and 100 μg/ml) substantially decreased the H_2O_2-induced cell impairment (Fig. 3B, C). The protection seen by Pr-PM was similar to those of free Pr, showing the preservation of antioxidant activity by propolis micellar formulation. In contrast, the blank micelles (60–750 μg/ml) did not show any protection against oxidative stress damage (Fig. 3A). It should be noted that the protection was observed in the propolis concentrations up to 100 μg/ml, while at the higher concentration (250 μg/ml), both Pr and Pr-PM did not show an increase in cell viability, compared to H_2O_2-treatment.

In vivo *hepatoprotection in a model of carbon tetrachloride-induced toxicity*

Clinic observations

The repeated oral administration of PM (60 mg/kg b.w.), Pr (20 mg/kg b.w.) and Pr-PM (propolis, equivalent to 20 mg/kg b.w.) did not show any changes in the clinical appearance, reflections, respiration, behavior or mortality of the animals of all treated groups *vs* untreated controls. The average daily feed intake, body and liver weight in the treatment groups did not show statistically significant changes *vs* control group (Table 2).

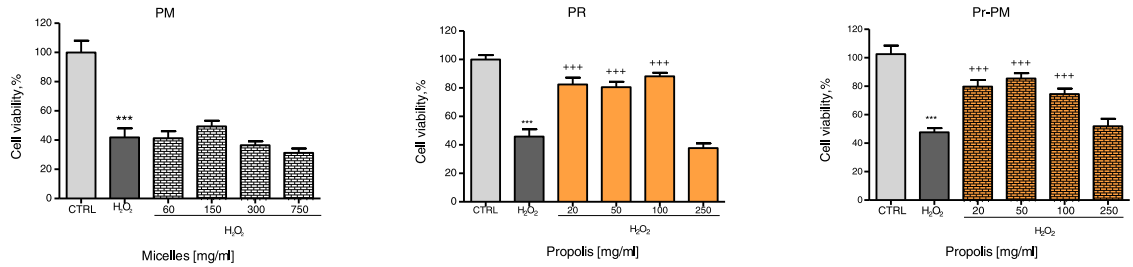

Fig. 3. Effect of blank $PEO_{26}PPO_{40}PEO_{26}$ micelles (PM), non-loaded propolis (Pr) and propolis-loaded $PEO_{26}PPO_{40}PEO_{26}$ micelles (Pr-PM) on cell the viability of HepG2 cells in a model of H_2O_2-induced toxicity. Mean ± SD ($n = 8$). Values of $^{***}p < 0.001$ *vs* control group; $^{+++}p < 0.001$ *vs* H_2O_2 group were considered statistically significant.

Table 2
Effects of blank $PEO_{26}PPO_{40}PEO_{26}$ micelles (60 mg/kg b.w.), non-loaded propolis (20 mg/kg b.w.) and propolis-loaded micelles (Pr-PM, propolis corresponding to 20 mg/kg b.w.) on daily feed intake, total body weight and liver weight after 14 days treatment (*p.o.*) in male Wistar rats.

Treatment	Body weight (g)	Liver weight (g)	Food intake (g)
Control	272.1 ± 3.2	6.2 ± 1.2	28.9 ± 3.1
PM	271.9 ± 5.1	5.8 ± 1.1	29.1 ± 2.9
Pr	268.3 ± 4.2	5.9 ± 2.2	30.1 ± 2.8
Pr-PM	270.9 ± 3.8	6.1 ± 2.3	28.9 ± 2.3
CCl_4	271.1 ± 4.1	5.7 ± 3.1	29.7 ± 2.9
Pr/CCl_4	269.2 ± 2.9	5.6 ± 2.8	30.0 ± 2.1
Pr-PM/CCl_4	272.2 ± 4.3	6.2 ± 1.8	29.3 ± 1.8

Data are presented as the mean ± SD ($n = 6$).
propolis (Pr); polymer micelles (PM)

Hematology and serum biochemistry analysis

Several hematological parameters were monitored in order to evaluate the eventual hematology impairment (Table 3). As shown, no significant changes in hematology parameters were found in all treatment groups, before and after CCl_4-treatment, compared to the control animal. The effects of blank micelles, non-loaded propolis and propolis-loaded PEO_{26}–PPO_{40}–PEO_{26} micelles on serum biochemical parameters are presented in Table 4. As expected, significant increase in aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were detected in the CCl_4 group ($^{**}p < 0.01$ and $^{*}p < 0.05$), thus confirming the hepatotoxic injury. Animals orally received 20 mg/kg free or micellar propolis for 7 days prior CCl_4-treatment, followed by a post-treatment period of 7 days, showed significant improvement of the most biochemical markers toward the normal values.

Oxidative stress markers assay in liver

The mechanisms of CCl_4-induced toxicity include generation of unstable trichloromethyl ($CCl_3^{\bullet}$) and trichloromethyl peroxyl ($CCl_3O_2^{\bullet}$) radicals by CYP450 in liver endoplasmic reticulum, which triggers tissue and cell damage (Weber et al., 2003). Particularly, our attention was focused to the possible protective effects of micellar propolis on the levels of MDA and GSH as markers of non-enzymatic antioxidant defense system.

The repeated oral administration (14 day, *p.o.*) of PM and Pr-PM did not reveal any signs of toxicity, since no statistically significant changes in the level of GSH and MDA were observed (Fig. 4A, B). In contrast, CCl_4 treatment evidently caused hepatotoxicity. Compared to the control untreated group, CCl_4 administration induced significant pro-oxidant effects, discerned by a marked increase in MDA production by 65% ($p < 0.001$, *vs* untreated control) and a decrease in GSH levels by 46% ($p < 0.001$, *vs* untreated control). However, the toxic effects were attenuated in the groups pre-treated with propolis, either non-loaded or loaded in polymer

Table 3
Effects of blank $PEO_{26}PPO_{40}PEO_{26}$ micelles (60 mg/kg b.w.), non-loaded propolis (20 mg/kg b.w.) and propolis-loaded micelles (Pr-PM, propolis corresponding to 20 mg/kg b.w.) on hematology parameters in a model of CCl_4-induced liver injury.

Parameter	Control	PM	Pr	Pr-PM	CCl_4	CCl_4 + Pr	CCl_4 + Pr-M
White blood cell (10^9/l)	11.43 ± 2.69	7.45 ± 3.70	9.15 ± 3.14	6.05 ± 3.47	9.51 ± 2.62	7.85 ± 2.07	9.11 ± 4.64
Lymphocyte (10^9/l)	5.31 ± 2.28	4.45 ± 2.31	5. 01 ± 2.19	3.55 ± 2.48	5.42 ± 1.02	5.43 ± 1.11	5.28 ± 2.37
Granulocyte (10^9/l)	3.68 ± 0.43	2.78 ± 1.41	3.83 ± 0.91	2.38 ± 0.97[a]	3.81 ± 2.43	3.18 ± 1.08	3.53 ± 2.18
Red blood cell (10^{12}/l)	8.51 ± 0.54	9.06 ± 0.91	8.71 ± 0.67	7.49 ± 0.45	8.29 ± 0.31	7.68 ± 0.43	7.73 ± 1.61
Hemoglobin (g/l)	149.01 ± 10.52	164.10 ± 18.07	153.25 ± 9.91	139.75 ± 8.85	144.51 ± 4.79	131.75 ± 7.63	136.75 ± 27.84
Hematocrit (%)	47.53 ± 2.85	51.5 ± 5.71	48.125 ± 2.67	41.95 ± 2.65	45.63 ± 1.25	41.98 ± 2.58	47.25 ± 8.47
Red cell distribution width (%)	14.325 ± 1.01	13.43 ± 1.02	13.93 ± 0.96	13.31 ± 0.63	14.35 ± 0.44	14.28 ± 0.71	14.20 ± 0.61

Data are given as the mean ± SD ($n = 6$).
propolis (Pr); polymer micelles (PM)
[a] $p<0.05$ vs untreated controls.

Table 4
Effects of blank $PEO_{26}PPO_{40}PEO_{26}$ micelles (60 mg/kg b.w.), non-loaded propolis (Pr, 20 mg/kg b.w.) and propolis-loaded $PEO_{26}PPO_{40}PEO_{26}$ micelles (Pr-PM, propolis corresponding to 20 mg/kg b.w.) on serum biochemical parameters in a model of CCl_4-induced liver injury.

Parameter	Control	PM	Pr	Pr-PM	CCl_4	CCl_4 + Pr	CCl_4 + PrPM
Aspartate aminotransferase (U/l)	199.83 ± 67.22	203.35 ± 8.05	166.51 ± 20.60	149.125 ± 15.88	328.35 ± 28.23[a]	193.51 ± 23.24[c]	170.05 ± 23.11[c]
Alanine aminotransferase (U/l)	79.75 ± 14.60	77.60 ± 10.95	79.46 ± 20.31	68.41 ± 5.06	368.18 ± 65.01[b]	75.42 ± 12.21[d]	69.18 ± 13.78[d]
Total protein (g/l)	63.73 ± 3.78	63.88 ± 2.29	65.23 ± 1.12	64.93 ± 2.99	64.91 ± 1.2	64.93 ± 3.39	63.08 ± 2.64
Alkaline phosphatase (U/l)	551.01 ± 13.29	530.75 ± 58.36	557.51 ± 58.48	575.25 ± 72.51	732.25 ± 29.07[a]	535.25 ± 57.73[c]	533.25 ± 60.38[c]

Data are given as the mean ± SD ($n = 6$).
[a] $p < 0.05$ *vs* untreated controls.
[b] $p < 0.01$ *vs* untreated controls.
[c] $p < 0.05$ *vs* CCl_4-treatment group.
[d] $p < 0.01$ *vs* CCl_4-treatment group.

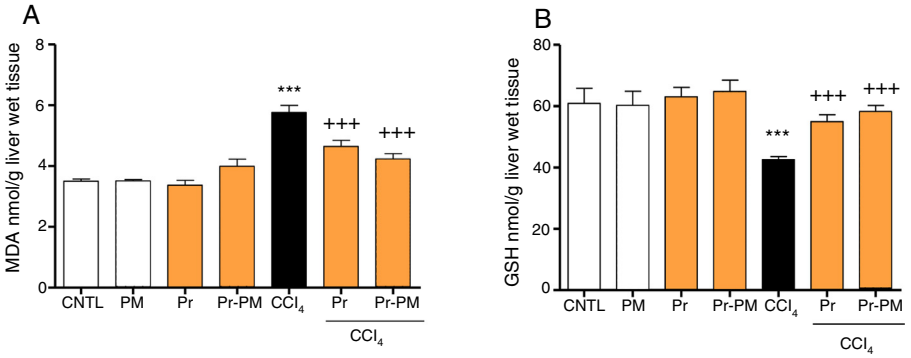

Fig. 4. Protective effects of blank $PEO_{26}PPO_{40}PEO_{26}$ micelles (PM), non-loaded propolis (Pr) (20 mg/kg b.w.) and propolis-loaded in polymeric micelles (Pr-PM) (propolis, corresponding to 20 mg/kg b.w.) on the contents of MDA (A) and GSH (B) in a model of CCl_4-induced (10%, 1.25 ml/kg b.w.) liver injury in male *Wistar* rats. Data are presented as the mean ± SD for 6 animals in each group ($n = 6$). $^{***}p < 0.001$ *vs* control value (non-treated rats); $^{+++}p < 0.001$ *vs* carbon tetrachloride (CCl_4) treated group.

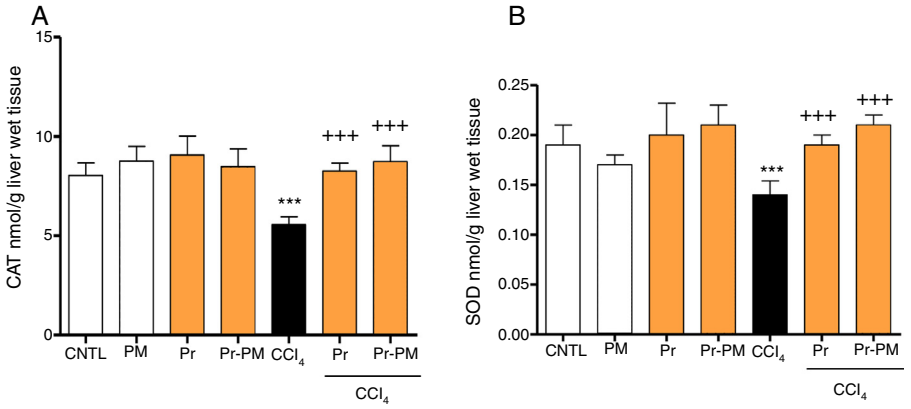

Fig. 5. Catalese (CAT) and superoxide dismutase (SOD) activities in rat liver microsomes after CCl_4-induced (10%, 1.25 ml/kg b.w.) liver damage in rats. Blank $PEO_{26}PPO_{40}PEO_{26}$ micelles (PM), non-loaded propolis (Pr) (20 mg/kg b.w.) and propolis-loaded in polymeric micelles (Pr-PM) (propolis, corresponding to 20 mg/kg b.w.) were administered orally, as described in Materials and methods. Data are presented as the mean ± SD for 6 animals in each group ($n = 6$). $^{***}p < 0.001$ *vs* control values; $^{+++}p < 0.001$ *vs* CCl_4-treatment.

micelles (Pr/CCl_4; $Pr\text{-}PM/CCl_4$), and then challenged with CCl_4. The measured parameters were close to/or restored to the control level, thus showing a significant protection (Fig. 4A). MDA levels were significantly decreased by micellar propolis (26% decrease *vs* CCl_4 group) followed by non-loaded propolis (19.7% decrease *vs* CCl_4 group).

The evaluation of GSH levels in liver homogenate showed significant protection against CCl_4-induced depletion in both experimental groups pre-treated with Pr (46% GSH increase *vs* CCl_4 group, $p < 0.001$) and Pr-PM (49% GSH increase *vs* CCl_4-group, $p < 0.001$) (Fig. 4B). The protective effect of propolis-loaded micelles was superior to that of non-loaded propolis.

Liver antioxidant enzyme assay

In attempt to elucidate in depth the protective mechanisms of propolis-loaded micelles on toxic hepatic injury and lipid peroxidation, we also evaluated the effects on antioxidant enzymes activity of CAT and SOD (Fig. 5). The treatment with blank micelles, propolis or the propolis-loaded micellar formulation (14 day, *p.o.*) did not result in any changes of the antioxidant enzymes. In contrast, CCl_4 administration induced a significant decrease in antioxidant activity of both CAT (by 30%, $p < 0.001$) and SOD (by 26%, $p < 0.001$), compared to the control untreated group. However, in the groups pre-treated with propolis, either non-loaded or loaded in polymeric micellar formulation and then challenged with CCl_4, the antioxidant enzyme activities were close to controls. Furthermore, the treatment with propolis-loaded micelles led to a significant increase in both antioxidant enzyme activities, CAT by 37% and in SOD by 50% ($p < 0.001$), *vs* CCl_4-treatment group.

Liver histopathological examination

The liver segments were evaluated according the acinar structure related to the central venous and terminal portal circulation, parenchymal cells and reticular system changes and biliary canalicular network (Fig. 6). Pr and Pr-PM samples showed no signs of necrosis, regeneration changes and cell replacement (Fig. 6B, C). In contrary, the CCl_4-treated group showed severe pericentral fatty degeneration, necrosis of hepatocytes and onset of fibrous sinusoidal regeneration (Fig. 6D). The livers of the animals pre-treated with Pr and Pr-PM and after that challenged to CCl_4, showed normal periportal acinar structures with several focuses of pericentral sinusoid congestion (Fig. 6E, F). Lack of necrosis, regeneration changes and cell replacement, as well as intact biliary canalicular network were established. Thus, the histopathological analysis shows high hepatoprotection in both propolis and micellar propolis treatment groups. These findings confirmed the beneficial effects already observed on oxidative stress biomarkers GSH and MDA, and on enzymes SOD and CAT.

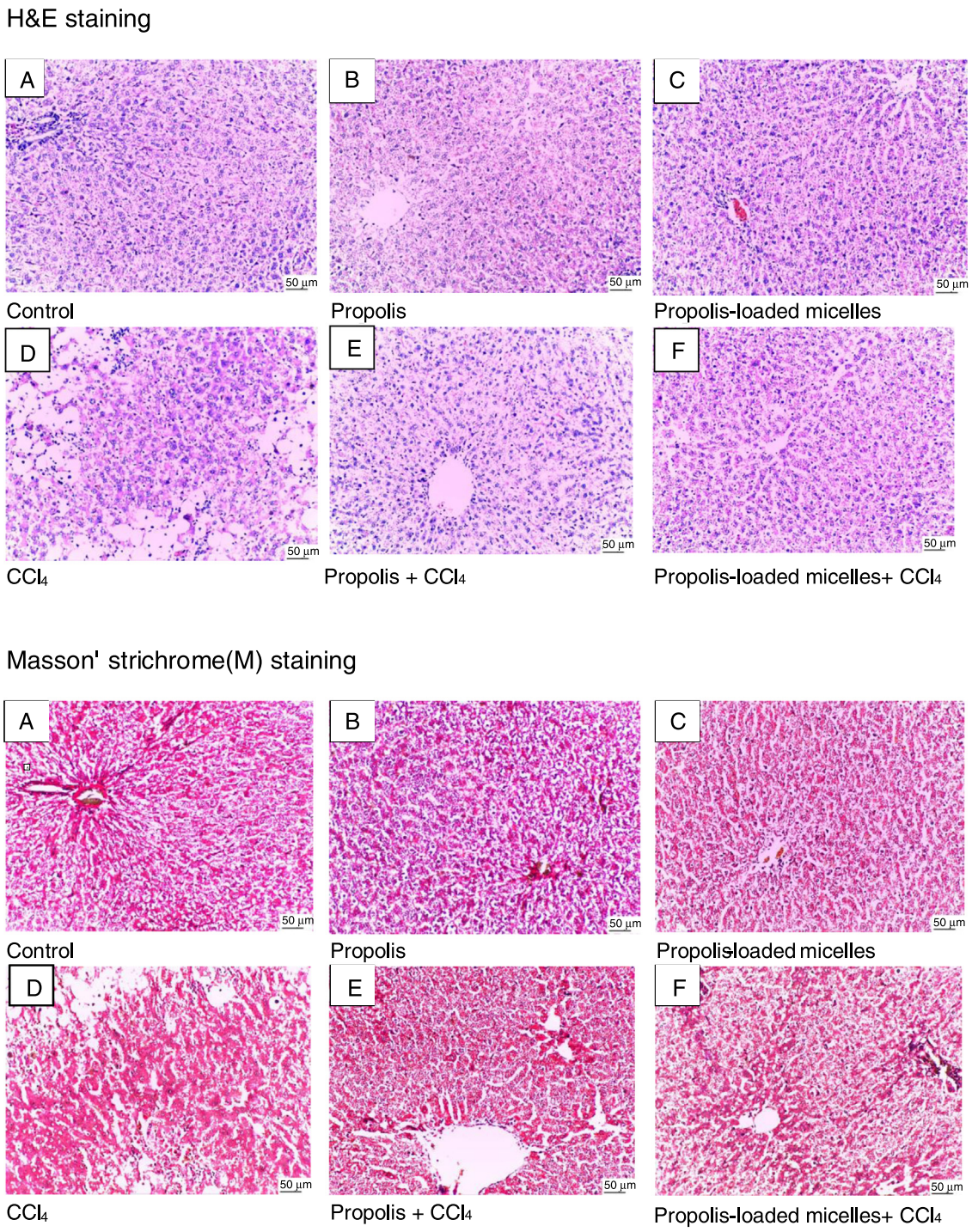

Fig. 6. Rat liver sections after H&E staining and Masson's trichrome (M) staining (scale bars = 50 µm). The treatments were as described: (A) untreated controls; (B) non-loaded propolis (Pr; 20 mg/kg b.w.); (C) propolis-loaded PEO–PPO–PEO micelles; (D) CCl_4-treated group (10%, 1.25 ml/kg b.w.); (E) non-loaded propolis (20 mg/kg b.w.) + CCl_4; (F) propolis-loaded $PEO_{26}PPO_{40}PEO_{26}$ micelles (propolis, corresponding to 20 mg/kg b.w.) + CCl_4. The liver segments are evaluated according the acinar structure related to the central venous and terminal portal circulation, parenchymal cells and reticular system changes and biliary canalicular network.

Discussion

Poplar propolis and the major constituents, and especially caffeic acid and its phenethyl ester (CAPE), galangin, pinocembrin are well-known antioxidants (Boisard et al., 2014; Bacanlı et al., 2016; Coban et al., 2017; Yang et al., 2018). Poplar propolis has also demonstrated hepatoprotective properties (Banskota et al., 2000). However, propolis active constituents are lipophilic and poorly soluble in water and aqueous/physiological media and this is a serious obstacle for application of propolis in therapy. The therapeutic effects of natural antioxidants, such as propolis, might be hindered by inadequate delivery and low bioavailability. The strategy for improvement of antioxidant delivery includes a choice of an appropriate carrier with gradual, sustained release rate, thus avoiding burst effects. Additionally, a possible pro-oxidant toxicity risk might be prevented by application of the antioxidants at low effective concentrations. The carrier would allow efficient intracellular delivery and protection of the loaded antioxidant from

inactivation. Recently, new propolis-loaded PEO_{26}–PPO_{40}–PEO_{26} copolymer micelles were developed and reported by our team (Petrov et al., 2016). The main advantages of the propolis-loaded micelles *vs* well known alcoholic extracts include the solubilization in aqueous media of biologically active lipophilic constituents of propolis, as well as achievement of sustained release which would give an opportunity for its use in broad spectrum of pathologies, including liver impairments. The present study demonstrates high hepatoprotective and antioxidant activities and a good safety profile of the novel micellar form of propolis *in vitro* and in a model of CCl_4-induced liver injury *in vivo*.

H_2O_2 is generated from different sources and could easily enter the body tissues and cells, allowing free radicals formation (*i.e.* hydroxyl radical formed by Fenton's reaction), which further interact directly with cellular and subcellular structures causing damage to proteins, lipids, DNA, *etc. In vitro*, we showed a significant antioxidant protection of propolis-loaded micelles at concentrations <100 μg/ml. Nevertheless, we found that at higher concentrations (250 μg/ml) propolis-loaded micelles did not increase HepG2 cell viability. These results were not surprising having in mind the numerous reports regarding propolis mediated suppression of cell proliferation. Many studies have shown an anti-proliferative activity of both propolis and its bioactive compounds (Watanabe et al., 2011; Sawicka et al., 2012; Tyszka-Czochara et al., 2014; Turan et al., 2015). Recently, it was reported that Chinese poplar propolis (100 and 300 μg/ml; ethanolic extract) or brown Cuban propolis (La Habana) significantly inhibited proliferation of HepG2 cells (Andreu et al., 2015; Zhao et al., 2014).

Further, we performed *in vivo* evaluation of the hepatoprotective potential of propolis-loaded polymer micelles (Pr-PM) in a model of CCl_4-induced toxicity in male *Wistar* rats. The elevated levels of liver serum transaminases ALT, AST and also ALP were considered to be evidence of CCl_4-induced liver injury. *In vivo* pre-treatment with Pr-PM restored the levels of serum transaminases. The protective mechanisms might include regeneration of parenchymal cells in response to the beneficial effects of different biologically active constituents of poplar propolis (*i.e.* flavonoids, phenolic acids and their esters). Thus, preventing the cell membrane destruction, the natural antioxidants subsequently might decrease the leakage of liver transaminase in blood circulation. The performed histopathological observation sustained biochemical data and confirmed the protective effects of Pr-PM against CCl_4-induced liver damage. Significant morphological changes coherent with the onset of an acute hepatocellular injury with hepatic necrosis in the CCl_4-treatment group were highly prevented by pre-treatment with Pr-PM.

Considerable lowering of the GSH level and increase in MDA in the liver clearly reflects a fall in the antioxidant status. This reaction is a response to CCl_4 administration and might be due to its increased utilization by hepatocytes in attempt to scavenge CCl_3. and other toxic radicals. In a model of CCl_4-induced hepatotoxicity, we observed protective antioxidant effects of propolis-loaded micelles, as measured by the elevated levels of endogenous GSH and by decreased MDA production in liver. The effect of micellar propolis was superior of those of non-loaded propolis. The observed propolis protection correlates with other *in vivo* studies. Lin et al. (1998) found that propolis improves the antioxidant defense system by increasing the total GSH pool. Antioxidant enzymes, such as SOD, CAT, glutathione peroxidase and peroxireductase act as liver defense system against free radical induced damage. In conditions of liver injury (*i.e.* CCl_4-intoxication), the ability of the endogenous antioxidant system is impaired and the membrane disintegration of hepatocytes is discerned by a decrease in antioxidant enzyme activities. However, we found that the pre-treatment with Pr-PM prevented the CCl_4-induced oxidative damage by preserving antioxidant enzyme activities of SOD and CAT; the effects resemble those of propolis. Our observations about protective effects of propolis against carbon tetrachloride (CCl_4)-induced injury are consistent with other reports (Mahran et al., 1996; Bhadauria et al., 2008; El-Khatib et al., 2014). For example, Bhadauria et al. (2008) evaluated the hepatoprotective effect of propolis (50–400 mg/kg b.w.) in a model of CCl_4-induced liver toxicity. They found an effective protection only after administration of higher doses of propolis (200 and 400 mg/kg, b.w.). It should be mentioned, that we observed considerable hepatoprotective effects of micellar propolis using much lower propolis doses (20 mg/kg, b.w.). Most probably, the high stability and sustained release micellar propolis, contribute to its favorable effects (Petrov et al., 2016). The therapeutic use of low dose propolis could be considered advantageous taking into account that high antioxidant concentrations would exert pro-oxidant effects.

Conclusion

The present study demonstrates antioxidant and hepatoprotective effects of micellar formulation of poplar propolis, based on the biocompatible triblock copolymer PEO_{26}–PPO_{40}–PEO_{26} in oxidative stress toxicity models *in vitro* and *in vivo*. The antioxidant activity and hepatoprotection was proven by restoration of the glutathione levels, by normalizing the serum transaminase activities, and by significant protection of liver antioxidant enzymes SOD and CAT. A significant improvement in the liver histoarchitecture by micellar propolis was observed, suggesting liver protection. Our findings suggest that the newly developed propolis micellar nanoformulation might be considered as a promising and safe delivery system against oxidative stress-induced injury in liver.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

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Authors' contribution

VT mainly contributed to conception, study design and interpretation of the results and writing of the manuscript, VB has substantial contribution in propolis preparation and chemical characterization, PP prepared and characterized the propolis loaded micellar nanoformulation and contributed to writing of the manuscript, DA and YY (PhD student) have a substantial contribution to acquisition of experimental *in vitro* and *in vivo* data (biochemical and hematology parameters), MKB contributed to non-enzyme biomarker analysis *in vivo*, RS and VV contributed in acquisition of experimental *in vivo* data and oxidative enzyme biomarker analysis, FO and AA have performed histopathology analysis and results interpretation, BT and KY contributed to the

release studies, interpretation of the results and critical revision of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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