ORIGINAL ARTICLE

Annals of Agricultural and Environmental Medicine
www.aaem.pl

Evaluation of antioxidant activity of NG-R1 saponin against bacterial cells in induced oxidative stress – a preliminary study

Anna Lichota[1,A-F] ✉, **Monika Sienkiewicz**[1,D-F]

[1] *Department of Pharmaceutical Microbiology and Microbiological Diagnostic, Medical University of Lodz, Łódź, Poland*
A – Research concept and design, B – Collection and/or assembly of data, C – Data analysis and interpretation, D – Writing the article, E – Critical revision of the article, F – Final approval of the article

Lichota A, Sienkiewicz M. Evaluation of antioxidant activity of NG-R1 saponin against bacterial cells in induced oxidative stress – a preliminary study. Ann Agric Environ Med. doi: 10.26444/aaem/161339

Abstract

Introduction and Objective. Notoginsenoside R1 (NG-R1) is isolated from *Panax notoginseng*, a medicinal herb well-known for its long use in traditional Chinese medicine. NG-R1 is relatively under-studied in research on bacteria. The aim of the study was to investigate antioxidant properties of NG-R1 saponin of selected bacterial strains of intestinal microbiota that may be involved in the pathogenesis of thromboembolic diseases. *Enterococcus faecalis* and *Escherichia coli* were used in the study.

Materials and method. The study determined the concentration of hydroperoxides, the level of lipid peroxidation, as well as carbonyl groups and free thiol groups. The research carried out in this way will allow determination of the influence of the above factors on bacteria living in intestinal microbiota.

Results. An evaluation of selected parameters of oxidative stress allowed to check whether the tested compound could reduce the pro-thrombotic activity of bacteria that were stimulated with H_2O_2. It was found that NG-R1 reduced hydroperoxide levels in both types of bacteria. In turn, lipid peroxidation initiated by H_2O_2 was suppressed by NG-R1. Hydrogen peroxide led to a strong increase in the level of carbonyl groups in *Enterococcus faecalis* and, to a lesser extent, in *Escherichia coli*. The addition of NG-R1 to the medium significantly reduced the level of carbonyls. Additionally, NG-R1 also induced a significant increase in the level of free thiol groups.

Conclusions. Obtained results indicate that NG-R1 may have a protective effect on the intestinal microbiom through mechanisms involving changes in the redox state.

Key words

thromboembolism, gut microbiota, saponin, oxidative stress, notoginsenoside R1

INTRODUCTION

In recent years, a correlation between many diseases and intestinal dysbiosis has been increasingly observed. According to the literature, disturbances in the intestinal microbiota may also contribute to the occurrence of cardiovascular diseases [1]. Due to their antioxidant activity, plants of the genus *Panax*, including *Panax notoginseng*, are of particular interest [2, 3]. Active secondary metabolites of this plant include, among others, notoginsenoside R1 (NG-R1) (Fig. 1), belonging to the group of saponins [2, 4, 5]. NG-R1 is isolated from the *Panax notoginseng*, a medicinal herb well-known for its long use in traditional Chinese medicine. NG-R1 is characterized by its numerous pharmacological properties, including anti-cancer, anti-inflammatory, cardioprotective, and neuroprotective effects [6, 7, 8].

The significant cardioprotective effect of NG-R1 on ischemia/reperfusion-induced damage has been elucidated. This saponin inhibited oxidative stress and cell apoptosis in the I/R injury of heart model *in vivo* and *in vitro*. In addition, saponin inhibits Tunicamycin-induced cell death and cardiac dysfunction in rats [9]. Saponin NG-R1 has anticoagulant properties related to inhibition of platelet aggregation, as well as increasing fibrinolytic potential, which is related to secretion of the tissue plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA). In turn, the cardioprotective effect of NG is related to protection of myocardial cells against ischemia. Moreover, saponin can be used in the treatment of vascular restenosis caused by limited intimal vascular hypertrophy inhibited proliferation of vascular smooth muscle cells (VSMC) [10, 11]. Saponin also has anti-inflammatory effects which involve limiting the expression of integrin genes, interleukins IL-18, IL-1B, as well as the expression of matrix metalloproteinases (MMPs), such as MMP-2, MMP-9 [5, 11]. In addition, NG-R1 saponin exhibits anticoagulant properties by extending the coagulation time. This compound stimulates the angiogenesis process by activating VEGF-KDR/Flk-1 and PI3K-Akt-eNOS in signaling pathways [12], antioxidant enzymes (SOD, CAT, GPx) [9] and glutathione (GSH) [12].

Cardiovascular disease is linked to oxidative stress associated with gut microbiota. Elevated levels of uric acid in the blood may lead to increased production of ROS and predispose to endothelial dysfunction of blood vessels [13, 14]. Gut microbiota may be involved in the formation of blood clots in arteries, as well as in the development of atherosclerosis. Trimethylamine-N-oxide (TMAO) formed as a result of metabolism of phosphatidylcholine, carnitine and choline with the participation of intestinal bacteria, can initiate the

✉ Address for correspondence: Anna Lichota, Department of Pharmaceutical Microbiology and Microbiological Diagnostic, Medical University of Lodz, Łódź, Poland
E-mail: anna.lichota@umed.lodz.pl

Received: 03.01.2023; accepted: 17.02.2023; first published: 02.03.2023

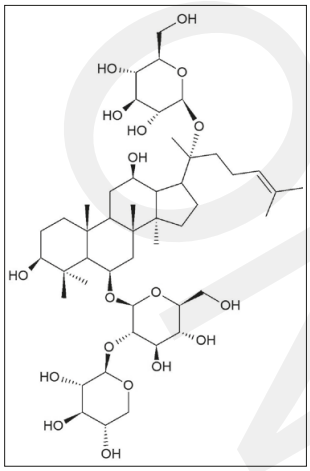

Figure 1. Structure of notoginsenoside R1 (NG-R1).

formation of foam cells and atherosclerotic plaques [15, 16]. In addition, pathogens of intestinal microbiota increase proadhesion and activate vascular endothelial cells, as well as increase the level of factor VIII, von Willebrand factor (vWF) and Weibel-Palade bodies. In addition, intestinal pathogens release metabolites that promote platelet activation [15]; for example, lipopolysaccharide (LPS) found in the cell wall of Gram-negative bacteria reduces the flow rate of erythrocytes, due to leukocyte adhesion to the venous wall, damage to mast cells, and secretion of cytokines [10, 17]. LPS correlates with FVIII, which increases the level of thrombin formation [18]. Besides, lipopolysaccharide activates the defence system with Toll-like receptors (TLR). The bacterially-induced TLR2 signalling pathway promotes thrombus formation in mice [19]. Inflammation, platelet adhesion and aggregation, as well as formation of neutrophil extracellular traps (NETs), are mediated by the action of TLRs. Neutrophils are a key component of innate immunity and are also involved in the clotting process. Together with thrombocytes, they accumulate at the site of blood vessel damage and contribute to the formation of vascular thrombosis [18, 20].

As an element of the intestinal microbiota, *Escherichia coli* is involved in the pathogenesis of civilization diseases, such as cardiovascular or kidney diseases, by disrupting the blood barrier and inhibiting the expression of tight junction proteins [19]. Moreover, the uropathogenic strain of *Escherichia coli*, which has external vesicles on the cell membrane, contributes to the development of heart disease affecting myocardial cardiomyocytes, both *in vitro* and *in vivo*. The uropathogenic strain of this bacterium is responsible for cytotoxicity, inflammatory response, and cardiomyocyte dysfunction. The outer vesicles of the cell membrane lead to inflammation and damage to myocardial tissue, which impairs heart efficiency. *Escherichia coli* can interact with platelets through receptors on their surface (P-selectin, glycoproteins and TLR450). It was observed that platelet exposure to *Escherichia coli* K12 increased the surface expression of P-selectin and CD63 activation markers.

In addition, the above bacteria indirectly react with plasma proteins, releasing Shiga toxin or LPS [21]. *Enterococcus faecalis* is another pathogen colonizing the human digestive tract. A significant number of strains of these microorganisms are able to produce extracellular superoxide ($O_2^{\cdot-}$). Under physiological conditions of the large intestine, superoxide dismutation leads to the formation of hydrogen peroxide (H_2O_2). The H_2O_2 formed near the colonic epithelium can penetrate cell membranes of colonocytes and generate hydroxyl radicals [22]. *Enterococcus faecalis* also lyses intestinal epithelial cells and the bacteria enter the bloodstream, allowing colonization of distant organs in the host. A good example is the colonization of heart valves. Substances such as gelatinase and capsular polysaccharides, produced by pathogens, enable them to bypass the host defence systems [23].

OBJECTIVE

The aim of the study is to investigate the antioxidant properties of NG-R1 saponin on selected bacterial strains of the intestinal microbiota which may be involved in the pathogenesis of thromboembolic diseases. *Enterococcus faecalis* and *Escherichia coli* were used in the study. An evaluation of selected parameters of oxidative stress will allow to check whether the tested compound could reduce the pro-thrombotic activity of bacteria that will be stimulated with H_2O_2. The work determined the concentration of hydroperoxides, the level of lipid peroxidation, as well as carbonyl groups and free thiol groups. Research carried out in this way will allow determination of the influence of the above factors on bacteria living in intestinal microbiota.

MATERIALS AND METHOD

Bacterial strains isolation and growth conditions. The reference strains Enterococcus faecalis ATCC 51299 and Escherichia coli NCTC 10538 were used in the study. The bacterial strains were propagated on Brain Heart Infusion (BHI) agar in Erlenmeyer flasks on a rotary platform shaker at 250 rpm for 24 h aerobically at 37 °C. Subsequently, the culture was diluted to optical density (OD) 1.0 at 600 nm. 300 ml of log-phase cells was harvested at 4 °C by centrifugation for 1 h at 9,500×g and washed once with 50 mM potassium phosphate (pH 7.8), containing 1 mM EDTA. The washed cells were resuspended in 10 ml of buffer and enzymatic lysis with lysozyme at 37 °C for 2 h. Cell debris was removed by centrifugation at 9,500×g for 2 h at 4 °C. The investigated oxidative stress parameters were compared with the control group (bacteria), in bacteria after incubation with saponin (40 μM), H_2O_2 (0.5 mM and 1.5 mM) and in bacteria after incubation with H_2O_2 and saponin NG-R1.

Quantification of hydroperoxides. To determine the level of peroxides, the FOX2 assay with xylenol orange was applied. The reaction was based on rapid oxidation of Fe^{2+} to Fe^{3+} in the presence of peroxides [24, 25]. 250 μM of ammonium iron (II) sulfate in 25 mM H_2SO_4 and 100 μM xylenol orange were used to prepare a working solution. The samples were then mixed with a working reagent and incubated for 30 min in the dark. Reaction of Fe^{3+} with xylenol orange yielded a violet-coloured complex, which was then quantified spectrophotometrically at 560 nm. The absorbance were measured on a spectrophotometer (Varian Cary 1 UV-Visible Spectrophotometer). The peroxide concentration was calculated using the calibration curve for different concentrations of tert-butyl hydroperoxide as a standard.

Lipid peroxidation. The level of lipid peroxidation in lysates was measured on the basis of the content of peroxidation products reacting with thiobarbituric acid (0.37% TBA) in 0.25 M HCl [26, 27]. The lysates were mixed with trichloroacetic acid (20% TCA) in 0.25 M HCl and TBA, and boiled for 15 min. After cooling, the samples were centrifuged at 10,000×g for 5 min. Absorbance of the supernatant was measured at 535 nm. The absorbance was measured on a spectrophotometer (Varian Cary 1 UV-Visible Spectrophotometer). TBARS levels were calculated using the MDA absorption coefficient of ε=156,000 $M^{-1} \cdot cm^{-1}$.

Detection of protein carbonyl groups. Protein carbonyl content was calculated using 2,4-dinitrophenylhydrazine (DNPH) [28–30]. Samples of cells lysate were mixed with 10 mM DNPH in 2 M HCl and kept at room temperature for 1 h. Precipitation of the protein was carried out with 20% TCA. In order to remove free reagent, the precipitate was washed several times with a solution of ethanol-ethyl acetate (1:1).The protein samples were then tissolved in a 6 M guanidine hydrochloride solution and the absorbance was measured at 370 nm on a spectrophotometer (Varian Cary 1 UV-Visible Spectrophotometer). Carbonyl concentration was calculated using the molar absorption coefficient of ε=22,000 $M^{-1} \cdot cm^{-1}$.

Thiol groups assay. The concentration of thiol groups was calculated using Ellman's reagent (5.5'-dithiobis-(2-nitrobenzoic acid); DTNB) [27, 31, 32]. The product of the reaction of thiol groups with Ellman's reagent is yellow 2-nitro-5-thiobenzoate (NTB), which exhibits absorbance at 412 nm. Colour intensity in NTB-containing lysates directly corresponds to the concentration of thiol groups in the sample. Lysates were mixed with phosphate buffer (100 mM, pH 8.0) and the absorbance was measured at 412 nm on a spectrophotometer (Varian Cary 1 UV-Visible Spectrophotometer). Subsequently, DTNB (5 mM in phosphate buffer 100 mM, pH 8.0) was added to the mixture and incubated for 1 h at 37˚C in the dark. The NBT^{2-} molar extinction coefficient of ε=13,600 $M^{-1} \cdot cm^{-1}$ was used to calculate the concentration of monothiol produced.

Statistical Analysis. Results are shown as mean values and standard deviations, obtained from 5 independent experiments. Multiple comparisons were made by one-way analysis of variance (ANOVA), followed by the Tukey's *post-hoc* test. Values of $p < 0.05$ were considered statistically significant. Statistical analysis was conducted using STATISTICA software ver.13 (StatSoft Inc., Tulsa (OK) USA).

RESULTS

Determination of peroxides concentration. Quantification of hydroperoxides generated after saponin NG-R1 and/or H_2O_2 treatment of *Enterococcus faecalis* and *Escherichia coli* were performed using FOX2 assay (Fig. 2). Figure 2 shows quantitative changes of hydroperoxides after 24 h incubation with saponin NG-R1 (40 µM) and/or H_2O_2 (0.5 mM and 1.5 mM). There was a slight increase in the concentration of hydroperoxides in samples of both bacteria after 0.5 mM and 1.5 mM H_2O_2 incubation. In response to NG-R1, statistically significant changes in the level of hydroperoxide were observed for the control, as well as for the combination of H_2O_2 (1.5 mM) with saponin for both the bacteria.

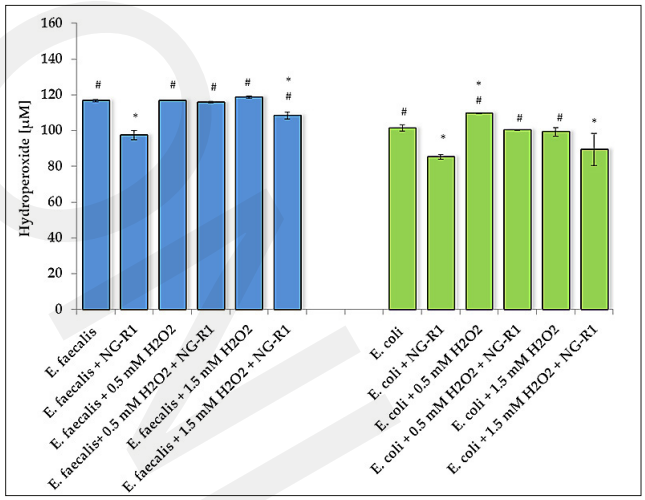

Figure 2. Effect of saponin NG-R1 (40 µM) and H_2O_2 (0.5 mM and 1.5 mM) on the level of hydroperoxides on *Enterococcus faecalis* and *Escherichia coli*. Samples were incubated for 24 h aerobically at 37 °C. Measurements were made using FOX2 assay. Bars – mean values with standard deviations; * – values statistically different in comparison to those observed in untreated bacteria (*$p < 0.05$); hashes – values statistically different compared with values obtained in samples containing bacteria with NG-R1 (# $p < 0.05$). All experiments were repeated 3–4 times

Measurement of lipid peroxidation. ROS cause direct oxidative modification of unsaturated lipids. Lipids can be modified by oxidative reactions leading to changes in their structure and loss of function. Malondialdehyde (MDA) and other products are produced in cells during the process of polyunsaturated fatty acid peroxidation. Accordingly, this compound is a marker of oxidative stress. TBA is used in order to detect the level of lipid peroxidation in the cells [33]. Quantification of lipid peroxidation was performed with thiobarbituric acid reactive substances (TBARS) assay. Obtained results are presented in Fig. 3. After the treatment

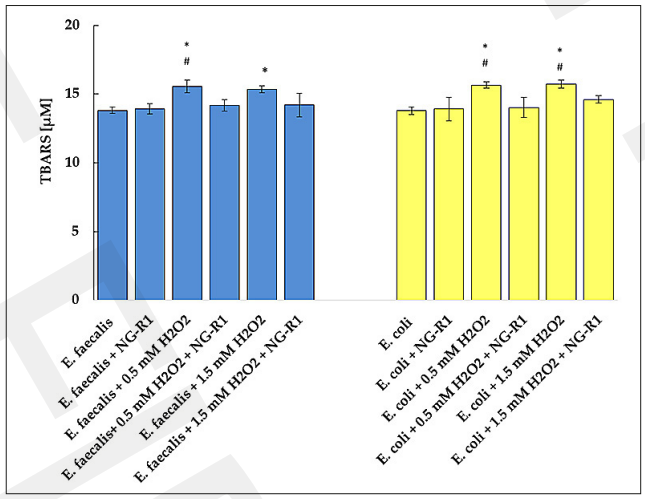

Figure 3. Effect of saponin NG-R1 (40 µM) and H_2O_2 (0.5 mM and 1.5 mM) on the level of thiobarbituric acid reactive substances (TBARS) on *Enterococcus faecalis* and *Escherichia coli*. Samples were incubated for 24 h aerobically at 37 °C. Measurements were made using TBARS assay.
Bars – mean values with standard deviations; * – values statistically different in comparison with those observed in untreated bacteria (control) (* $p < 0.05$); hashes – values that are statistically different compared with values obtained in samples containing bacteria with NG-R1 (# $p < 0.05$). All experiments were repeated 3 times

of H_2O_2 TBARS levels in *Enterococcus faecalis* and *Escherichia coli* were statistically significant. The TBARS levels were significantly lower in both groups of bacteria treated with combination of H_2O_2 (0.5 and 1.5 mM H_2O_2) and NG-R1.

Determination of concentration of carbonyl groups. Protein CO groups are biomarkers of oxidative stress as they form early and their presence proves the stability of carbonylated proteins. Most tests used to detect carbonyl groups in proteins involve reaction of the groups with 2,4-dinitrophenylhydrazine (DNPH), which leads to formation of a stable product of dinitrophenyl (DNP) hydrazone [29]. Fig. 4 shows quantitative changes of carbonyl content after 24 h incubation of *Enterococcus faecalis* and *Escherichia coli* with saponin NG-R1 (40 μM) and/or H_2O_2 (0.5 mM and 1.5 mM). Increasing H_2O_2 concentration was accompanied by an increase in the level of carbonyl groups. The highest level of carbonyl groups was observed for *Enterococcus faecalis* (25.03 μM) and *Escherichia coli* (7.21 μM) in response to application of 1.5 mM H_2O_2. Saponin NG-R1 reduced carbonyl content and significantly decreased the effect of 0.5 mM H_2O_2 and 1.5 mM H_2O_2. With regards to *Escherichia coli*, there was no statistically significant difference after application of 1.5 mM H_2O_2 and NG-R1 saponin.

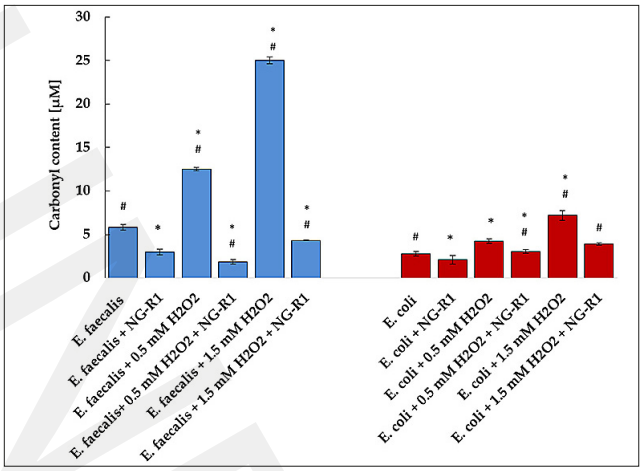

Figure 4. Effect of saponin NG-R1 (40 μM) and H_2O_2 (0.5 mM and 1.5 mM) on carbonyl groups on *Enterococcus faecalis* and *Escherichia coli*. The samples were incubated for 24 h aerobically at 37 °C. Measurements were made with the use of 2,4-dinitrophenylhydrazine (DNPH).
Bars – mean values with standard deviations; * – values that are statistically different in comparison with those observed in untreated bacteria (control) (* $p < 0.05$); hashes – values statistically different compared with values obtained in samples containing bacteria with NG-R1 (# $p < 0.05$). All experiments were repeated 3–4 times

Assessment of free thiol groups concentration. Thiol-containing compounds play an important role in protecting biological systems from oxidative damage. The thiol content in samples was determined by spectrophotometric measurements using the Ellman's method. As shown in Fig. 5, thiol groups levels measured in H_2O_2-treated bacteria were comparable to those measured in untreated cells. The differences between control and H_2O_2-treated cells were not statistically significant. These data were significantly different with respect to –SH group levels measured for bacteria treated with NG-R1. The highest level of thiol groups was observed in *Enterococcus faecalis* after treatment with NG-R1 alone. The correlation of NG-R1 with H_2O_2 resulted

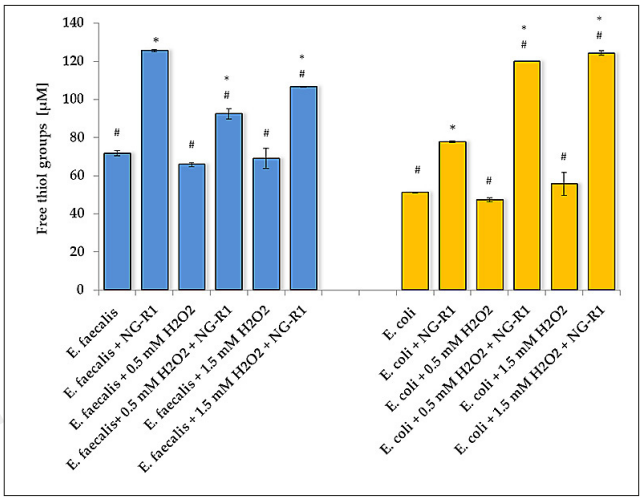

Figure 5. Effect of saponin NG-R1 (40 μM) and H_2O_2 (0.5 mM and 1.5 mM) on free thiol groups on *Enterococcus faecalis* and *Escherichia coli*. The samples were incubated for 24 h aerobically at 37 °C. Measurements were made with the use of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB).
Bars – mean values with standard deviations; * – values that are statistically different compared with those observed in untreated bacteria (control) (* $p < 0.05$); hashes – values statistically different compared with values obtained in samples containing bacteria with NG-R1 (# $p < 0.05$). All experiments were repeated 3 times

in a significant increase in free thiol groups, compared to the effect of H_2O_2 alone on both strains of bacteria.

DISCUSSION

Gut microbiota is increasingly considered as an environmental factor promoting the progression of cardiovascular disease (CVD) states and arterial thrombosis. In recent years, gut microbiota has been identified as a chronic inflammatory stimulus [34, 35]. It has been shown that the gut of patients with coronary artery disease (CAD) contains increased levels of *Lactobacillus*, *Streptococcus*, *Escherichia*, *Shigella* and *Enterococcus* species [36]. Gut microbiota impacts the development and function of hepatic vascular endothelium and the function of prothrombotic platelet. Moreover, gut-derived microbial-associated molecular patterns could stimulate hepatic vWF synthesis and plasmatic vWF levels through the Toll-like receptor-2 (TLR2). Recent studies suggest that gut microbial composition also affects the efficacy of cardiovascular pharmacotherapy.

The impact of different treatment methods on gut microbiota may help to establish novel strategies in preventing cardiovascular diseases [34, 35]. Platelets are able to interact with different bacteria directly via platelet surface receptors, such as P-selectin, glycoproteins and TLR4; indirectly, through plasma proteins, or via bacteria releasing exotoxins. The interaction of platelets with Gram-negative bacteria is not so well characterized. However, it has been shown that Gram-negative *E. coli*, *Helicobacter pylori* and *Klebsiella pneumoniae* have an activating or aggregating effect on platelets [21]. Patients with symptomatic stroke and transient ischemic attack demonstrate altered gut microbiota. Furthermore, the gut microbiota can substantially contribute to variable blood lipid composition [37].

Gut microbiota dysbiosis, a result of a disruption to the overall state, is associated with increased inflammation which, in turn, is linked with hypertension and vascular dysfunction

[14]. Some gut bacteria metabolites are found to be directly associated with increased ROS levels [38]. ROS include radical species, such as superoxide ($O_2^{\bullet -}$), hydroxyl radical ($HO^{\bullet}$) and hydrogen peroxide (H_2O_2), which play important roles in hypertension, ischemic heart disease, ischemia-reperfusion injury, and other vascular diseases, including thrombosis. Perturbation of the redox state leads to ROS interaction with proteins, enzymes, nucleic acids, which can lead to vascular pathology. In pathological conditions, inflammatory cells that release ROS are continuously recruited [39]. ROS can be formed during exposure of bacteria to endogenous or exogenous factors; for example, lipid hydroperoxides can be generated due to an attack of ROS on the bacterial membrane. To alleviate the oxidative damage, bacterium induces synthesis of a variety of antioxidant defense enzymes (catalases, superoxide dismutases, peroxidases). Additional defences against bacteria include alkyl hydroperoxide reductase (AhpC), bacterioferritin-comigratory protein (BCP), and periplasmic thiol peroxidase (p20). P20 has been defined as a periplasmic protein that exists in Gram-negative bacteria, such as *E. coli*, whereas AhpC and BCP, being cytoplasmic proteins, have been found in all bacterial species [40].

In the current study, an attempt was made to determine the effect of saponin NG-R1 on *E. faecalis* and *E. coli* and its protective role on induced oxidative stress. The study investigated antioxidant properties of NG-R1 saponin on selected bacterial strains of intestinal microbiota that may be involved in the pathogenesis of thromboembolic diseases. An evaluation of selected parameters of oxidative stress made it possible to check whether the tested compound can reduce H_2O_2-stimulated oxidative stress.

E. coli is a representative biological model for Gram-negative bacteria. This type of bacteria is characterized by a cell wall that is structurally far more complex than that of Gram-positive bacteria. In addition to the phospholipid bilayer of the plasma membrane, only Gram-negative bacteria have an outer membrane comprising phospholipid bilayer, lipoproteins and lipopolysaccharides [41]. *E. faecalis* is a bacterium naturally inhabiting the gastrointestinal tract of humans and other mammals. Yan et al. [42], revealed that H_2O_2 (1.5 – 2 mM) can affect the survivability of bacteria which are able to successfully respond to the activity of H_2O_2. Results of a study carried out by Rodriguez-Rojas et al. [43] indicated that concentration (0.1 mM) is the maximum dose that does not contribute to differences in the growth of *E. col,i* compared to non-treated cells.

Microbes have developed defence mechanisms against oxidative stress. Many pathogens in response to excessive ROS levels can trigger synthesis and/or accumulate antioxidant substances, as well as increase the activity of antioxidant enzymes [44]. It has been shown that treatment of *E. coli* with KS400 H_2O_2 increased the levels of TBARS and carbonyl groups. The TBARS levels increased significantly after treatment with 5 mM H_2O_2. In contrast, a significant increase in carbonyl groups was observed after treating *E. coli* with KS400 and 100 mM H_2O_2. In turn, in *E. coli* AB1157 cells, an application of 20 mM hydrogen peroxide after 20 min led to a significant increase in TBARS and carbonyl group levels. These results indicate different oxidative damage to lipids and proteins induced by H_2O_2 for different strains *E. coli*. The level of TBARS in AB1157 cells was about 4 times higher than that noted for KS400. However, the level of carbonyl groups is more than twice as low in AB1157 than in KS400. These data show the differences between the 2 bacterial strains in their response to oxidative damage to lipids, compared to proteins. This may be related to their different defence mechanisms against oxidative stress [30]. In the current study, 0.5 and 1.5 mM concentrations of H_2O_2 were used.

As demonstrated in Figures, exposure of cells to H_2O_2 changed the level of various biomarkers of oxidative stress, including the level of hydroperoxide (Fig. 2), TBARS level – marker of lipid peroxidation (Fig. 3), protein carbonylation (Fig. 4), and free thiol groups (Fig. 5). A study by Cong et al. [45] revealed that NG-R1 in low concentration (below 50 μM) had no effects on human lung fibroblast viability. Moreover, it has been shown that 30 or 40 μM NG-R1 treatment remarkably alleviated the LPS-induced cell viability loss.

The current study shows that NG-R1 significantly reduced the levels of peroxides in both *E. faecalis* and *E. coli* in the control and in bacterial suspensions treated with hydrogen peroxide. A slight increase in the hydroperoxide level after treatment with both bacteria with H_2O_2 was accompanied by a slight but significant increase in the TBARS level. NG-R1 led to a significant decrease in the TBARS level in both bacterial suspensions treated with H_2O_2. These small changes in hydroperoxides and TBARS levels may be related to a small amount of unsaturated fatty acids in bacterial lipids, which contain mostly monounsaturated acids. However, bacterial lipids are mainly monounsaturated and therefore not so prone to oxidation [41].

A similar effect of NG-R1, which significantly decreased the amount of lipid peroxidation products (TBARS), was demonstrated in the serum of rats [46]. NG-R1, which has a multi-substituted triterpenoid (steroid) ring in its chemical structure, is able to directly scavenge H_2O_2. An *in vitro* study showed that NG-R1 removes H_2O_2 in a dose-dependent manner [47], and protects human proximal tubular epithelial cells against inflammatory damage induced by LPS [5]. Moreover, *Panax notoginseng* has been reported to be effective in treating myocardial infarction in an animal model and cardiovascular disease in humans [48]. Various studies have suggested that biological functions of NG-R1 involves protection against oxidation. Results of the current study are consistent with other reports indicating that *Panax notoginseng* and its active compounds showed an inhibitory effect on lipid peroxidation. Unsaturated fatty acids in lipids of the cell membrane are prone to peroxidation. This chain reaction is initiated by hydroxyl radicals attacking lipids and by generated free radicals of lipid hydroperoxide. Ginseng extract was shown to exhibit DPPH radical scavenging activity *in vitro* and hydroxyl radical scavenging activity, thereby protecting membranes against lipid peroxide formation, and consequently protecting the cell membrane from oxidative damage [49].

In biological systems, proteins are the main target for reactive oxygen species because they are present in large amounts and exhibit a high rate of constants. ROS can lead to fragmentation of the protein side chain and backbone through formation of covalent bonds with other proteins, and protein cross-linking [49]. Oxidative modifications can lead to increased hydrophilicity of the side chain, which leads to conformational changes in proteins, resulting in altered interactions with biological partners [50]. Besides, as a result of protein oxidation (peroxidation), aldehydes, ketones, and

other compounds are formed as products (carbonyl protein) which enable determination of the level of carbonyl groups.

The current study has also shown that treatment with H_2O_2 leads to a significant increase in the level of carbonyl groups in *E. faecalis* and, to a lesser extent, in *E. coli*, but the results were also statistically significant. NG-R1 highly protected proteins against oxidative stress by strongly reducing the formation of carbonyl groups in *E. faecalis*. A similar effect, however not that strong, was observed for *E. coli*.

Thiol groups present in proteins, bound directly to cysteine and to a lesser extent to methionine, are most sensitive to oxidation, also by H_2O_2, and change the activity and function in these proteins [51, 52]. In this study, it was observed that the treatment of *E. faecalis* and *E. coli* with 2 concentrations of H_2O_2 decreased the level of thiol groups. It is noteworthy that the action of NG-R1 led to a strong growth of the level of -SH group in *E. faecalis,* and to a lesser extent, in *E. coli*. This increase may possibly be related to a change in protein conformation under the influence of saponin and exposure of new thiol groups that react with Ellman's reagent. NG-R1 restored levels of -SH groups after treatment of bacteria with hydrogen peroxide, and their levels were higher than those noted in the control. A greater effect of NG-R1 was observed in *E. coli* than in *E. faecalis*. *E. faecalis* is one of pathogens which is highly resistant to stress.

CONCLUSIONS

In this study, *Enterococcus faecalis* and *Escherichia coli* were exposed to NG-R1 and/or H_2O_2. Results indicate that NG-R1 exhibited protective properties against hydrogen peroxide-induced oxidative stress by reducing lipid peroxidation and carbonyl formation. Interestingly, NG-R1 induced the growth of the level of -SH groups in both bacteria, and protected the thiols from oxidation by hydrogen peroxide. However, the role of NG-R1 in modulation of the intracellular redox mechanism of intestinal microbiota requires further clinical research.

Acknowledgments

The authors would also like to thank Ms Dorota Wawrzyniak, from the Foreign Language Centre of the Medical University of Lodz, Poland for a language consultation.

REFERENCES

1. Jansen VL, Gerdes VE, Middeldorp S, et al. Gut microbiota and their metabolites in cardiovascular disease. Best Pract Res Clin Endocrinol Metab. 2021;35(3):101492. https://doi.org/10.1016/j.beem.2021.101492
2. Meng X, Sun G, Ye J, et al. Notoginsenoside R1-mediated neuroprotection involves estrogen receptor-dependent crosstalk between Akt and ERK1/2 pathways: A novel mechanism of Nrf2/ARE signaling activation. Free Radic Res. 2014;48(4):445–460. https://doi.org/10.3109/10715762.2014.885117
3. Park SK, Hyun SH, In G, et al. The antioxidant activities of Korean Red Ginseng (Panax ginseng) and ginsenosides: A systemic review through in vivo and clinical trials. J Ginseng Res. 2021;45(1):41–47. https://doi.org/10.1016/j.jgr.2020.09.006
4. Chen XJ, Zhang XJ, Shui YM, et al. Anticancer Activities of Protopanaxadiol- and Protopanaxatriol-Type Ginsenosides and Their Metabolites. Evidence-based Complement. Altern Med. 2016;2016:5738694. https://doi.org/10.1155/2016/5738694
5. Liu H, Yang J, Yang W, et al. Focus on notoginsenoside R1 in metabolism and prevention against human diseases. Drug Des Devel Ther. 2020;14:551–565. https://doi.org/10.2147/DDDT.S240511
6. Tang Z, Yang C, He Z, et al. Notoginsenoside R1 alleviates spinal cord injury through the miR-301a/KLF7 axis to activate Wnt/β-catenin pathway. Open Med. 2022;17(1):741–755. https://doi.org/10.1515/med-2022-0461
7. Dong Y, Yan X, Yang X, et al. Notoginsenoside R1 suppresses miR-301a via NF-κB pathway in lipopolysaccharide-treated ATDC5 cells. Exp Mol Pathol. 2020;112:104355. https://doi.org/10.1016/j.yexmp.2019.104355
8. Fan C, Qiao Y, Tang M, et al. Notoginsenoside R1 attenuates high glucose-induced endothelial damage in rat retinal capillary endothelial cells by modulating the intracellular redox state. Drug Des Devel Ther. 2017;11:3343–3354. https://doi.org/10.2147/DDDT.S149700
9. Yu Y, Sun G, Luo Y, et al. Cardioprotective effects of Notoginsenoside R1 against ischemia/reperfusion injuries by regulating oxidative stress- and endoplasmic reticulum stress-related signaling pathways. Sci Rep. 2016;6:21730. https://doi.org/10.1038/srep21730
10. Fang H, Yang S, Luo Y, et al. Notoginsenoside R1 inhibits vascular smooth muscle cell proliferation, migration and neointimal hyperplasia through PI3K/Akt signaling. Sci Rep. 2018;8(1):7595. https://doi.org/10.1038/s41598-018-25874-y
11. Yang X, Xiong X, Wang H, et al. Protective effects of panax notoginseng saponins on cardiovascular diseases: A comprehensive overview of experimental studies. Evidence-based Complement. Altern Med. 2014;2014:204840. https://doi.org/10.1155/2014/204840
12. Zhao H, Han Z, Li G, et al. Therapeutic potential and cellular mechanisms of Panax notoginseng on prevention of aging and cell senescence-associated diseases. Aging Dis. 2017;8(6):721–739. https://doi.org/10.14336/AD.2017.0724
13. Murphy K, O'donovan AN, Caplice NM, et al. Exploring the gut microbiota and cardiovascular disease. Metabolites. 2021;11(8):493. https://doi.org/10.3390/metabo11080493
14. Kazemian N, Mahmoudi M, Halperin F, et al. Gut microbiota and cardiovascular disease: opportunities and challenges. Microbiome 2020;8(1):36. https://doi.org/10.1186/s40168-020-00821-0
15. Formes H, Reinhardt C. The gut microbiota–a modulator of endothelial cell function and a contributing environmental factor to arterial thrombosis. Expert Rev Hematol. 2019;12(7):541–549. https://doi.org/10.1080/17474086.2019.1627191
16. Luz PL da, Haas EA, Favarato D. Intestinal Microbiota and Cardiovascular Diseases. Int J Cardiovasc Sci. 2020;33(5):462–471. https://doi.org/10.36660/ijcs.20200043
17. Sun K, Wang C-SS, Guo J, et al. Protective effects of ginsenoside Rb1, ginsenoside Rg1, and notoginsenoside R1 on lipopolysaccharide-induced microcirculatory disturbance in rat mesentery. Life Sci. 2007;81(6):509–518. https://doi.org/10.1016/j.lfs.2007.06.008
18. Hasan RA, Koh AY, Zia A. The gut microbiome and thromboembolism. Thromb Res. 2020;189:77–87. https://doi.org/10.1016/j.thromres.2020.03.003
19. Suganya K, Son T, Kim K-W, et al. Impact of gut microbiota: How it could play roles beyond the digestive system on development of cardiovascular and renal diseases. Microb Pathog. 2021;152:104583. https://doi.org/10.1016/j.micpath.2020.104583
20. Lichota A, Gwozdzinski K, Szewczyk EM. Microbial modulation of coagulation disorders in venous thromboembolism. J Inflamm Res. 2020;13:387–400. https://doi.org/10.2147/JIR.S258839
21. Fejes AV, Best MG, van der Heijden WA, et al. Impact of Escherichia coli K12 and O18:K1 on human platelets: Differential effects on platelet activation, RNAs and proteins. Sci Rep. 2018;8(1):16145. https://doi.org/10.1038/s41598-018-34473-w
22. Huycke MM. Enterococcus faecalis produces extracellular superoxide and hydrogen peroxide that damages colonic epithelial cell DNA. 2002;23(3):529–36. https://doi.org/doi.org/10.1093/carcin/23.3.529
23. Van Tyne D, Martin MJ, Gilmore MS. Structure, function, and biology of the Enterococcus faecalis cytolysin. Toxins (Basel). 2013;5(5):895–911. https://doi.org/10.3390/toxins5050895
24. Nogueira A, Peixoto F, Oliveira MM, et al. The Effects of Long-Term Chaetomellic Acid A Administration on Renal Function and Oxidative Stress in a Rat Model of Renal Mass Reduction. Biomed Res Int. 2017;2017:5125980. https://doi.org/10.1155/2017/5125980
25. Lopes D, Melo T, Santos N, et al. Evaluation of the interplay among the charge of porphyrinic photosensitizers, lipid oxidation and photoinactivation efficiency in Escherichia coli. J Photochem Photobiol B Biol. 2014;141:145–153. https://doi.org/10.1016/j.jphotobiol.2014.08.024
26. Álvarez MC, Caldiz C, Fantinelli JC, et al. Is cardiac hypertrophy in spontaneously hypertensive rats the cause or the consequence of oxidative stress? Hypertens Res. 2008;31(7):1465–1476. https://doi.org/10.1291/hypres.31.1465

27. Czubak-Prowizor K, Trelinski J, Stelmach P, et al. Increased Oxidative Stress in Acute Myeloid Leukemia Patients after Red Blood Cell Transfusion, but Not Platelet Transfusion, Results Mainly from the Oxidative/Nitrative Protein Damage: An Exploratory Study J Clin Med. 2021;10(7):1349. https://doi.org/10.3390/jcm10071349
28. Stefek M, Trnkova Z, Krizanova L. 2,4-Dinitrophenylhydrazine carbonyl assay in metal-catalysed protein glycoxidation. Redox Rep. 1999;4(1–2):43–48. https://doi.org/10.1179/135100099101534738
29. Dalle-Donne I, Rossi R, Giustarini D, et al. Protein carbonyl groups as biomarkers of oxidative stress. Clin Chim Acta 2003;329(1–2):23–38. https://doi.org/ 10.1016/s0009-8981(03)00003-2
30. Semchyshyn H, Bagnyukova T, Storey K, et al. Hydrogen peroxide increases the activities of regulon enzymes and the levels of oxidized proteins and lipids in. Cell Biol Int. 2005;29(11):898–902. https://doi.org/10.1016/j.cellbi.2005.08.002
31. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys. 1959;82(1):70–77. https://doi.org/ 10.1016/0003-9861(59)90090-6
32. Gwozdzinski K, Pieniazek A, Brzeszczynska J, et al. Alterations in Red Blood Cells and Plasma Properties after Acute Single Bout of Exercise. Sci World J. 2013;2013:168376. https://doi.org/10.1155/2013/168376
33. Girgin Ersoy Z, Barisci S, Dinc O. Mechanisms of the Escherichia coli and Enterococcus faecalis inactivation by ozone. LWT 2019;100:306–313. https://doi.org/10.1016/j.lwt.2018.10.095
34. Reinhardt C. The Gut Microbiota as an Influencing Factor of Arterial Thrombosis. Hamostaseologie 2019;39(2):173–179. https://doi.org/10.1055/s-0038-1675357
35. Haghikia A, van Mens TE, Pontarollo G, et al. Editorial: Impact of the gut microbiota on cardiovascular medicine. Front Med. 2022;9:939890. https://doi.org/10.3389/fmed.2022.939890
36. Masenga SK, Hamooya B, Hangoma J, et al. Recent advances in modulation of cardiovascular diseases by the gut microbiota. J Hum Hypertens. 2022;36(11):952–959. https://doi.org/10.1038/s41371-022-00698-6
37. Fu J, Bonder MJ, Cenit MC, et al. The Gut Microbiome Contributes to a Substantial Proportion of the Variation in Blood Lipids. Circ Res. 2015;117(9):817–824. https://doi.org/10.1161/CIRCRESAHA.115.306807
38. Shandilya S, Kumar S, Kumar Jha N, et al. Interplay of gut microbiota and oxidative stress: Perspective on neurodegeneration and neuroprotection. J Adv Res. 2022;38: 223–244. https://doi.org/10.1016/j.jare.2021.09.005
39. Lichota A, Szewczyk EM, Gwozdzinski K. Factors Affecting the Formation and Treatment of Thrombosis by Natural and Synthetic Compounds. Int J Mol Sci. 2020;21(21):7975. https://doi.org/10.3390/ijms21217975
40. Cha M-K, Hong S-K, Lee D-S, et al. Vibrio cholerae Thiol Peroxidase-Glutaredoxin Fusion Is a 2-Cys TSA/AhpC Subfamily Acting as a Lipid Hydroperoxide Reductase. J Biol Chem. 2004;279(12):11035–11041. https://doi.org/10.1074/jbc.M312657200
41. Alves E, Santos N, Melo T, et al. Photodynamic oxidation of Escherichia coli membrane phospholipids: New insights based on lipidomics. Rapid Commun. Mass Spectrom. 2013;27(23):2717–2728. https://doi.org/10.1002/rcm.6739
42. Yan X, Budin-Verneuil A, Verneuil N, et al. Response of Enterococcus faecalis V583 to Low Hydrogen Peroxide Levels. Curr Microbiol. 2015;70(2):156–168. https://doi.org/10.1007/s00284-014-0691-8
43. Rodríguez-Rojas A, Kim JJ, Johnston PR, et al. Non-lethal exposure to H2O2 boosts bacterial survival and evolvability against oxidative stress. PLOS Genet. 2020;16(3):e1008649. https://doi.org/10.1371/journal.pgen.1008649
44. Peters LP, Carvalho G, Martins PF, et al. Differential Responses of the Antioxidant System of Ametryn and Clomazone Tolerant Bacteria. PLoS One 2014;9(11):e112271. https://doi.org/10.1371/journal.pone.0112271
45. Cong S, Xiang L, Yuan X, et al. Notoginsenoside R1 up-regulates microRNA-132 to protect human lung fibroblast MRC-5 cells from lipopolysaccharide-caused injury. Int Immunopharmacol. 2019;68:137–144. https://doi.org/10.1016/j.intimp.2018.12.065
46. Gui D, Wei L, Jian G, et al. Notoginsenoside R1 Ameliorates Podocyte Adhesion Under Diabetic Condition Through α3β1 Integrin Upregulation in Vitro and in Vivo. Cell Physiol Biochem. 2014;34(6):1849–1862. https://doi.org/10.1159/000366384
47. Zhang H-SS, Wang S-QQ. Notoginsenoside R1 inhibits TNF-α-induced fibronectin production in smooth muscle cells via the ROS/ERK pathway. Free Radic Biol Med. 2006;40(9):1664–1674. https://doi.org/10.1016/j.freeradbiomed.2006.01.003
48. Xu H, Zhang X, Shi Y, et al. Notoginsenoside R1 relieves the myocardial infarction via activating the JAK2/STAT3 signaling pathway in vivo and in vitro. Bioengineered. 2022;13(3):5653–5662. https://doi.org/10.1080/21655979.2022.2037366
49. Zhang W, Xiao S, Ahn DU. Protein Oxidation: Basic Principles and Implications for Meat Quality. Crit. Rev. Food Sci Nutr. 2013;53(11):1191–1201. https://doi.org/10.1080/10408398.2011.577540
50. Davies MJ. Protein oxidation and peroxidation. Biochem J. 2016;473(7):805–825. https://doi.org/10.1042/BJ20151227
51. Luo D. Smith SW, Anderson BD, Kinetics and Mechanism of the Reaction of Cysteine and Hydrogen Peroxide in Aqueous Solution. J Pharm Sci. 2005;94(2):304–316. https://doi.org/10.1002/jps.20253
52. Sjöberg B, Foley S, Cardey B, et al. Methionine oxidation by hydrogen peroxide in peptides and proteins: A theoretical and Raman spectroscopy study. J Photochem Photobiol B Biol. 2018;188:95–99. https://doi.org/10.1016/j.jphotobiol.2018.09.009