

Article

Effects of bioflavonoids from *Taxus media* var. *Hicksii* on superoxide generation, phosphorylation of proteins and translocation of cytosolic compounds to the cell membrane in human neutrophils

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Abstract: In present work, the effects of bioflavonoids (ginkgetin and sciadopitysin) on stimulus-induced superoxide generation, tyrosyl and serine/threonine phosphorylation of proteins in human neutrophils, and the translocation of cytosolic compounds (p47phox, p67phox and Rac) to cell membrane were studied, which were isolated from the needles of *Taxus media* var. *Hicksii*. Meanwhile, three normal flavonoids (apigenin, quercetin and isoquercetin) were involved as contrasts. The results indicated that ginkgetin and sciadopitysin were capable of concentration-dependently inhibitory effects on the superoxide generation induced by N-formyl-methionyl-leucyl-phenylalanine (fMLP), arachidonic acid (AA) and phorbol-12-myristate 13-acetate (PMA). And they also suppressed fMLP- and AA- induced tyrosyl or PMA-induced serine/threonine phosphorylation and the translocation of cytosolic compounds (p47phox, p67phox and Rac) to cell membrane, which were in parallel with the suppression of the stimulus-induced superoxide generation. The effect of these compounds on the radical-scavenging was also investigated. Ginkgetin and sciadopitysin did not show remarkable effect on DPPH radical-scavenging activity, and they didn't display the radical-scavenging activity on superoxide anion generated by phenazine methoxysulfate (PMS)-NADH system. Apparently, ginkgetin and sciadopitysin had great performance in pharmacological value and they are worthy of in-depth study.

Keywords: bioflavonoids; superoxide generation; oxidative phosphorylation; translocation

1. Introduction

Paclitaxel (commercial name, Taxol) is a famous natural antitumor drug isolated initially from *Taxus canadensis* and *Taxus media* var. *Hicksii* [1, 2]. Most researchers have been devoted to studying on paclitaxel and its homologues from genus *Taxus* owing to their outstanding anticancer effects[3]. When it comes to bioflavonoids in *Taxus*, there's only a few research. However, several investigations have confirmed that biflavonoids has significant pharmacological activity, even though they are rare in nature and [4]. Specifically, the extract of Ginkgo biloba leaves (approximately contains 0.65% of ginkgetin) has been demonstrated through basic researches and clinical trials that it is capable of treating hyperlipidemia and cardiovascular diseases, and it's popular in Asia, North America and Europe[4-8].

We have reported that tricyclic diterpenoid compounds isolated from *T. media* suppressed stimulus-induced superoxide generation in human neutrophils[9]. However, the corresponding research on bioflavonoids from *T. media* have not been reported. Therefore, in present study we evaluated the inhibitive activities of biflavonoids, namely ginkgetin and sciadopitysin, for oxidative phosphorylation and its underlying pharmacological mechanisms by studying their effects on superoxide generation, phosphorylation of proteins and translocation of cytosolic compounds to the cell membrane in human neutrophils. Moreover, the effects of them on the scavenging function of

generation radicals were also investigated. Here, other three normal flavonoids (apigenin, quercetin and isoquercetin) were involved as contrasts.

Neutrophils, a kind of white blood cells, are specific effector cells in the innate immune system and usually serve as the first barrier against pathogen invasion by phagocytosis in the host[10]. Superoxide generation in human neutrophils, a key process related to innate immunity and inflammation, is stimulated not only during phagocytosis, but also by the treatment of the cells with various stimuli, such as certain chemoattractants (the complement fraction C5a, interleukin-8, platelet activating factor, etc.) and activators of protein kinase[11]. In the activated state, neutrophils can produce an enormous amount of highly responsive superoxide anions and reactive oxygen species (ROS), which lead to the damage and death of pathogenic microbes through redox reactions with bacterial cell membranes, nucleic acid and proteins[12, 13]. In this process large amounts of superoxide anions and toxic oxygen metabolites are produced, which is mediated by the activation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a multi-component enzyme, localized in the plasma membrane of phagocytic leucocytes[14, 15]. The core enzyme consists of five components: p67phox, p47phox, p40phox, p22phox and gp91phox. In the resting cell, three cytosolic components remain as a complex (p67phox, p47phox and p40phox) and the other components p22phox and gp91phox are located in the membrane of secretory vesicles as a heterodimeric flavohemoprotein known as cytochrome b558. When the cell is exposed to stimuli, p47phox together with p67phox migrate to the membrane associating with cytochrome b558 under the control of Rac via a cytoskeletal scaffold[16-18]. Moreover, the response of neutrophils to an activating stimulus is potentiated sometimes by prior exposure to a priming agent, and a variety of proinflammatory stimuli are observed to exercise this effect[19, 20].

In present study, we investigated the inhibitive activity and mechanism of oxidative phosphorylation of biflavonoids from the needles of *T. media* through examining the effects of these compounds on stimulus-induced superoxide generation, tyrosyl and serine/threonine phosphorylation of proteins in human neutrophils, and the translocation of cytosolic compounds (p47phox, p67phox and Rac) to cell membrane. And the radical-scavenging activity was also investigated. And three normal flavonoids (apigenin, quercetin and isoquercetin) were involved as contrasts.

2. Results and Discussion

2.1 Inhibitory effect of five compounds on superoxide generation

All the five compounds significantly suppressed the fMLP-induced superoxide generation in a concentration-dependent manner as shown in Fig. 1A. Among the compounds, apigenin suppression of superoxide generation was significantly higher compared with the other compounds. Besides, the compounds also suppressed the AA-induced superoxide generation in a concentration-dependent manner. However, this suppression was not as significant as that of fMLP (Fig. 1B). The order of suppression was as following: isoquercetin > ginkgetin \approx sciadopitysin > quercetin > apigenin. The suppression of PMA-induced superoxide generation by quercetin was significantly higher than other compounds (Fig. 1C). The order of suppression was quercetin > isoquercetin > ginkgetin \approx apigenin > sciadopitysin.

It's clear that the inhibitory effects of these five compounds on superoxide generation induced by various stimuli were extremely different. Quercetin was more effective on PMA-induced superoxide generation, and apigenin was more effective on fMLP-induced superoxide generation. In addition, ginkgetin and sciadopitysin were more effective on AA-induced superoxide generation. These inconsistent inhibitory effects may be due to different inhibition mechanisms based on different chemical structures, but in general, the more phenolic hydroxyl, the stronger the activity. Many researches also suggested that the phenolic hydroxyl is the active part for antioxidant activity, and their position also could affect the activity[21, 22]. Moreover, the 3- hydroxyl of quercetin also contributed to its activity. Roughly, quercetin and isoquercetin had strong inhibitory activities, and the effects of two bioflavonoids (ginkgetin and sciadopitysin) were not inferior. According to their

chemical structures, B-3', 4'- dihydroxyl of isoquercetin and quercetin perhaps was the primary cause to their fine activities; the glucose of isoquercetin at the position of C-3 makes its activity weaker than quercetin; and the 4'-methoxy of sciadopitysin perhaps limited its activity. Although ginkgetin and sciadopitysin have weaker inhibitory effects on superoxide generation than quercetin, they are likely to have better activities in vivo. That's because quercetin may be produced after the metabolism of these two bioflavonoids based on the C-C bond cleavage between C-8 and its substituent (dimethoxyl apigenin). In addition, their greater liposolubility makes them more likely to pass through biological barriers after oral administration. For example, medicines with ginkgetin and its metabolites usually played its excellent role in the clinical treatments of cardiovascular diseases for their good liposolubility[23].

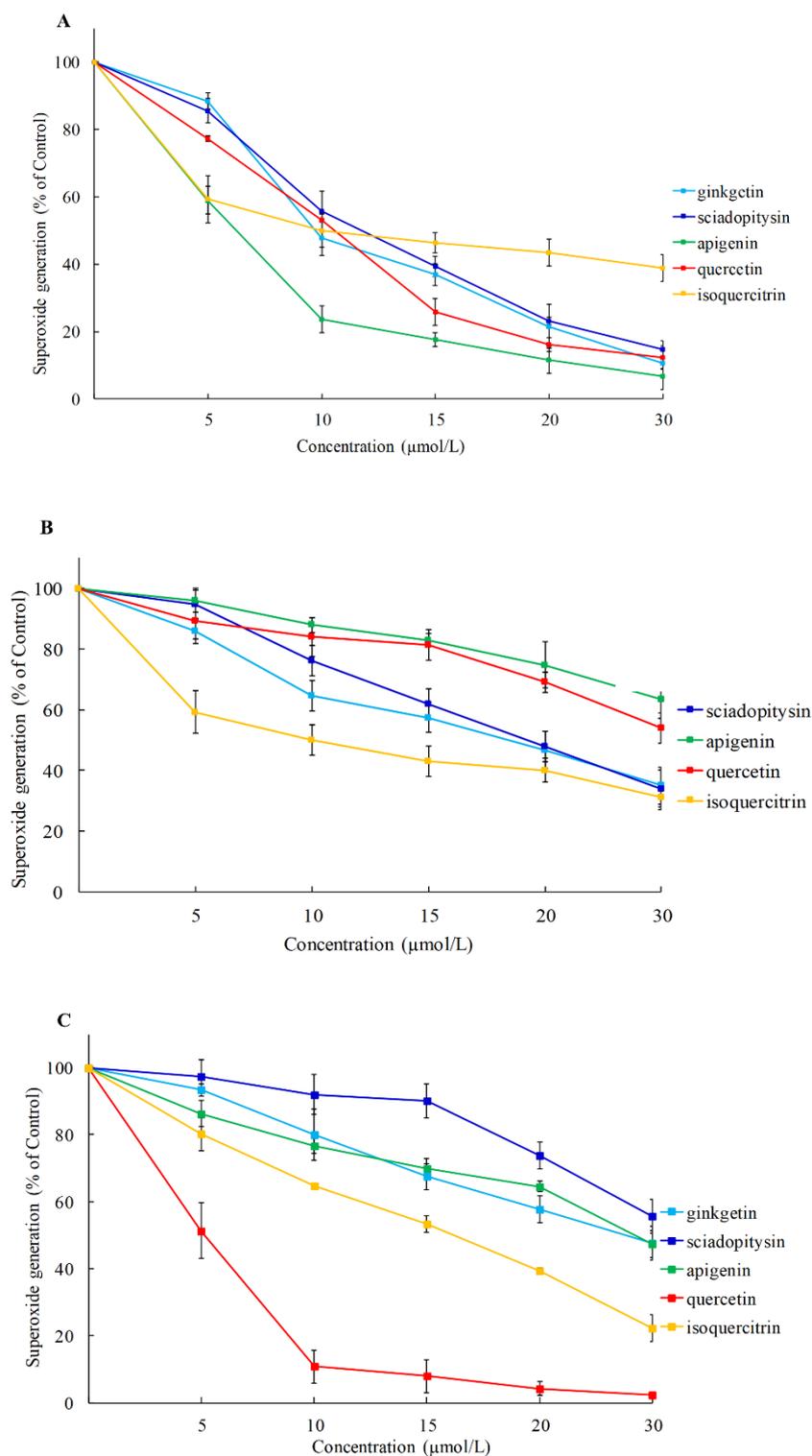


Fig. 1. Inhibitive activities of five compounds on superoxide generation(A: fMLP-induced superoxide generation; B: AA-induced superoxide generation; C: PMA-induced superoxide generation)

2.2 Biflavonoids inhibition of tyrosyl or serine/threonine phosphorylation of neutrophil proteins and translocation of p47phox, p67phox and Rac to the neutrophil membrane

The results indicated that all the compounds affected the tyrosyl or serine/threonine phosphorylation and the translocation of cytosolic p47phox, p67phox and Rac to the cell membrane induced by fMLP, AA and PMA in human neutrophils. Tyrosyl phosphorylation of 94.6- and 79.9-

kDa proteins was induced when the neutrophils were incubated with fMLP. Fig. 2A showed that tyrosyl phosphorylation was suppressed in a dose-dependent manner in the presence of ginkgetin and apigenin. These results were consistent with the changes of the superoxide generation, which means that tyrosyl phosphorylation was involved in the biflavonoids-mediated suppression of superoxide generation in human neutrophils. When these compounds bond to 94.6- and 79.9-kDa proteins, they caused the changes in the conformation of the two proteins and the tyrosyl phosphorylation of them was inhibited. The translocation of cytosolic p47phox, p67phox, and Rac to the cell membrane decreased in a dose-dependent manner when neutrophils were incubated with fMLP as shown in Fig. 3A. These results were consistent with the effects of the compounds on superoxide generation and the tyrosyl phosphorylation of neutrophil proteins in fMLP-stimulated neutrophils.

Tyrosyl phosphorylation of 94.6- and 79.9-kDa proteins was induced when neutrophils were incubated with AA in the presence of the compounds. The tyrosyl phosphorylation suppression by the compounds was in a dose-dependent manner (Fig. 2B). And the translocation of p47phox, p67phox and Rac to cell membrane also decreased in a dose-dependent manner in the presence of the compounds as shown in Fig. 3B. Serine/threonine phosphorylation of 71.2-kDa proteins was induced when neutrophils were incubated with APA in the presence of the compounds. The suppression of Serine/threonine phosphorylation by the compounds occurred in a dose-dependent manner (Fig. 2C). The translocation of p47phox, p67phox, and Rac to the cell membrane also decreased in a dose-dependent manner in the presence of the compounds as shown in Fig. 3C. These results were also in parallel to that of AA-induced superoxide generation.

In brief, the suppression of fMLP- and AA- induced tyrosyl or PMA-induced serine/threonine phosphorylation and the translocation of cytosolic compounds to cell membrane were all in parallel to the suppression of the stimulus-induced superoxide generation. It has been reported that p47phox and p67phox are key subunits of NADPH oxidase, and they mainly regulate the generation of reactive oxygen species by the translocation of them from cytoplasm to cell membrane[24-26]. Rac is also involved in the regulation of NADPH oxidase activity, and it also regulates the translocation of p47phox and p67phox[27, 28]. Furthermore, previous studies also suggested that the phosphorylation of p47phox is critical for NADPH oxidase hyperactivation and priming, and the serine/threonine phosphorylation usually related to oxidative stress[11, 29]. And it could conclude that the tyrosyl or serine/threonine phosphorylation and the translocation of cytosolic p47phox, p67phox and Rac are closely related in the process of superoxide generation.

In addition, the effect of five compounds on hydroxyl radical-induced lipid peroxidation of erythrocyte membrane ghosts is shown in Fig. 4. Quercetin, isoquercetin, ginkgetin and apigenin reduced the level of lipid peroxidation of erythrocyte membrane ghosts at 0-30 $\mu\text{mol/L}$ and this occurred in a concentration-dependent manner. However, sciadopitysin did not show any effect on lipid peroxidation. Studies suggested that quercetin can individually suppress lipopolysaccharide-induced oxidative responses and enhance the antioxidative stress pathway, and the hydroxyl of apigenin could have antioxidant activity by binding free radicals[30, 31]. It could be deduced that ginkgetin may be quite pretty positive to the inhibition on hydroxyl radical-induced lipid peroxidation.

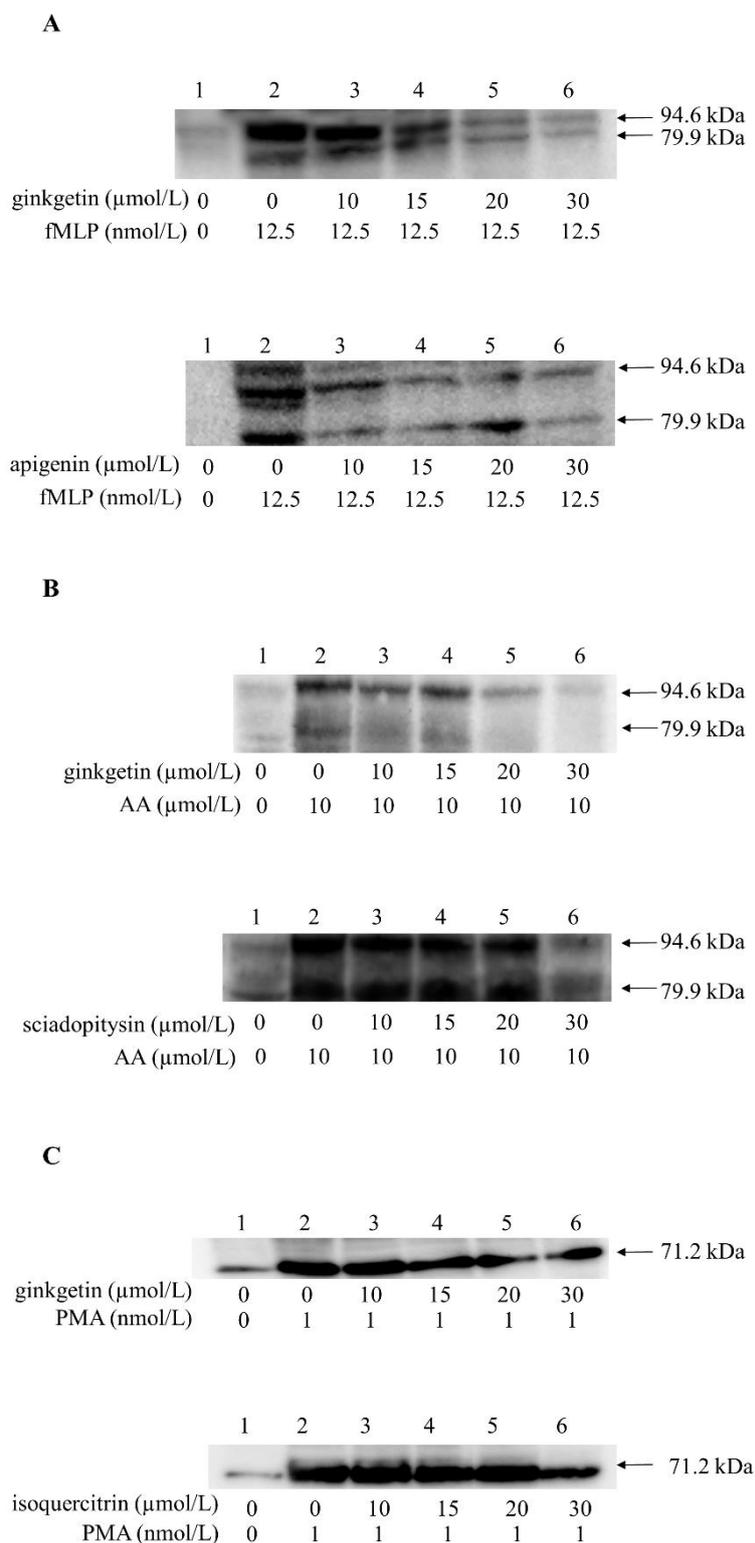


Fig. 2. Inhibitive effects of four compounds on tyrosyl or serine/threonine phosphorylation of neutrophil proteins(A: neutrophils were incubated with fMLP; B: incubated with AA; C: incubated with PMA)

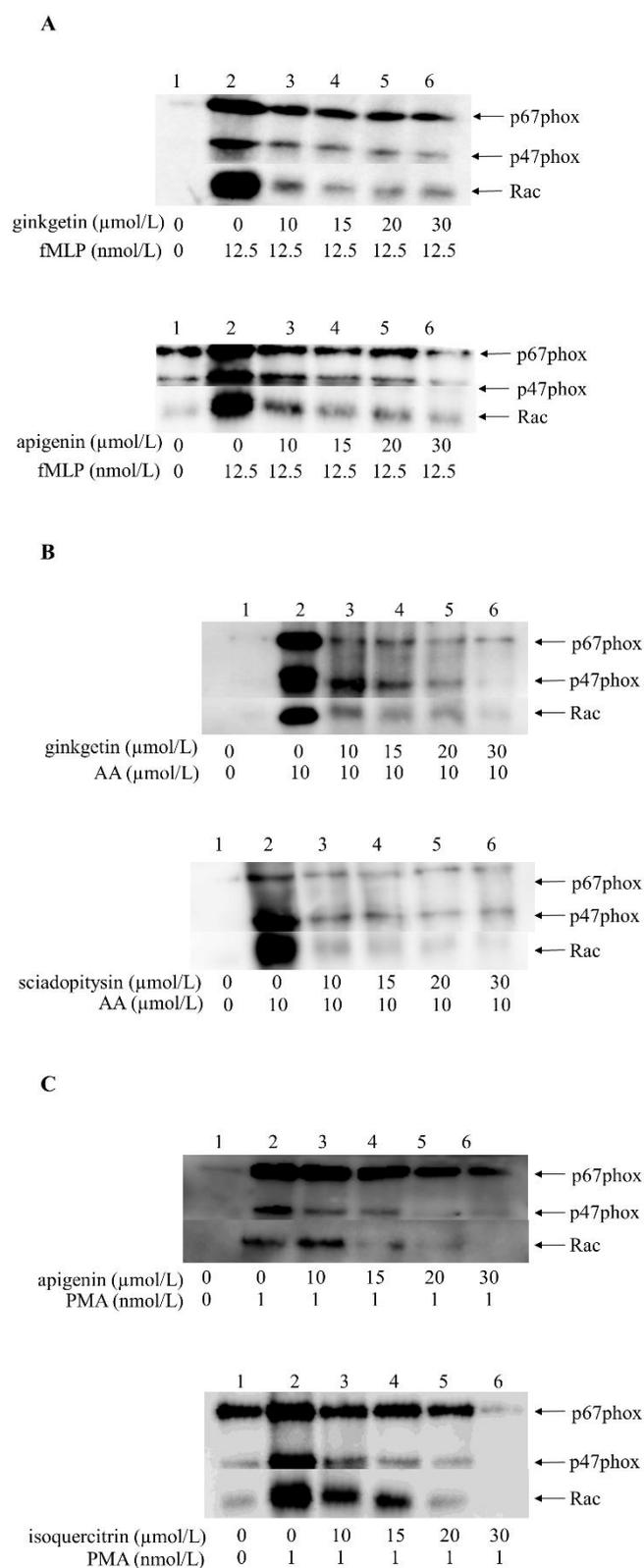


Fig. 3. The translocation of cytosolic p47phox, p67phox and Rac to cell membrane after neutrophils were incubated with various stimuli (A: incubated with fMLP; B: incubated with AA; C: incubated with PMA)

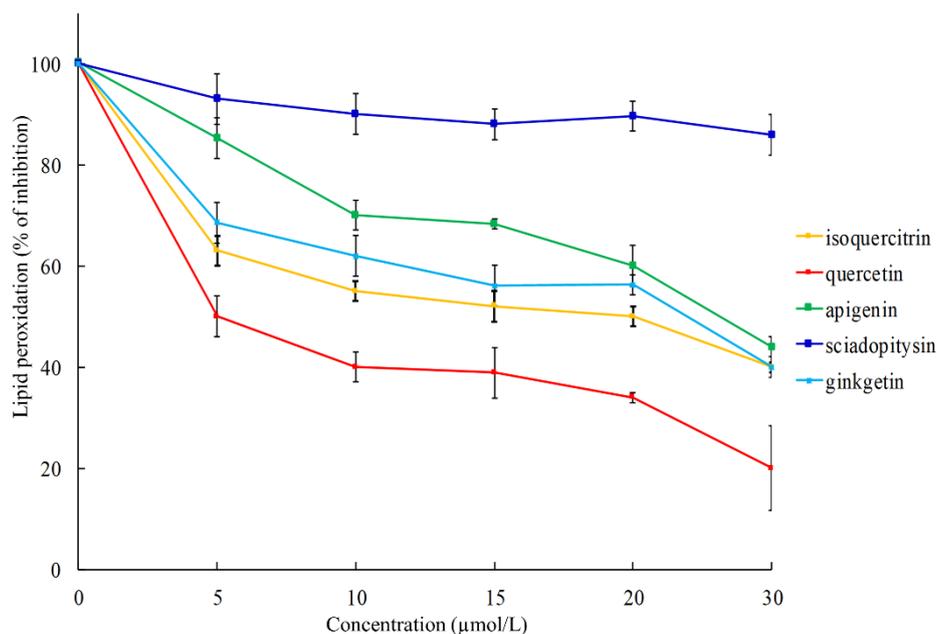


Fig. 4. The effect of five compounds on hydroxyl radical-induced lipid peroxidation of erythrocyte membrane ghosts

2.3 Radical-scavenging activity on DPPH and superoxide anion generated by phenazine methosulfate (PMS)-NADH system

The effects of the five compounds on the scavenging activity of generated radicals were also investigated. Quercetin and isoquercitrin showed significant radical-scavenging activity at 0-30 μmol/L in a concentration-dependent manner. However, the other compounds did not show any remarkable effect on DPPH radical-scavenging activity as shown in Fig. 5. The radical-scavenging activity on superoxide anion generated by phenazine methoxysulfate (PMS)-NADH system was also investigated. Quercetin showed weak radical scavenging activity, while the other compounds did not show any effect on superoxide anion radical-scavenging activity (Fig. 6). As though it is unclear why the free radical scavenging ability of quercetin was so different with the other tested compounds in the two cases, previous studies have found that the dependence of antiradical capacity of quercetin on the composition of solvent systems where the reactions are performed and also on the surrounding temperature[32], and it may be a new path to study in future.

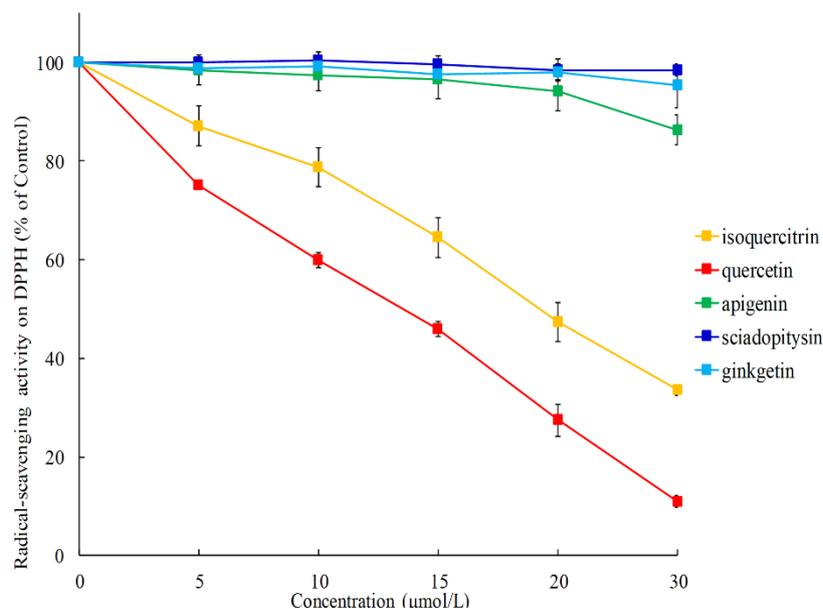


Fig. 5. The effects of the five compounds on the DPPH radical-scavenging activity (Results showed that quercetin and isoquercitrin had a significant radical-scavenging activity at the concentration of 0-30 µmol/L in a concentration-dependent manner, and ginkgetin and sciadopitysin have almost no effect on DPPH radical-scavenging activity)

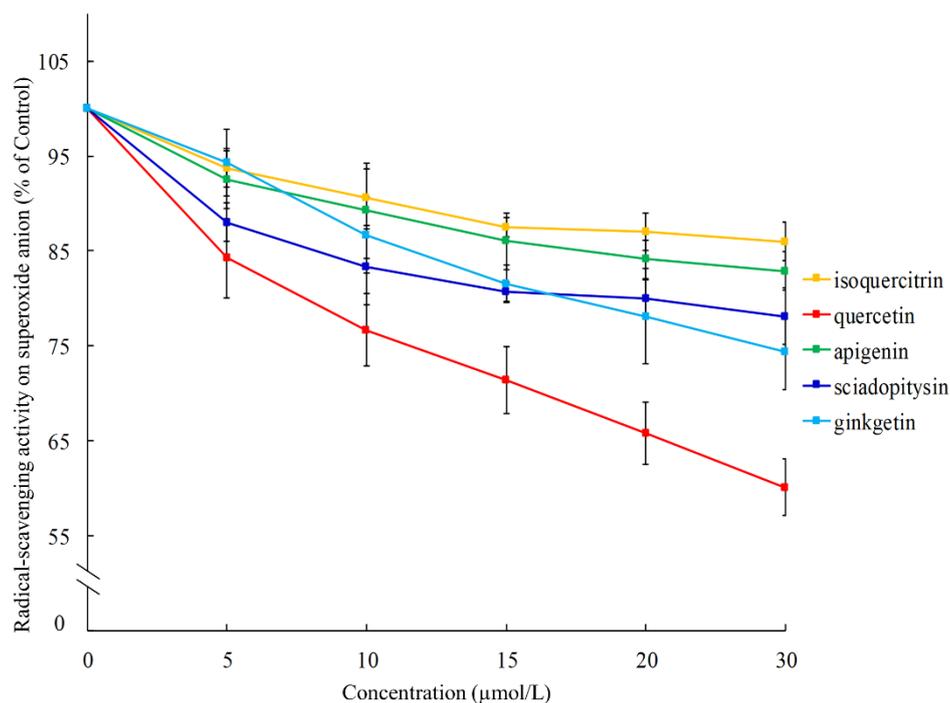


Fig. 6. The radical-scavenging activity of five compounds on superoxide anion generated by phenazine methoxysulfate (PMS)-NADH system (Results revealed that quercetin had weakly radical-scavenging activity, and other compounds were less than quercetin)

2.4 Hemolytic activity of biflavonoids

Considering the possibility of clinical applications of the compounds, their hemolytic effect was investigated. These compounds showed no effect on hemolysis at 30 µmol/L and 60 µmol/L as shown in Fig. 7, which is starting to support for the non-toxicity of bioflavonoids in present study.

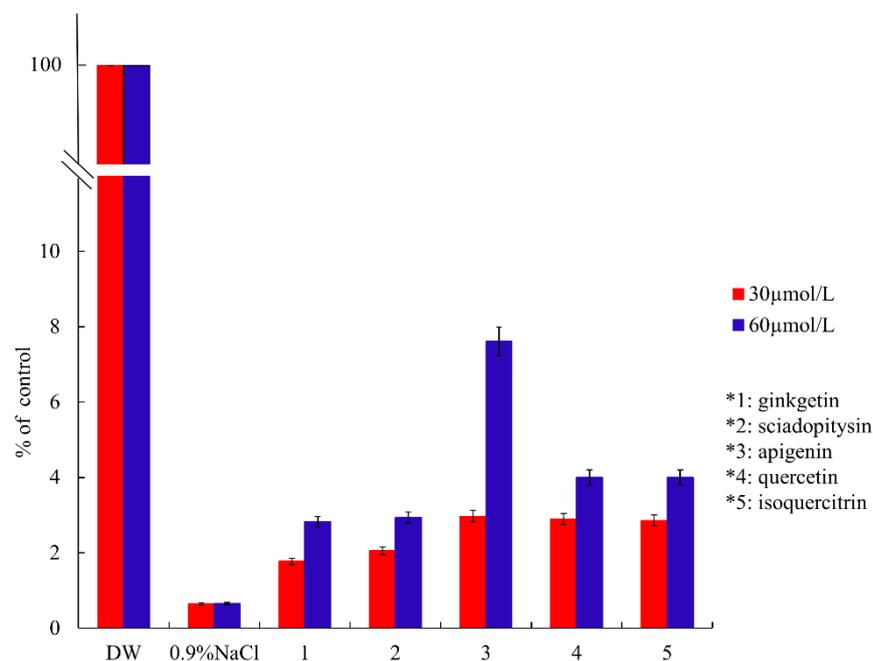


Fig. 7. Comparison of hemolytic effect of five compounds (results indicated that all of two bioflavonoids and three normal flavonoids didn't show an obvious effect on hemolysis at 30 μmol/L and 60 μmol/L)

3. Materials and Methods

3.1. Chemicals and reagents

Five compounds (ginkgetin, sciadopitysin, apigenin, quercitrin and isoquercitrin) were isolated from *Taxus media* var. *Hicksii* collected from Liaoning Province (China) and identified by Professor Jincai Lu (School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University). The extraction method referred to the previously reported method [33]. The voucher specimen was stored in the Herbarium of Shenyang Pharmaceutical University. The structures of these five compounds (Fig. 8) were determined by ^1H - and ^{13}C -NMR analyses, and all the purities of them were more than 96%. NADPH, ferricytochrome c (cyt.c), N-formyl-methionyl-leucyl-phenylalanine (fMLP), arachidonic acid (AA) and phorbol-12-myristate 13-acetate (PMA) were from Sigma (St. Louis, MO, USA). All other reagents used were analytical grade.

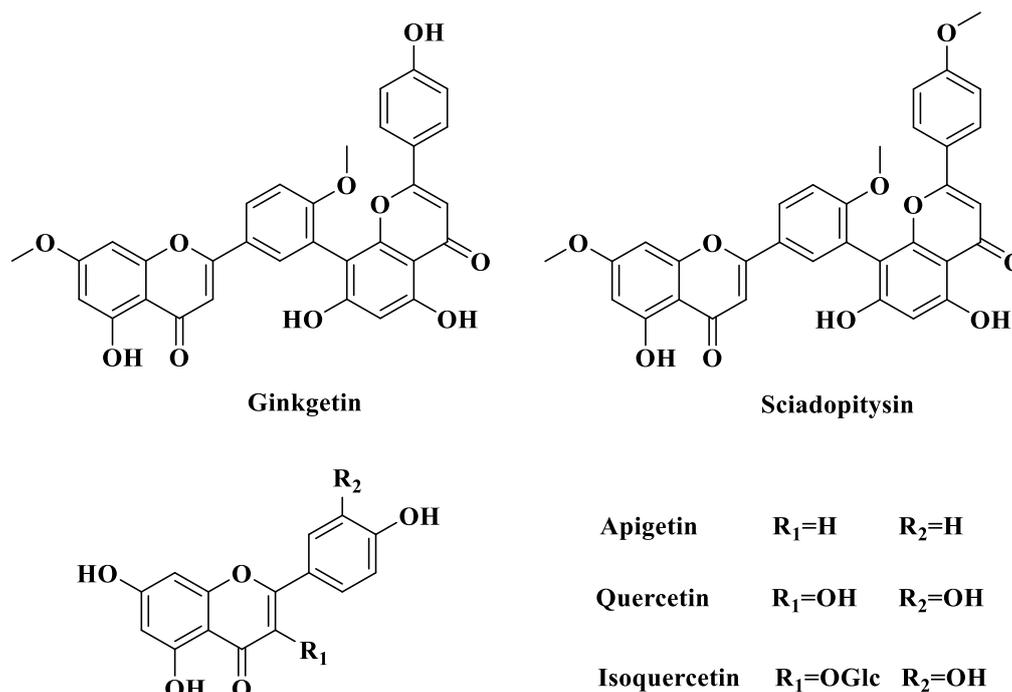


Fig. 8. The structures of five compounds

3.2. Isolation of neutrophils

Human polymorphonuclear neutrophils were isolated from peripheral and fresh blood of healthy human subjects by Ficoll-Hypaque (Flow Laboratories; Irvine, CA, USA) density gradient centrifugation and were washed twice with Krebs-Ringer-phosphate solution free of Ca²⁺ and glucose (free KRP, pH 7.4)[34]. These cells were counted and resuspended in KRP at a concentration of 1×10⁶ cells/mL.

3.3. Assay of superoxide generation

The superoxide generation was assayed by measuring the reduction of cyt.c at 37°C using a dual-beam spectrophotometer (Shimadzu UV-3000; Shimadzu, Kyoto, Japan) under continuous stirring. The standard assay mixture consisted of 1×10⁶ cells/mL neutrophils, 20 μmol/L cyt.c, 0-30 μmol/L biflavonoid compounds and stimulus (12.5 nmol/L fMLP, 10 μmol/L AA or 1 nmol/L PMA) in 2 mL free KRP solution containing 1 mmol/L CaCl₂ and 10 mmol/L glucose (KRP, pH 7.4). After preincubation for 3 min at 37°C, the reaction was started by adding a stimulus and the absorbance change at 550-540 nm (ΔA₅₅₀₋₅₄₀) was monitored for 4 min. The difference in absorbance before and after the incubation was calculated. Quercetin was used as positive control, which is widely used as inhibitor of superoxide generation.

3.4. Detection of tyrosyl or serine/threonine phosphorylation of neutrophil proteins

Human neutrophils (1×10⁶ cells/mL) were preincubated in 1 mL of KRP solution, 0-30 μmol/L flavonoid compounds for 3 min at 37°C. A stimulus (12.5 nmol/L fMLP or 10 μmol/L AA or 1 nmol/L PMA) was added for 4 min at 37°C. Then, 0.5 mL of ice-cold 45% trichloroacetic acid containing 1 mmol/L sodium vanadate and 2 mmol/L phenyl-methylsulfonyl fluoride was added to stop the reaction. The mixture was then incubated for 30 min and centrifuged at 10,000×g for 15 min at 4°C. The precipitate was washed twice with diethylether-ethanol (1:1, v/v), dissolved in 50 μL of 62.5 mmol/L Tris-HCl (pH 6.8) containing 2% sodium dodecyl sulfate (SDS), 0.7 mol/L 2-mercaptoethanol and 10% glycerol.

For immunoblot analysis, samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with a 10% gel. The electrophoresed proteins were transferred onto an Immobilon-P membrane (Nippon Millipore Ltd.; Minatoku Tokyo, Japan) using a semidry blotting apparatus for

90 min at 20V, and the tyrosyl or serine/threonine phosphorylated proteins were detected using specific phosphotyrosine (PY-20; ICN Biochemicals; Irvine, CA, USA) or phosphoserine/threonine (BD Biosciences) monoclonal antibodies, followed by horseradish peroxidase-conjugated rabbit anti-mouse IgG (E.Y. Laboratories Inc.; San Mateo, CA, USA) on an ECL Western blotting Detection System (Amersham; Piscataway, NJ, USA). Protein molecular mass was determined using prestained molecular weight standards (14,300-200,000 molecular weight range; Gibco-BRL; Carlsbad, CA, USA).

3.5. Translocation of p47phox, p67phox and Rac to the neutrophil membrane

In this section, the translocation of the cytosolic components to cell membrane was performed, as described below. Isolated PMNs were preincubated in a phosphate-buffered saline glucose solution containing 4 mmol/L glucose, 1.2 mmol/L MgCl₂, 2 mmol/L NaN₃ and 0-30 μmol/L flavonoid compounds for 3 min at 37°C. After that a stimulus (12.5 nmol/L fMLP, 10 μmol/L AA or 1 nmol/L PMA) was added and incubated for 4 min at 37°C. The mixture was cooled on ice for 20 min and all the following procedures were performed at 4°C. The cells were spun at 1,500×g for 5 min at 4°C and resuspended in buffer A [100 mmol/L KCl, 3 mmol/L NaCl, 3.5 mmol/L MgCl₂, 10 mmol/L PIPES (pH 7.4)] after standing on ice for 20 min. To separate the postnuclear supernatants (PNS), cells were first disrupted by sonication and spun at 500×g for 5 min at 4°C. PNS fractions were then separated into cell membrane and cytosol at 200,000×g for 20 min at 4°C. The pellet was resuspended by 50 μL sample buffer, which composed of 109 mmol/L Tris-HCl (pH 7.4) containing 3.5% SDS, 0.0087% bromophenol blue and 17.4% glycerol and sonicated for 5 min to obtain membrane fractions.

For immunoblot analysis, the membrane fraction was subjected to SDS-PAGE with 10% gel. The electrophoresed proteins were transferred onto an immobilon-P membrane using a semidry blotting apparatus for 90 min at 20V. The transferred proteins were probed with a mixture of p47phox, p67phox and Rac primary monoclonal antibody (BD Biosciences) followed by horseradish peroxidase-conjugated rabbit anti-mouse IgG on an ECL Western blotting Detection System.

3.6. Determination of lipid peroxidation of erythrocyte membrane ghosts by hydroxyl radicals

Fresh human blood was collected, which composed of nine parts blood and one part 3.8% sodium citrate. Then the red blood cells (RBC) were centrifuged and washed three times in PBS buffer[35]. White erythrocyte membrane ghosts were prepared by repeated washing and lysis at 4°C in 5 mmol/L sodium phosphate buffer (pH 8.0). The ghosts were diluted with PBS buffer to get a final concentration of 1 mg/mL. Hydrogen peroxide (3 mmol/L) and FeSO₄ (5 mmol/L) were added to the ghost suspensions (1 mL) with each of the flavonoid compounds (0-30 μmol/L) in five separate experiments. The suspensions were incubated for 30 min at 37°C, and the hydroxyl radical-induced lipid peroxidation of the ghosts was determined by measuring thiobarbituric acid-reactive substances[36].

3.7. Radical-scavenging activity on DPPH

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity assay was carried out[15]. The reaction mixture consisted of 1 mL of 0.1 mol/L acetate buffer (pH 5.5), 1.87 mL of ethanol and 0.1 mL of 3 mmol/L DPPH in ethanol, and 0-30 μmol/L five compounds were mixed together, and then recorded the absorbance of each sample at 517 nm for 20 min. The activity of control with water was expressed as 100%.

3.8. Radical-scavenging activity on superoxide anion generated by phenazine methosulfate (PMS)-NADH system

Then, we measured the radical-scavenging activity on superoxide anion generated by PMS-NADH system by improving the existing methods[37]. After the addition of 0.03 mL 0.5% bovine serum albumin, 0.03 mL 5 mM nitroblue tetrazolium (NBT), 0.03 mL 7.8 mmol/L NADH, 0-30 μmol/L five compounds, 2.82 mL 40 mmol/L sodium carbonate buffer containing 0.1 mmol/L EDTA (pH

10.0), the mixture was incubated at room temperature for 3 min. And then, 3.1 $\mu\text{mol/L}$ PMS was added to the mixture and recorded the difference of absorbance at 560 nm for 2 min. The mixture in the absence of the five compounds was used as a control.

3.9. Hemolysis measurement

Fresh human blood from a healthy human (9 parts of blood: 1 part of 3.8% sodium citrate) was collected in plastic tube, and red blood cells (RBCs) were separated by centrifugation at 2500 rpm for 10 min. The RBCs were washed twice with 2 volume of 0.9% saline solution, and then resuspended in 0.9% saline solution to give a 10% RBCs concentration. Hemolysis effect of biflavonoids was carried out using the RBCs suspension as reported in previous paper[38-40].

4. Conclusions

The results indicated that the two bioflavonoids of ginkgetin and sciadopitysin isolated from the needles of *T. media* were capable of concentration-dependently inhibitory effects on the superoxide generation induced by fMLP, AA and PMA by means of suppressing tyrosyl or serine/threonine phosphorylation and translocation of p47phox, p67phox and Rac to cell membrane in human neutrophils. Particularly, although the inhibitive effects of ginkgetin and sciadopitysin on oxidative phosphorylation were not as good as normal flavonoids in vitro, they should have better effects in vivo through the formulation improvement. In terms of the effects of these compounds on the radical-scavenging, two biflavonoids did not show remarkable effect on DPPH radical-scavenging activity, and they didn't display the radical-scavenging activity on superoxide anion generated by phenazine methoxysulfate (PMS)-NADH system.

Author Contributions: Conceptualization, methodology, data curation, writing-original draft preparation, funding acquisition, C.L.; writing—review and editing, X.N.; supervision, project administration, funding acquisition, J.L. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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