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Dracocephalum moldavica L. extract ameliorates intestinal inflammation by regulating gut microbiota and repairing the intestinal barrier in 2K1C rats

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Abstract: When used in ethnomedicine, *Dracocephalum moldavica* L. (DML) and its bioactive extract protect against several diseases caused by inflammation and oxidative stress. However, the effect of DML on intestinal inflammation in hypertension has not been thoroughly investigated. This study focused on the effects of DML extract on intestinal inflammation in two-kidney, one clip (2K1C) rats. Male Wistar rats were divided into three groups with daily intragastric administration of either saline (control group and model group) or DML extract (treatment group, 50 mg/kg per day) for 4 weeks. In the model and treatment groups, 2K1C hypertension was induced by clipping the left renal artery. The profiles of stool microbiota, the levels of short-chain fatty acids (SCFAs), the integrity of the gut barrier, oxidative stress biomarkers and inflammatory factors were analyzed in rats supplemented with or without DML extract. This study revealed the alleviation of high blood pressure in 2K1C rats treated with DML extract. The DML extract attenuated gut microbiota dysbiosis in the 2K1C rats by increasing the abundance of the genus *Lactobacillus* and by decreasing the abundance of *Papillibacter*, *Desulfovibrio* and *Anaerobiospirillum* genera. Treatment with the DML extract increased the levels of SCFAs and repaired the intestinal barrier, it reduced the release of oxidative stress biomarkers and inflammatory factors and suppressed the expression of TLR4 and NF-κB mRNAs. We conclude that the DML extract may alleviate intestinal inflammation by adjusting the profile of gut microbiota and enhancing the intestinal barrier.

Keywords: hypertension; two-kidney, one clip rats; *Dracocephalum moldavica* L.; gut microbiota; intestinal inflammation

INTRODUCTION

Hypertension is a major public health problem worldwide, and more than 30% of adults have high blood pressure [1]. A better understanding of the underlying mechanisms of hypertension could open avenues for more successful prevention and control of high blood pressure for individuals [2]. Numerous studies have demonstrated that human microbiota comprises trillions of microorganisms, including bacteria, viruses, protozoa, archaea and fungi, which are integral to multiple physiological processes of the host [3]. Microbial dysbiosis is often defined as an imbalance in the composition and function of the microbiota. Alterations in the gut microbiota composition have been identified in various chronic inflammatory disorders [4]. To date, there are several studies that have focused on the relationship between intestinal bacteria and hypertension, which have been identified as systemic activators of the inflammatory response [5,6]. Dysbiosis of gut microbiota contributes to hypertension via inflammatory mediators and metabolites [7,8]. Therefore, the therapeutic rationale for targeting the microbiome may be a potential treatment for hypertension [9].

The recent developments in sequencing technologies and bioinformatics have enabled researchers to explore the mechanisms underlying gut microbiota-mediated effects on disease progression, leading to developments in novel therapeutics, such as prebiotics, probiotics, fecal transplantation and drugs [10]. In recent years, more and more studies have focused on traditional Chinese medicines that can modulate gut microbiota [11]. *Dracocephalum moldavica* L. (DML), a traditional ethnomedicinal remedy, possesses a broad spectrum of pharmacological effects,

including antiinflammatory, antioxidant, cardioprotective and antimicrobial actions [12-14]. DML contains different biologically active compounds that belong to different chemical classes such as flavonoids, steroids, glycosides, saponins, tannins, phenols and essential oils [15]. We speculated that it might modulate the gut microbiota to achieve its diverse effects. The present study was designed to explore the effect of DML on blood pressure and changes in the composition of gut microbiota and the structure of the intestinal barrier in response to DML treatment in 2K1C rats. We also assessed the changes in the expression of the TLR4/NF-κB signaling pathways.

MATERIALS AND METHODS

Ethics statement

This study was approved by the Animal Ethics Committee of Baotou Medical College (Ethics number: 2021040).

Plant material, extract preparation and physicochemical property analysis

The aerial parts of DML were collected in the county of Tongliao (latitude 43°39' north and longitude 122°14' east), Inner Mongolia, China. A voucher specimen (IMKLDRB-2022-01) was deposited in the Inner Mongolia Key Laboratory of Disease-Related Biomarkers, Baotou Medical College (Baotou, Inner Mongolia, China). The plant samples were air-dried before being ground into a fine powder. DML extracts were prepared as described [14]. Briefly, the plant powder was subjected to extraction twice with 65% ethanol (EtOH) for 120 min at a temperature of 60°C. After removing the ethanol by vacuum distillation, the obtained extracts were separated with ethyl acetate (EtOAc) using separating funnels. The total phenolic and total flavonoid contents of the resultant extracts were determined using the Folin-Ciocalteu and spectrophotometric methods, respectively [16]. The results are shown in the Supplementary Materials (Supplementary Table 1). Moreover, high-performance liquid chromatography was used to simultaneously quantify the resultant extracts with the standards tallianine, rosmarinic acid, luteolin, apigenin and diosmetin as references [14]. The chromatograms showed that rosmarinic acid, tilianin, luteolin, apigenin and diosmetin were present in the DML extract (Supplementary Fig. S1).

Animal experiments

Male Wistar rats (body weight 160 to 180 g) were obtained from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All rats were kept in a pathogen-free environment, with free access to water and rodent chow. Animals were maintained in a temperature-controlled environment and on a 12-h light/dark cycle. To induce a two-kidney, one clip (2K1C) model, the Wistar rats were anesthetized with pentobarbitone sodium (50 mg/kg by intraperitoneal injection). As previously reported, the left renal artery was constricted with a silver clip to obtain the renal hypertension model [17]. The sham-operated rats underwent a similar operation, except for the silver clip application. The blood pressure was measured weekly by non-invasive tail-cuff plethysmography (Chengdu Instrument Factory, Sichuan, China). The animals were divided into the following three groups: sham group, model group and treatment groups. In the treatment groups, the 2K1C rats were treated with DML extract for 4 weeks by oral gavage. At the end of the experiments, the investigated animals were anesthetized with an anesthetic overdose (4% pentobarbital sodium), before being euthanized.

Sample collection

Stool samples were collected from the rats' cecum and immediately frozen at -80°C for intestinal flora analysis. Blood was collected from the abdominal aorta and serum was collected for D-lactate determination. The intestinal tissue was quickly removed and placed on ice, quickly frozen in liquid nitrogen and stored at -80°C for later use.

Fecal DNA extraction

Microbial DNA was extracted from feces specimens using the MoBio Power Fecal DNA Isolation kit (Mo Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's protocol. The quality of the extracted DNA was examined by denaturing agarose gel electrophoresis (Solarbio, Beijing, China), and the OD 260/280 was measured by a spectrophotometer.

16S ribosomal RNA gene sequencing

The V3-V4 of the bacteria 16S ribosomal RNA genes were amplified by PCR (94°C for 3 min, followed by 30 cycles at 94°C for 30 s, 56°C for 45 s, and 72°C for 42 s, with a final extension at 72°C for 10 min) using primers 341F (5'-ACT CCT ACG GGA GGC AGC AG-3') and 806R (5'- GGA CTA CHV GGG TWT CTA AT-3'). PCR products were purified using AMPure XP beads (Beckman Coulter, Fullerton, CA, USA) and quantified using the Agilent 2100 bioanalyzer (Agilent, USA). The amplicon library was used for sequencing on the Illumina MiSeq platform (BGI, Shenzhen, China). Operational taxonomic units (OTUs) were clustered with a 97% similarity cutoff using UPARSE (version 7.0.1090). Diversity analyses (alpha and beta diversity) were calculated using Mothur (version 1.31.2) and QIIME (version 1.8.0) software, respectively. Linear discriminant analysis (LDA) effect size (LEfSe) analysis was performed using LEfSe software.

Analysis of fecal SCFAs

The fecal sample was diluted with 350 μL of water and centrifuged at 14,000 × g for 5 min. Then 100 μL of the supernatant was mixed with 20 μL of ethyl butyrate (8.6 mM), 270 μL of 1 M sodium hydroxide and 280 μL of 0.36 M perchloric acid. After freeze-drying, the samples were reconstituted by adding 400 μL of acetone and 100 μL of 5 M formic acid. After centrifugation at 14,000 × g for 5 min, 1 μL of the organic phase was used for the determination of SCFA using an Agilent 5890 series II gas chromatograph (Waldbronn, Germany) equipped with an HP-20 M column and a flame ionization detector (FID).

Measurement of antioxidant activity

The supernatant was obtained after the homogenization of intestinal tissue. The superoxide anion radical scavenging activity, hydroxyl radical scavenging activity, superoxide dismutase (SOD) activity and the concentration of malondialdehyde (MDA) were assayed using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA) analysis

The serum samples were used for detecting the concentration of D-lactate with a commercial ELISA kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Reverse transcription-quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from intestinal tissue using an RNAprep Pure Tissue Kit (TIANGEN, Beijing, China). The first cDNA template was synthesized using the Prime Script RT Master Mix (TaKaRa, Beijing, China). qPCR was performed using the TB Green Premix Ex Taq II (TaKaRa, Beijing, China) on the ABI PRISM 7500 system (Applied Biosystems, Foster City, USA). The relative number of transcripts was calculated by the $2^{-\Delta\Delta Ct}$ method using GAPDH as the internal normalized reference. The primer sequences are shown in Supplementary Table S2.

Statistical analysis

SPSS 21.0 software was used to analyze the data. The significant differences between multiple groups were analyzed using one-way ANOVA, and two groups were calculated using the Student's unpaired t-test. Results are expressed as means±SEM, and a value of $P<0.05$ indicated statistical significance.

RESULTS

Application of the DML extract improved the arterial blood pressure in 2K1C rats

We examined the effect of DML on systolic blood pressure (SBP) levels in experimental rats. The *in vivo* experiment scheme is shown in Fig 1A. The results showed that there were no significant differences in SBP among all groups at baseline. Compared with the sham group (115.5±7.2 mmHg), SBP in the 2K1C group was found to be significantly higher (184.4±20.1 mmHg) after 4 weeks of operation ($P<0.05$). DML reduced SBP in the treatment group compared to the model group (Fig. 1B). The treatments had not yet started on the 4th week and the SBP increased in

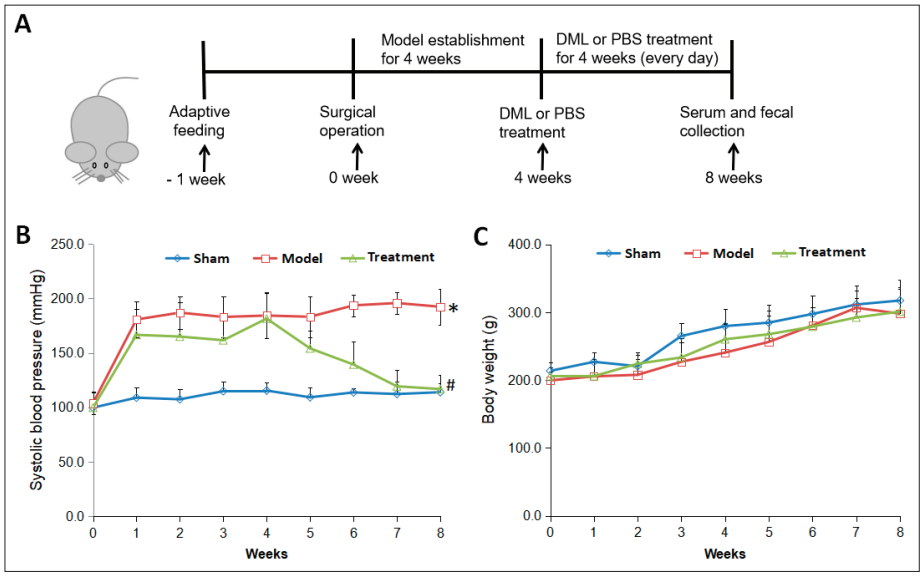

Fig. 1. Therapeutic evaluation of DML extract in ameliorating hypertension in 2K1C rats. **A** – *In vivo* experimental scheme. **B** – Systolic blood pressure. **C** – Body weight change. Values are expressed as the mean±SEM analyzed by one-way ANOVA followed by Duncan's multiple comparison test; n = 8. *P<0.05 vs sham group, #P<0.05 vs the model group.

the treatment group as compared to the sham group (P<0.05). In contrast, SBP was significantly reduced in the treatment group after 2 weeks of treatment with DML extract (P<0.05) (Fig. 1B). No significant change in body weight was observed among these groups at the end of the experimental period (P>0.05) (Fig. 1C). The data indicated that treatment with the DML extract ameliorated hypertension.

DML extract attenuated gut microbiota dysbiosis in 2K1C rats

The abundance and diversity of the bacterial community were assessed by the α-diversity indices (Shannon, Simpson and the observed species). The results revealed no difference across the different groups (Fig. 2A-C), indicating that treatment with the DML extract has no obvious effect on the α-diversity of gut microbiota. Subsequently, we assessed the β-diversity of gut microbiota across different groups. A significant difference was observed in β-diversity based on the unweighted UniFrac (P<0.001) but not the weighted UniFrac (P=0.295) distance between the model group and the treatment group (Fig. 2D and E). Additionally, the unweighted pair group method with the arithmetic mean (UPGMA) clustering method classified the model and sham rats into two distinct groups, suggesting that the gut microbial profile was different in the sham and model groups. UPGMA clustering analysis also revealed that the gut microbiota composition between the sham and treatment groups tended to be more similar compared with the model group at the phylum level. The results suggest that the β-diversity of the gut microbiota was affected by the treatment with the DML extract (Fig. 2F).

The composition of gut microbiota across the different groups was further compared at the phylum and genus levels. At the phylum level, *Firmicutes* and *Bacteroidetes* were the dominant phyla in the three groups (Fig. 3A). *Proteobacteria* represented the most abundant bacterial group, accounting for an average of 1.66%, 3.57% and 1.73% of sequences in the sham, model and treatment groups, respectively. 2K1C rats showed an increased abundance of *Bacteroidetes* and *Proteobacteria* and a lower number of *Firmicutes*. However, the changes in the fraction of *Firmicutes*, *Bacteroidetes* and *Proteobacteria* were attenuated by treatment with the DML extract (Fig. 3A). At the genus level, *Lactobacillus* was the dominant genus in the three groups (Fig. 3B). 2K1C rats exhibited a decreased abundance of *Lactobacillus*. In contrast, the abundance of this genus was reversed by treatment with the DML extract. In addition, the differences in the relative abundance of gut microbiota across diverse groups were compared using the Metastatic method with Fisher's exact test. Compared with the sham group, two genera (*Papillibacter* and *Desulfovibrio*, members of the phyla *Firmicutes* and *Proteobacteria*, respectively) displayed an increase in the model group (Fig. 3C). In contrast, DML-extract treatment attenuated the 2K1C-mediated upregulation of these two genera (Fig. 3C). LEfSe analysis was used to identify differentially abundant features that responded to hypertension and the treatment with the DML extract and a microbe biomarker was identified. The model group showed higher enrichment of this taxon at each level from phylum to genus than the sham group (Fig.

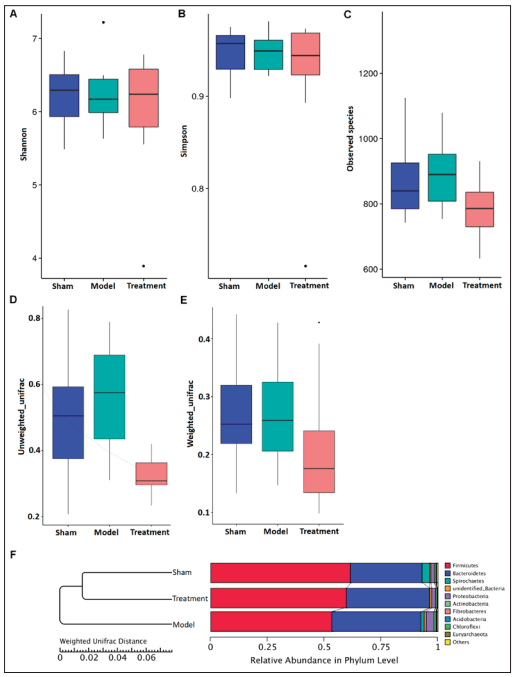

Fig. 2. Effects of treatment with the DML extract on alpha and beta diversity of gut microbiota of 2K1C rats. **A-C** – Alpha diversity indices (Shannon, Simpson and observed species, respectively). **D-E** – Beta diversity (unweighted and weighted UniFrac distance metrics, respectively). F – UPGMA cluster tree.

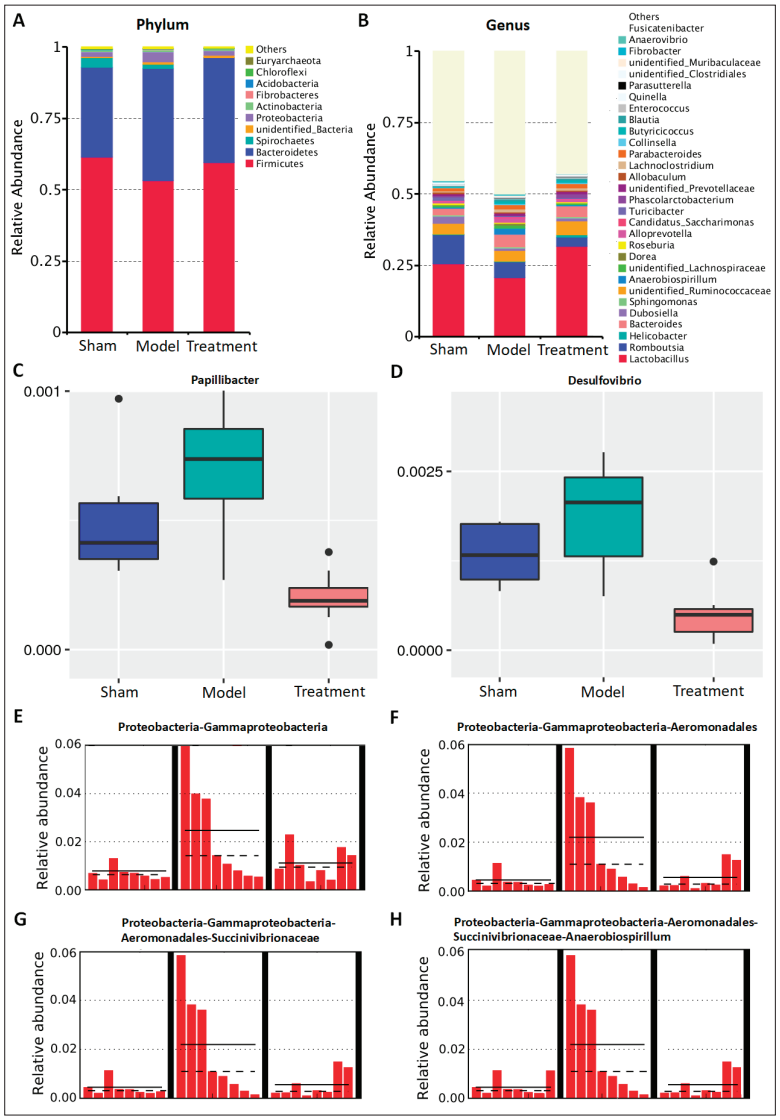

Fig. 3. Treatment with the DML extract modulated the imbalance of gut microbiota in 2K1C rats. **A** – Composition of the gut microbiota at the phylum level. **B** – Composition of the gut microbiota at the genus level. **C** – Metastatic analysis showing the relative abundance of the significant two bacteria at the genus level in the different groups. **D** – Biomarker taxa obtained from LEfSe analysis presenting different levels (class, order, family, genus).

3D). These changes were counteracted by the administration of the DML extract (Fig. 3D).

DML extract increased the short-chain fatty acid (SCFA) content in 2K1C rats

The content of SCFA across the three groups was compared. The amounts of acetate and butyrate in the model group were decreased when compared with those of the sham group ($P<0.05$) (Fig. 4A and B). However, the changes in the content of acetate and butyrate were attenuated by the DML-extract intervention ($P<0.05$) (Fig. 4A and B).

DML extract ameliorated intestinal oxidative stress in 2K1C rats

The hydroxyl and superoxide anion radical scavenging activities of the DML extract were 87.71%±4.37% U/mL and 90.23% ± 5.91%, respectively. In addition, we measured the levels of oxidative stress biomarkers in the intestinal tract. Rats in the model group exhibited significantly higher intestinal MDA and lower intestinal SOD levels (903.7±37.7 mmol/mg and 4.7±0.3 U/mg, respectively) than those in the sham group (191.7±15.9 mmol/mg and 12.3±0.8 U/mg, respectively) ($P<0.05$) (Fig. 5A and B). DML-extract administration significantly decreased the intestinal MDA level and increased the intestinal SOD level in 2K1C rats ($P<0.05$) (Fig. 5A and B).

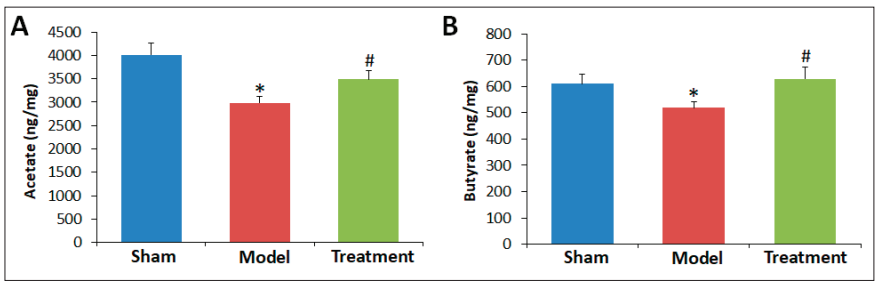

Fig. 4. Treatment with the DML extract restored the levels of SCFA in 2K1C rats. **A** – Fecal level of acetate and butyrate. **B** – Fecal level of butyrate. Values are expressed as the mean±SEM and analyzed by unpaired t-test analysis; n = 8. *P<0.05 vs the sham group, #P<0.05 vs the model group.

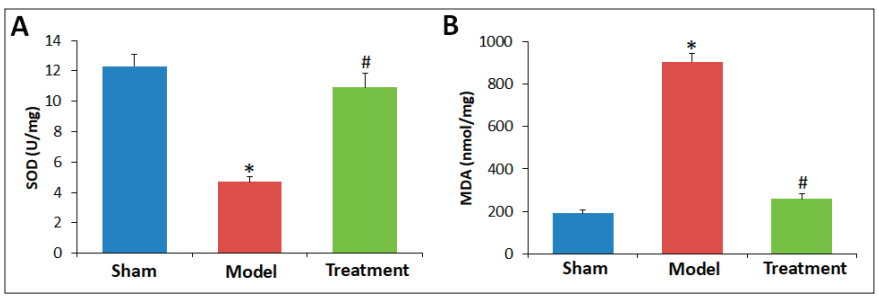

Fig. 5. Treatment with the DML extract ameliorated intestinal oxidative stress in 2K1C rats. **A** – Intestinal level of SOD specific activity. **B** – Intestinal concentration of MDA. Values are expressed as the mean±SEM and analyzed by unpaired t-test analysis; n=8. *P<0.05 vs the sham group, #P<0.05 vs the model group.

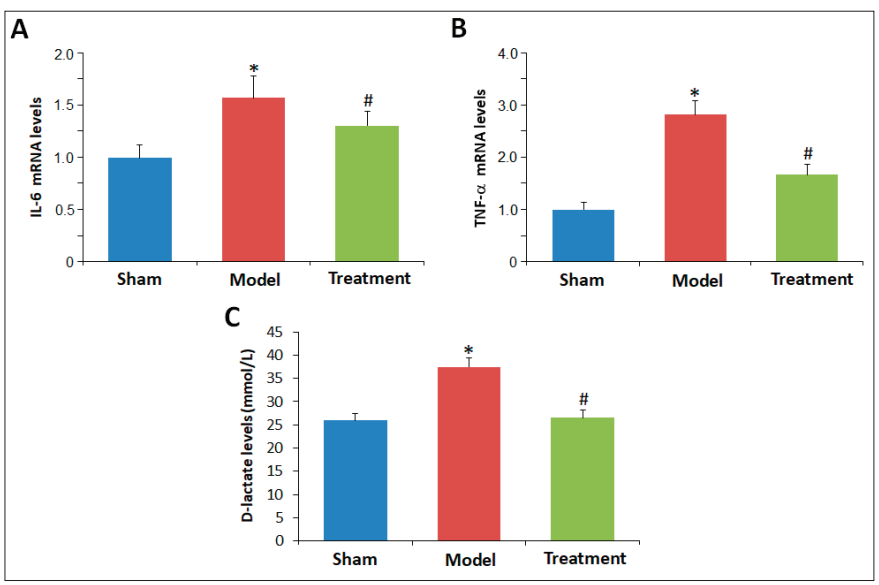

Fig. 6. Treatment with the DML extract ameliorated intestinal inflammation and barrier dysfunction in 2K1C rats. **A** – Relative expression of IL-6 mRNA in intestinal tissue. **B** – Relative expression of TNF-α mRNA in intestinal tissue. **C** – The serum level of D-lactate. Values are expressed as the mean±SEM and analyzed by unpaired t-test analysis; n=8, *P<0.05 vs the sham group, #P<0.05 vs the model group.

DML extract ameliorated intestinal inflammation in 2K1C rats

The intestinal levels of IL-6 and TNF-α were significantly higher (1.7±0.2- and 2.8±0.2-fold, respectively) in the 2K1C rats than those in the sham rats, and the increase in inflammatory cytokines was attenuated by DML-extract administration (P<0.05) (Fig. 6A and B).

DML extract ameliorated maintained intestinal barrier function in 2K1C rats

Intestinal permeability was evaluated by measuring the serum levels of D-lactate by ELISA. D-lactate content was significantly higher in the 2K1C rats (37.3±1.9 mmol/L) compared to the controls (25.9±1.3 mmol/L) (P<0.05) (Fig. 6C). In rats treated with the DML extract, the levels of D-lactate were similar to the control values (P<0.05) (Fig. 6C).

DML extract reduced TLR4/NF-κB signaling pathway expression in 2K1C rats

To determine whether the TLR4/NF-κB signaling pathway is involved in the inflammatory response, relative changes in the expression levels of TLR4 and NF-κB mRNAs were determined. The expression of TLR4 and NF-κB mRNAs in the model group was significantly higher than that in the sham group (P<0.05) (Fig. 7A and B). DML-extract administration significantly blocked the hypertensive enhancement of the expression of TLR4 and NF-κB mRNAs (P<0.05) (Fig. 7A and B).

DISCUSSION

The incidence of hypertension is increasing worldwide [18]. Various

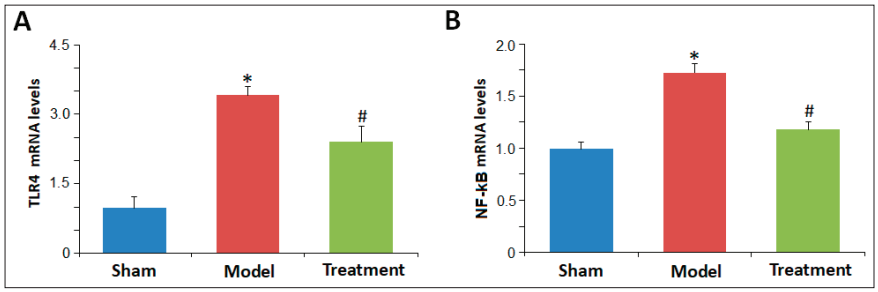

Fig. 7. Treatment with the DML extract lowered TLR4/NF-κB signaling pathway expression in 2K1C rats. **A** – Relative expression of TLR4 mRNA in intestinal tissue. **B** – Relative expression of NF-κB mRNA in intestinal tissue. Values are expressed as the mean±SEM and analyzed by unpaired t-test analysis; n = 8. *P<0.05 vs the sham group, #P<0.05 vs the model group.

mechanisms for hypertension have been put forward, including impaired functioning of the renin-angiotensin-aldosterone system, sympathetic nervous system dysfunction, the effects of gut microbiota, metabolic disorders and possibly also inflammatory and oxidative stress processes [19,20]. Our study has demonstrated that DML, a traditional Chinese medicine used for centuries, can protect against hypertension [21]. However, the detailed mechanism is still unclear and needs further investigation.

Studies have underscored the role of the gut microbiome in maintaining intestinal inflammation and thus contributing to improving hypertension [22,23]. In the present study, we evaluated the effects of DML extract in the experimental model of 2K1C rats. We found that DML noticeably reduced blood pressure in 2K1C rats. After 2 weeks of treatment with DML extract, SBP decreased by 54.3 mmHg (P<0.05), and the blood pressure levels returned to normal after 4 weeks of treatment. The change could be interpreted as being caused by changes in the gut microbiota. We found that *Firmicutes* and *Bacteroidetes* were the dominant phyla, which is consistent with a previous report [24]. At the genus level, a decrease in the relative abundance of *Lactobacillus* and an increase in the relative abundance of *Desulfovibrio* were detected in 2K1C rats when compared with the controls. *Lactobacillus* has been widely used as a microorganism capable of reducing blood pressure [25]. According to the literature, the level of *Desulfovibrio* species was increased in hypertensive rats [26]. Additionally, the LEfSe algorithm was used to identify key microbial taxa. Surprisingly, the rats in the model group were mainly characterized by a higher enrichment of *Anaerobiospirillum* at each level from phylum to genus. It has been reported that the relative proportion of *Anaerobiospirillum* was significantly associated with gut inflammation in rats with Alzheimer's disease [27]. Thus, alteration of the *Anaerobiospirillum* level might contribute to an inflammatory response in 2K1C rats. Interestingly, the intervention of the DML extract caused a reduction in the abundance of *Desulfovibrio* and *Anaerobiospirillum* as well as a significant increase in the abundance of *Lactobacillus*, suggesting its potential role in shaping the gut microbiota; thus, DML might ameliorate inflammation in 2K1C hypertensive rats by improving the gut microbiota.

The SCFAs acetate and butyrate in the feces of untreated 2K1C rats were significantly lower than in the sham group. However, the levels of acetate and butyrate in the treatment group were restored, consistent with the change in the SCFA-producing bacteria *Lactobacillus*. Previous studies have shown that SCFAs regulate the intestinal microenvironment and affect systemic inflammation [28]. SCFAs can suppress the production of proinflammatory cytokines and reinforce the intestinal epithelium [29]. Numerous studies have demonstrated that the protective effect of probiotics or natural active products on hypertension is associated with the ability to reduce inflammation and oxidative stress and restore damaged intestinal epithelium barriers, which may be the result of reshaping the composition of the gut microbiota and restoration of the levels of SCFAs [30,31]. We observed that DML had a profound effect on intestinal oxidative stress, inflammation and intestinal permeability, as evidenced by reduced levels of the proinflammatory cytokines IL-6 and TNF-α, MDA, the marker of oxidative stress, D-lactate, as well as increased activity of SOD in 2K1C rats. Thus, DML might improve intestinal inflammation by reversing intestinal microbial dysbiosis and regulating SCFA production in 2K1C rats.

Studies indicate that TLR4 dysfunction plays an important role in initiating oxidative stress and inflammation pathways, which subsequently contribute to the pathogenesis of hypertension [32-34]. Recently, it

was demonstrated that TLR4 blockade significantly reduces the expression of IL-6 and TNF-α, decreases ROS levels and regulates the progression of hypertension [33]; the levels of TLR4 were shown to correlate closely with the disruption of intestinal permeability in a rat model [34]. Moreover, it has been reported that SCFAs augment inflammation through the TLR4 pathway [35], and our results are consistent with these findings. The results presented herein show that DML intervention improved gut microbiota disorder, barrier function and decreased inflammation, at least in part by inhibiting the activation of the TLR4/NF-κB pathway in 2K1C rats. The results of our research point to alternative treatment options for early intervention in patients with hypertension.

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Author contributions: HY and ZC contributed equally to this work. Conceptualization, HY and ZW; methodology, HY; validation, HY, ZC and HC; investigation, ZC; resources, HY and ZW; data curation, ZW; writing – original draft preparation, HY; writing, review and editing, ZW; visualization, ZC; supervision, ZW; project administration, ZW; funding acquisition, HY and ZW. All authors have read and agreed to the published version of the manuscript.

Conflict of interest disclosure: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability: The data presented in this study are available in the Supplementary Material as a raw data set that can be accessed via the following link: https://www.serbiosoc.org.rs/NewUploads/Uploads/Yu%20et%20al_8502-Data%20Set.zip

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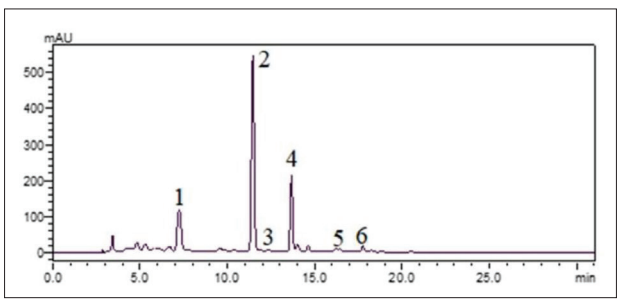
SUPPLEMENTARY MATERIAL

Supplementary Table S1. Total phenolic content (TPC) and total flavonoid content (TFC) of the DML ethanol (EtOH) extract.

Fractions	Yield (mg/g)	TFC (mg RE/g)	TPC (mg GAE/g)
EtOAc	9.33±	65.04±	511.05±

Supplementary Table S2. Specific primer sequences used for real-time PCR.

Primer name	Forward (5–3')	Reverse (3–5')
IL-6	GATGGATGCTTCCAAACTGG	AGGAGAGCATTGGAAGTTGG
TNF-α	AACACACGAGACGCTGAAGT	TCCAGTGAGTTCCGAAAGCC
TLR4	GATCTGAGCTTCAACCCCCT	TTGTCTCAATTTCACACCTGGA
NF-κB	AAAGCCCTGACAGTCCATTG	TTGCTAGACACCGTCTGTGC
GAPDH	ACAGCAACAGGGTGGTGGAC	TTTGAGGGTGCAGCGAACTT



Supplementary Fig. S1. HPLC-DAD chromatograms of the EtOAc fraction of the DML ethanol extract visualized at 330 nm. 1 – rosmarinic acid; 2 – tilianin; 3 – luteolin; 4 – unknown compound; 5 – apigenin; 6 – diosmetin.