doi.org/10.36721/PJPS.2020.33.1.REG.141-148.1

# Pine (*Pinus sylvestris* L.) bark proanthocyanidins affords prevention of peroxynitrite-induced l-tyrosine nitration, DNA damage and hydroxyl radical formation

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**Abstract**: Peroxynitrite is known as a strong deleterious species that may readily trigger several geriatric diseases via injuring cellular constituents. Proanthocyanidins, a biological flavonoids constituent of *Pinus sylvestris* L. bark, has been attributed a large variety of pharmacological functions to its antioxidant potential. The results revealed that peroxynitrite could cause the generation of hydroxyl radical, the breakage of φX-174 plasmid DNA brand as well as the nitration of L-tyrosine. However, pine (*Pinus sylvestris* L.) bark proanthocyanidins extracts at low concentration range markedly inhibited the peroxynitrite -induced the formation of open circular DNA form ($IC_{50}$ = 5.03±0.39 mg/mL). The 3-Nitro-L-tyrosine generated by the reaction of peroxynitrite with L-tyrosine was reduced by PBP ($IC_{50}$ = 1.01±0.01 mg/mL). Besides, electron spin resonance spectroscopy data indicates that the intensive signal of dimethyl pyridine N-oxide hydroxyl radical adduct from peroxynitrite was reversed by pine bark proanthocyanidins extracts ($IC_{50}$=1.02±0.04 mg/mL). Moreover, the obtained data shows that PBP provides more efficient protection against peroxynitrite than that of ascorbic acid. Together, the present study suggests that pine bark proanthocyanidins could exert potent preventive activity against peroxynitrite -elicited cytotoxicity on the biomacromolecules, a study-worthy finding with pharmacological importance.

**Keywords**: Pine (*Pinus sylvestris* L.), antioxidant, proanthocyanidins, peroxynitrite.

## INTRODUCTION

Peroxynitrite anion ($ONOO^-$) is one of potent nitrating and strong pro-oxidant reactive nitrogen species (RNS). This radical can be generated through the diffusion-limited interaction (pKa=$6.7\times10^9$ $M^{-1}S^{-1}$) *in vivo* between nitric oxide anion ($^{\cdot}NO$) and superoxide anions ($^{\cdot}O_2$) in several activated cell types, for instance endotheliocyte, neurocyte, especially macrophage (Laskin *et al*., 2002). The steady-state production of $ONOO^-$ may be essential in terms of inflammatory reactions against intruding pathogenic microorganisms under physiological conditions. Despite of its non-radical property, $ONOO^-$ is more cytotoxic than its precursors. Hence, the uncontrolled overproduction of $ONOO^-$ plays a pivotal role in the etiopathogenesis of some diseases such as Alhzeimer disease, Parkinson's disease, ischemia reperfusion injury, arteriosclerosis, cancer (Bai *et al*., 2010; Ischiropoulos and Beckman, 2003; Trujillo *et al*., 2008). The negative effects of excess $ONOO^-$ and its derivatives on the most cellular biomolecules have been involved in these undesired effects, for example, antioxidant depletion by reaction with thiol, LDL lipid peroxidation (LPO) and DNA breakage by oxidation, protein modification by nitrification of amino acid residues. Notably, $ONOO^-$ elicited impairment on free/protein-bound tyrosine and DNA molecular are believed to be critical initial events (Ducrocq *et al*., 1999). In view of this, the defense system *in vivo* is undoubtedly responsible for alleviating the sinister $ONOO^-$ -related consequences. Certain small-molecule non-enzymatic antioxidants solely participate in the burden of defense against $ONOO^-$ in physiological condition because inherent enzymes that can neutralize $ONOO^-$ in the human body are generally deficient. Hence, the importance of naturally-occurring candidates specific to inactivating $ONOO^-$ has been highlighted in the fields of nutrition supplements and food additives.

Pine (*Pinus sylvestris* L.) bark (PB), a sub-product from Pine lumber, is traditionally used in China herbal-based medicine. PB has been substantially elucidated to exhibit diverse therapeutic effects in many previous scientific literatures (Bito *et al*., 2000; Shimada *et al*., 2011; Valls *et al*., 2016). PB and its extracts have also been proven to accumulate several bioactive constituents, including mainly proanthocyanidins (PCs, fig. 1) (Selga and Torres, 2005), bioflavonoids (Yesil-Celiktas *et al*., 2009), tannins (Chupin *et al*., 2013) and phenolic acids. Recently, pine bark PCs (PBP) has attracted increasing attention due mostly to its status of strong antioxidant and powerful free radical scavenger (Busserolles *et al*., 2006; Tourino *et al*., 2005). For instance, pycnogenol, a well-known commercial PCs -rich extract from French maritime pine (*Pinus marittima)* bark, has been reported to alleviate oxidative damage by some possible mechanisms, including the inhibition of xanthine oxidase activity (Moini *et al*., 2001), prohibiting the degradation of endogenous antioxidants such as tocopherol (Virgili *et al*.,

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1998) and reduced glutathione (GSH) (Rimbach *et al*., 1999), invalidating reactive oxygen species (ROS) (Guo *et al*., 1999; Packer *et al*., 1999) and nitric oxide synthase (NOS) (Kobuchi *et al*., 1999). However, the comprehensive information is hitherto scant as regards the abilities of PBP to protect tyrosine and DNA from the assault triggered by $ONOO^-$. This research chiefly aims to provide further insight on the influence of PBP on tyrosine nitration and DNA strand scission in order to understand its potential benefits to human health.

## MATERIALS AND METHODS

### *Materials*

PB crude extract was purchased from Xian changyue plant chemical co. LTD. φX-174 RF I plasmid DNA was obtained from Takara Biomedical Technology (Beijing) Co., Ltd. Standard PCs with the purity of over 98% was supplied from National Institutes for Food and Drug Control. All other chemicals from Sigma Chemical Co, including isoamyl nitrite, ascorbic acid, L-tyrosine (L-Tyr), standard 3-nitro tyrosine (3-NT), hydrogen peroxide, were of analytical grade unless otherwise specified.

### *Preparation of PBP*

Crude extract powder of PB was immersed in 70% (v/v) ethanol solution containing 0.1% (m/v) metaphosphoric acid as well as 0.5 (M/V) sodium bisulfite at 80°C for 90 min. The ethanol solution was evaporated by a rotary evaporation below 40°C, and then dried in a vacuum freeze-dryer to obtain crude PBP. The extract was dissolved in 50% ethanol and subjected to a chromatographic column (32×5cm i.d.) which was packed with activated AB-8 macroporous adsorption resin and conditioned by flushing it with 10-fold volumes of water to eliminate hydrosoluble compounds. The PBP-containing fractions were eluted by a gradient of ethanol (40 ~ 70% ~ 90%) on the conditioned column at a flow rate of 3 mL/min. The effluent was collected and concentrated under vacuum in order to obtain PBP samples for further use. Before each analysis, PBP was qualified by the regression line obtained from standard PCs solutions (0.3, 0.6, 1.0, 2.0, 4.0 mg/mL).

### *Synthesis of peroxynitrite*

$ONOO^-$ was synthesized by an isoamyl nitrite/ $H_2O_2$ two-phase system as proposed by Uppu and Pryor (Uppu and Pryor, 1996). $H_2O_2$ (30%, 2.2 mL) and deionized water (50 mL) was mixed and chilled on ice, followed by adding 4 mL NaOH (5M) and 0.5 mL diethylenetriaminepentaacetic acid (DTPA, 0.04 M,). Above 1 mL $H_2O_2$ solution and 2.7 mL isoamyl nitrite (0.04 M) was strongly stirred by vortex for 10 h at 4°C. Thereafter, the organic phase was removed, while the aqueous layer was prewashed three times with two volumes of ice-cold hexane in order to eliminate mainly isoamyl alcohol and isoamyl nitrite, and then removed unreacted $H_2O_2$ by use of activated manganese oxide. $ONOO^-$ solution was filtered with a 0.45 μm fibred resin membrane filter and stored at -80°C. The concentration of $ONOO^-$ was calculated using a molar extinction coefficient at 302 nm ($\varepsilon_{302}$=1670 $M^{-1}\cdot cm^{-1}$) by a UV-VIS spectrophotometer (HITACHI, U-3010, Japan) before every further analysis.

### *Inhibition of PBP on $ONOO^-$ -mediated L-Tyr nitration*

In this study, the formation of 3-NT was regarded as the specific index of $ONOO^-$ -mediated L-Tyr nitration and measured by the described method (Crow, 1999). Each desired concentration dilution of PCs and $ONOO^-$ solution was mixed with L-Tyr (100uM) and diluted with phosphate buffer solution (PBS, 0.2 mM, PH 7.4) to 1 mL final volume. The final concentrations of $ONOO^-$ and PCs in reaction mixture were 1 mM and 0.068 – 0.134 mM, respectively. Phosphate (0.2M) was used to maintain the pH during the reaction. Sample solution without the antioxidants was used as the controls. 3-NT was measured using a Waters high-performance liquid chromatography (HPLC) system with a UV –detector and a ODS reversed-phase (RP) HPLC column (5μm, 150 × 4.6 mm, SunFire RM, China). The program of HPLC analysis for 3-NT detection was: PBS (PH 7.4) /methanol (v/v=90:10); flow rate, 1 mL /min for 30 min; UV detection, 420 nm; column temperature, 20°C. 3-NT quantification was conducted in accordance with peak area using a linear calibration curve obtained from standard 3-NT solutions in the concentrations of 10, 100, 200, 500, 1000 μM. The effect of ascorbic acid on 3-NT formation was evaluated under the same experiment conditions. The inhibition ratio was calculated according to the followed equation:

$$\text{Inhibition ration} = (1-I_1/I_0) \times 100\% \quad (1)$$

Where, $I_1$ and $I_0$ are the concentrations of 3 -NT in the presence of antioxidant and control, respectively.

### *DNA strand cleavage assay*

In this study, the ability of PBP extracts to protect the supercoiled (SC) φX-174 plasmid DNA from the assault of $ONOO^-$-generated radicals was evaluated with the DNA nicking assay according to the previous reports (Jia *et al*. 2010). In brief, 200 μl of φX-174 plasmid DNA solution (1.7 μg/mL) was separately incubated with concentrations of PBP (0, 1, 5, 10, 15 μM), and then treated with pre–cooling $ONOO^-$ of 100 μM final concentration at 37 °C away from light for 30 min in PBS (pH 7.4) at a final volume of 50 μL. After incubation, 10 μL of each sample was loaded into a 1% agarose gel (40 mM Tris, 20 mM sodium acetate, 2 mM EDTA), and then electrophoresed in TAE (Tris/acetate/EDTA) buffer under 5 V/cm voltage for 38 min. Following staining with 0.5 μg/ mL ethidium bromide, each gel sample were subsequently photographed under UV illumination by a gel imager (Chemilmager TM 4400), and then quantified as the percentage of OC form by Image J software (version 1.82).

***Electron spin resonance (ESR) assay***

Spin trap ESR method (Qi *et al*., 2016) was used to measure ·OH scavenging ability of PBP. For ESR measurement, approximate 100µl analytical solution sample contained the following reagents: DMPO solution (20 µM), $ONOO^-$ (100 µM) and antioxidant (PBP or ascorbic acid, 0 – 2.5 mg/mL). The control solution also contained the equivalent amounts of reagents except antioxidant. The concentrations of all above reagents are final concentrations. At room temperature, 50 µl of sample was immediately placed into a ESR cuvette for measurement with a Bruker A-300 X-band spectrometer (Bruker, Karisruhe, Germany). The Instrument settings were: modulation frequency, 100 KHz; X band frequency, 9.856 GHz; microwave power, 19.32 mW; modulation amplitude, 10.0G; central magnetic field, 3506G; sweep time, 180 s; receiver gain, $4\times10^5$. The scavenging effect against $ONOO^-$ -induced ·OH was evaluated through the ESR signal inhibition percentage calculated by the following equation（2）:

$$\text{Inhibition (\%)}=[(H_{control} - H_{sample})/ \text{Hcontrol}]\times100\% \quad (2)$$

Where, $H_{control}$ and $H_{sample}$ are the signals of the 2nd peak in the ESR spectrum for the control solution and analytical solution, respectively.

## DATA ANALYSIS

Three independent experiments were carried out in triplicate for every parameter. All values are presented as mean ± standard deviation (SD). The statistically significance between groups were evaluated using ANOVA with the statistical software SPSS 19.0 (SPSS Inc., Chicago, IL), followed by Duncan's test for multiple comparisons. $p<0.05$ were considered the significant level.

## RESULTS

***Inhibition effects of PBP against $ONOO^-$ -induced tyrosine nitration***

Fig. 2A: (a)(b)(c) shows the absorption spectrum of PBP, the product of the reaction of L-Try with ONOO as well as L-tyrosine, respectively. According to HPLC, the reaction product of 100 µM L-Tyr with 100 µM $ONOO^-$ have the similar retention time at 420 nm with standard 3-NT (fig. 2B), indicating 3-NT formation by the addition of $ONOO^-$ to L-Tyr. fig. 2C shows that the presence of various doses of PBP (0.5 – 2.5 µg/mL) significantly ($p<0.05$) attenuated the 3-NT generation in a dose-dependent manner. Moreover, The $IC_{50}$ (1.01±0.01 mg/mL) of inhibition ratio of PBP on 3-NT formation was statistically ($p<0.05$) lower than that (1.43±0.02 mg/mL) of potent $ONOO^-$ scavenger control, i.e. ascorbic acid.

***Inhibition of PBP on $ONOO^-$ -mediated DNA strand breakage***

Fig. 3: A and C shows that both PBP and ascorbic acid at the range of 1-15 mg/mL could inhibited the formation of OC form of φX174 DNA. As shown in fig. 3B, the percentage of OC form was 68.2 ± 1.5, 59.6 ± 1.6, 43.6 ± 1.6 and 25.1 ± 1.4 % in presence of PBP at 1, 5, 10 and 15 mg/mL, respectively, and 65.5 ± 1.6, 61.7 ± 0.6, 58.6 ± 1.0 and 53.3 ± 2.6 % in the presence of ascorbic acid at the corresponding concentrations. The significant ($p<0.05$) reduction of OC form percentage was induced by 10, 15 mg/mL PBP as compared with ascorbic acid. The $IC_{50}$ (5.03±0.39 mg/mL) of PBP was lower ($p<0.01$) than that (58.37±8.19) mg/mL of ascorbic acid.

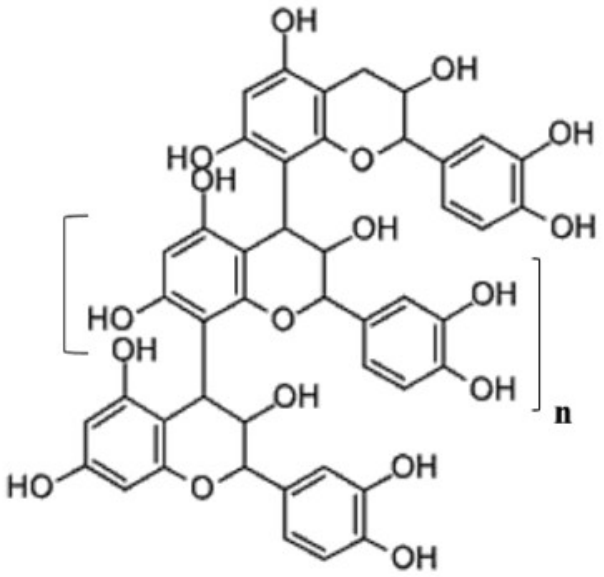


**Fig. 1**: Structure of procyanidins

***Inhibition of PBP on $ONOO^-$ -mediated hydroxyl radicals***

The well characteristic DMPO-OH ESR spectrums depicted as a group of peaks with 1:2:2:1 quartet were available. The 2nd peak amplitude in these spectrums was employed to quantify ·OH production from $ONOO^-$. As shown in fig. 4A line-a, no detectable signal intensity of the 2nd peak indicates few spin adducts in the non $ONOO^-$ reaction system. Whereas, fig. 4A line-b shows the maximum intensity of the 2nd peak, which was apparently associated with the abundance of DMPO-OH adduct in the condition of $ONOO^-$ exposure. The incubation of PBP (1-15 mg/mL) with 100 µM $ONOO^-$ for 10 min resulted in the concentration-dependent reduction in the intensity of DMPO-OH adduct ESR peak. In addition, the levels of the inhibition ratio were interestingly lower ($p<0.05$) in all concentrations of PBP samples compared to ascorbic acid. Moreover, The $IC_{50}$ (1.02±0.04 mg/mL) of PBP was lower ($p < 0.01$) than that (1.45±0.09) mg/mL of ascorbic acid (fig. 4B).

## DISCUSSION

Free or protein-bound tyrosine residues are reported to be especially susceptible to the nonselective attack of

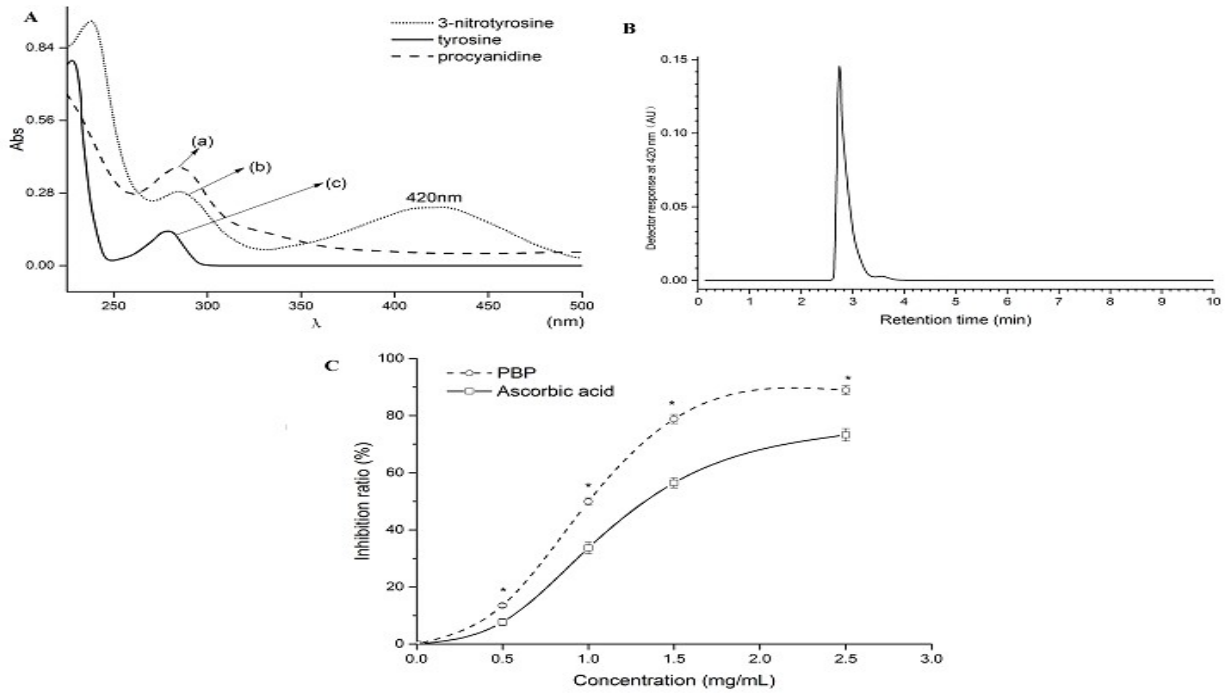


**Fig. 2**: A. Steady-state absorption spectra of (a) 5mg/mL procyanidine at pH7.0 and (b) the product of the reaction of 100 µM L –tyrosine (L-Try) with 100 µM $ONOO^-$ at pH7.0. The absorption spectrum of blank L -tyrosine is shown for comparison (c). Figure 2B. Representive HPLC chromatogram of 3-NT, HPLC conditions: ODS (SunFire RM, 5µm, 4.6x150mm), PBS (PH 7.4) /methanol (90:10), flow rate:1ml/min, colum temperature, 20 °C, UV detection, 420nm. Figure 2C. Effect of PBP or ascorbic acid on $ONOO^-$ dependent generation of 3-NT. L-Try (100µM) was incubated with $ONOO^-$ in the absence or in the presence of parallel concentrations of PBP and ascorbic acid. Relative inhibition (%) of 3-NT was calculated with reference to the control value. The results represent % of control value ± standard deviation (SD), n=3. * represents statistically different between PBP and ascorbic acid at same concentration ($p<0.05$).

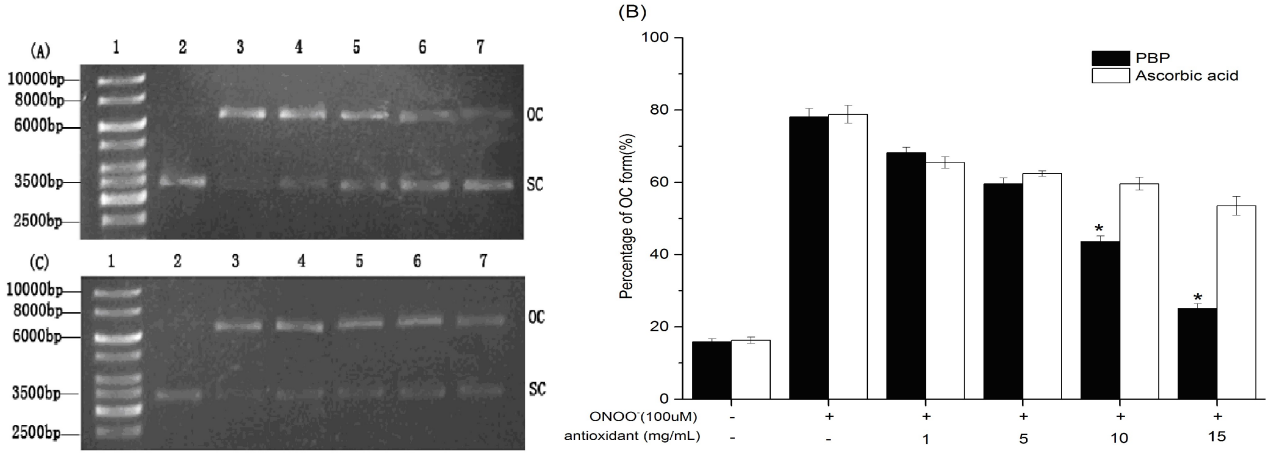


**Fig. 3**: Concentration – dependent effect of PBP and ascorbic acid on the open circular (OC) form of DNA strand damage by $ONOO^-$ in the φX-174 plasmid DNA. (A). The Agarose gel electrophoretic picture of φX174 DNA (0.34 mg) reacted with 100 µM $ONOO^-$ in the presence of various indicated concentrations of PBP (1, 5, 10, and 15 mg/mL for lane 4-7, respectively). (C) The Agarose gel electrophoretic picture of φX174 DNA (0.34 mg) reacted with 100 µM $ONOO^-$ in the presence of various indicated concentrations of ascorbic acid (1, 5, 10, and 15 mg/mL for lane 4-7, respectively). (B) Percentage of OC form of φX-174 DNA in the treatment of PBP and ascorbic acid. Bars with * for each treatment are significantly different ($p<0.05$), n =3.

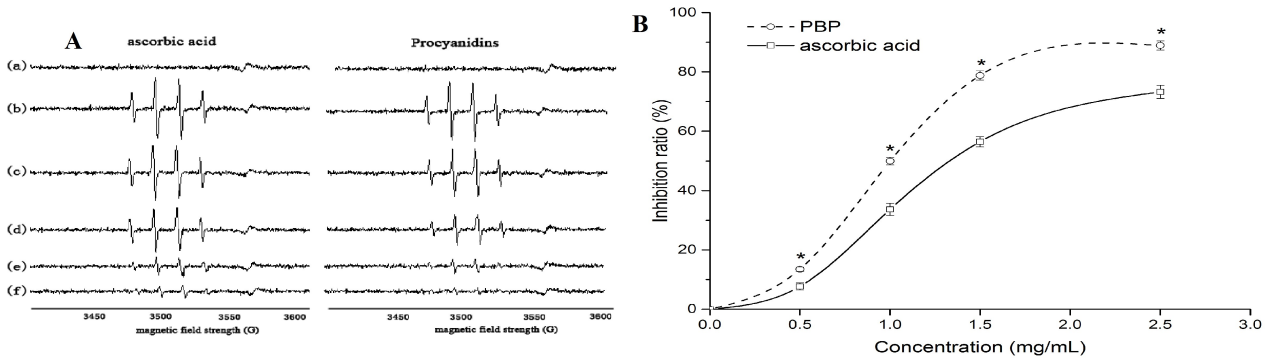


**Fig. 4**: Inhibition Effect of PBP on hydroxyl radical (·OH) formation induced by $ONOO^-$. ESR spectroscopy was utilized to measure the $OH^·$ scavenging ability of PBP. (A) Line a, without $ONOO^-$ and PBP; line b, 20 μM DMPO + 100 μM $ONOO^-$; line c, 0.5 mg/mL antioxidant + 20 μM DMPO + 100 μM ONOO- ; line d, 1.0 mg/mL antioxidant + 20μM DMPO + 100 μM $ONOO^-$ ; line e, 1.5mg/mL antioxidant + 20μM DMPO + 100 μM $ONOO^-$ ; line f, 2.0mg/mL antioxidant + 20μM DMPO + 100 μM $ONOO^-$ ; (B) Signal intensity at 3506 G was represented as mean ± standard deviation (SD). * represents statistically different between PBP and ascorbic acid at corresponding concentration ($p<0.05$), n =3.

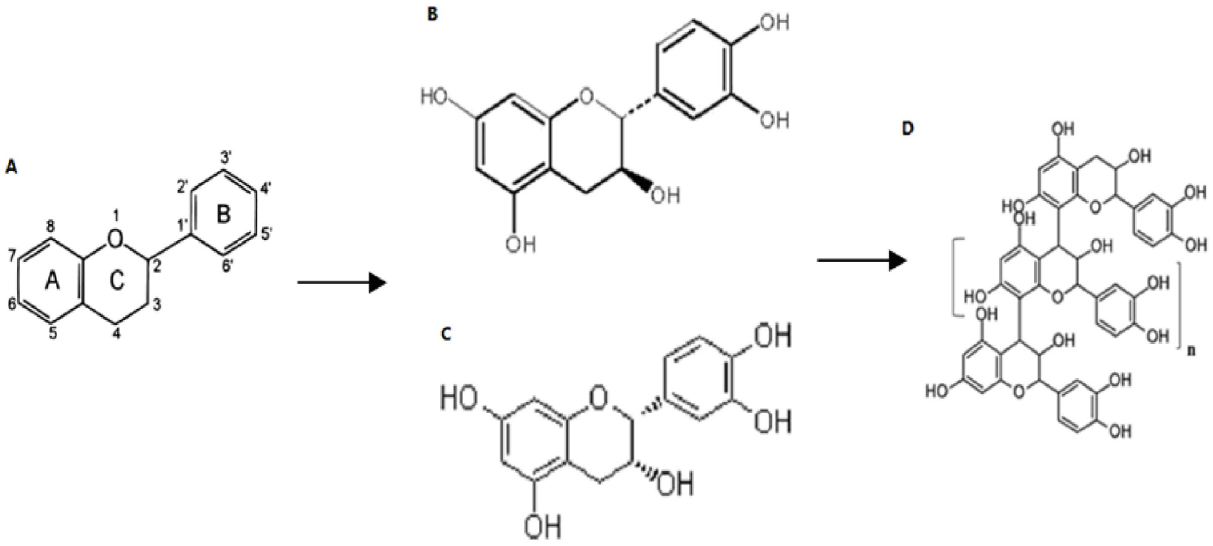


**Fig. 5**: The structure of basic flavonoid(A), (+)-catechi (B), (−)-epicatechin (C) and procyanidins(D)

$ONOO^-$, generating a stable product, namely 3-NT, which is widely used as a suitable biomarker of protein nitrification due to its convenience for analysis (Teixeira *et al*., 2016). The nitration of protein-bound tyrosine may disturb tyrosine phosphorylation/dephosphorylation dependent signaling pathways, which are associated with certain cellular physiology such as dopamine synthesis of neuron, proliferation and apoptosis (Schlessinger, 2000). Although several reports have also evaluated PBP detoxification activity against biologically relevant RNS (Tsuda *et al*., 2000), to the best of our knowledge, the effect of PBP on $ONOO^-$ -induced toxicity on cellular biomacromolecules remains unrevealed up to now. In the present research, as an *in vitro* model target, L-Tyr reacted with $ONOO^-$ to produce 3-NT, and HPLC coupled to ultraviolet–visible detection was used to measure 3-NT generation. Our results revealed that the addition of PBP (0.5–2.5 ug/ mL) to $ONOO^-$ dose-dependently suppressed the 3-NT production. Moreover, the inhibition ability of PBP showed stronger than that of ascorbic acid. The results demonstrated that PBP was accurately evaluated as regards its protective ability against $ONOO^-$ -mediated L-Tyr nitration. The possible mechanisms of PBP preventing against 3-NT include the action with $ONOO^-$ and the suppression of $ONOO^-$ availability.

$ONOO^-$ and its decomposers can stimulate poly (ADP-ribose) polymerase and eventually lead to apoptosis through gene mutation and DNA strand breakage (Szabo, 2003). In view of the vulnerability of φX-174 plasmid DNA to $ONOO^-$-mediated assault, the marked conversion of supercoiled (SC) φX-174 DNA plasmid DNA to relaxed open circular (OC) DNA is triggered by $ONOO^-$ (Graham *et al*., 2013). In this study, the incubation of 100 μM $ONOO^-$ with 34 μg φX-174 plasmid DNA led to the noteworthy breaks of the DNA brand, as evidenced by a

statistical increased percentage of OC DNA compared with the control. These observations confirm that the exposure of $ONOO^-$ could elicit DNA brands breaks as previous observations (Cao and Li, 2004; Chen *et al*, 2013). However, this tendency was reversed by low concentrations (1-15μg/mL) of PBP/ascorbic acid in a dose-dependent fashion, indicating that PBP certainly protects against DNA strand damage induced by $ONOO^-$. In addition, the results partially suggest that PBP would be more efficient in blocking $ONOO^-$ -mediated DNA fragmentation when compared with ascorbic acid.

The hemolysis of protonated peroxynitrous acid (ONOOH, pKa=6.8) can produce $^{\cdot}OH$, which is normally detected by ESR method based on principal spin-trapping compounds such as PBN, POBN, and DMPO (Szabo *et al*., 2007). In the current research, the possibility of PBP inhibiting $ONOO^-$-induced $^{\cdot}OH$ was assessed by ESR spectroscopy. $ONOO^-$-induced short-lived $^{\cdot}OH$ was trapped by DMPO to form a stable spin-trapped adduct (DMPO-OH) with half-life time of over 2h for analysis. $ONOO^-$ exposure evoked the abundance of DMPO-OH adduct, which undoubtedly sheds more light on the privatization of $ONOO^-$ to $^{\cdot}OH$ as previously reported (Frejaville *et al*., 1995; Vandjelovic *et al*., 2012). Whereas, PBP led to the significant reduction of DMPO-OH adduct ESR peak, which suggests the removal of $ONOO^-$ or its derived free radicals. Moreover, the potential of PBP on eliminating $ONOO^-$ and/or its decomposition might be higher than that of ascorbic acid. This argument was further supported by the evidence that PBP remitted $ONOO^-$ -mediated generation of DMPO-OH adduct as $IC_{50}$ value of 1.02±0.04 mg/mL, which was lower ( $p<0.05$) than that (1.45±0.09 mg/mL) of ascorbic acid. In addition to the direct removal effect, the inhibition on $^{\cdot}OH$ formation may be the probable protection mechanism of PBP against $ONOO^-$ -related destruction on biological macromolecules, which needs a further study.

Flavonoids are hydrophobic aromatic compounds and generally consist of flavan nucleus with three rings ($C_6$-$C_3$-$C_6$), which are labeled A, B and C (fig. 5A). The diversity of flavonoids mainly depends on the patterns of the ring substitution. The antioxidant capability of flavonoids is considerably determined by the different backbone structures and functional groups. More specifically, the major functional groups for radical-scavenging activity among flavonoids are (Ⅰ) a catechol structure in the ring-B, which can efficiently donate electron and is a target of various radicals (Dugas, Jr, *et al*., 2000), and (Ⅱ) the additional presence of a 3-OH group in the C-ring, which can enhance radical-scavenging activity through accomplishing the structural planarity of flavonoids (Pietta, 2000). Thus, flavonoids containing a catechol group in ring B and the 3-OH functionality in the C-ring are more active, for example (+)-catechin (fig. 5B) and (−)-epicatechin (fig. 5C). Proanthocyanidins is the typical class flavonoids in *Pinus* barks and largely consists of (+)-catechin and (−)-epicatechin units with a polymerization degree of up to heptamer (fig. 5D). Hence, the above-mentioned structure-activity that regulates the antioxidant potential of flavonoids would contribute to our understanding the role of PBP structure to inhibit $ONOO^-$.

## CONCLUSION

Taken together, the present work, firstly, demonstrates that PBP can efficiently alleviate $ONOO^-$-induced lesion on biological macromolecules. $ONOO^-$ is regarded as the critical pathogenesis of various forms of aging and chronic disorders. Furthermore, the inducement of biomolecular damage by $ONOO^-$ is considered to be a crucial for $ONOO^-$-regulated dysfunction. Accordingly, the potential of PBP to suppress $ONOO^-$ -elicited biological organic compounds damages, including tyrosine nitration, DNA damage and $^{\cdot}OH$ generation, as observed in this work, might make contribution to the understanding of meaning for PBP-mediated protection against $ONOO^-$.

## ACKNOWLEDGMENTS

This work was financially supported by the National Science-technology Support Plan Projects of China (No.2015BAK44B00) and the National Natural Science Foundation of China (No.20875006). The author wishes to acknowledge Beijing Key Laboratory of Environmental and Viral Oncology (College of Life Science and Bioengineering, Beijing University of Technology, Beijing 100124, P. R. China) for technical support.

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