

Original Research Article

Inhibition of Inducible Nitric Oxide Synthase, Cyclooxygenase-2 and Lipid Peroxidation by Methanol Extract of *Pericarpium Zanthoxyli*

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Abstract

Purpose: To explore the antioxidant properties of the methanol extract of *Pericarpium Zanthoxyli* and its effect on inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and lipopolysaccharides (LPS)-induced cell damage in macrophage cells.

Methods: Anti-oxidant activities were tested by measuring free radical scavenging activity (DPPH, NO) and lipid peroxidation levels. The mechanism of anti-oxidant action of *Pericarpium Zanthoxyli* extract was determined by Western blot analysis for iNOS and COX-2 expression in LPS-stimulated RAW 264.7 cells.

Results: *Pericarpium Zanthoxyli* extract contained anti-oxidant components including phenolics (2.456 mg/g), flavonoids (0.127 mg/g) and anthocyanins (20.34 mg/g). The extract exerted significant radical scavenging activity in a dose-dependent manner. It also inhibited lipid peroxidation and exerted dramatic reducing power (28.9-fold compared with control at a concentration of 1 mg/ml). Production of iNOS induced by LPS was significantly ($p < 0.05$) inhibited by the extract, suggesting that the extract inhibits nitric oxide (NO) production by suppressing iNOS expression. Strikingly, COX-2 induced by LPS was also significantly ($p < 0.05$) inhibited by the extract.

Conclusion: These results suggest that the methanol extract of *Pericarpium Zanthoxyli* exerts significant anti-oxidant activity via inhibiting free radicals, iNOS and lipid peroxidation as well as by inhibition of COX-2 enzyme.

Keywords: *Pericarpium Zanthoxyli*, Nitric oxide, iNOS, COX-2, Lipid peroxidation, Antioxidant

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INTRODUCTION

Pericarpium zanthoxyli refers to the rind of dried fruits of *Zanthoxylum bungeanum* Maxim., *Zanthoxylum schinifolium* Sieb. et Zucc., and *Zanthoxylum piperitum* A. P. DC, which are distributed in Korea and China. It contains a wide variety of components such as (+)-gamma-cadinene, (+)-beta-pinene, (-)-aromadendrene, (-)-isopulegol, (-)-N-acetylanonaine (R-type), hydroxy- α -sanshool, hydroxy- β -sanshool, hydro-

xyl- γ -sanshool, linalool, nerol, piperitone, skimmianine, terpinen-4-ol, zanthoxylin, zanthobungeanine, α -pinene, (+,-)- α -sanshool, α -terpineol, α -thujene, β -sanshool, β -sitosterol, γ -sanshool, trans-ocimene, etc. It has been widely used for cold perspiration in spleen and stomach, cold and painful stomach, diarrhea, chilly waist and knee, indigestion, acute and chronic gastritis, dysentery, toothache, etc, and generally known for antiparasitic and antibacterial effect [1-3]. Recently, it has been reported that

Pericarpium zanthoxyli has insecticidal activity [4].

Oxidative stress is caused by imbalance between free radical productions and their scavenging anti-oxidative defense system in the body, leading to damage to cellular components such as lipid, protein and DNA [5]. Most free radicals and their reactive metabolites, such as reactive oxygen species (ROS), are generated by the mitochondrial respiratory chain during cellular metabolism, playing essential roles in cellular signaling pathways. The mitochondrial respiratory chain can also produce nitric oxide (NO) and thus reactive nitrogen species (RNS) [6]. ROS and RNS induce excessive lipid peroxidation, producing other reactive species including malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) [7].

Antioxidants play crucial roles in preventing lipid peroxidation and removing free radicals. Evidence is mounting that a number of antioxidants such as phenolic acid and flavonoids can reduce the risk of tumors and heart diseases and they are helpful in scavenging ROS which involved in the onsets of those diseases [8]. Inducible nitric oxide synthase (iNOS), is a key enzyme responsible for the production of NO and it plays an important role in the oxidative stress and inflammation [8]. There are numerous reports that cyclooxygenase-2 (COX-2) is implicated in inflammation via production of various prostaglandins [9].

To explore the anti-oxidant activity of *Pericarpium zanthoxyli*, the total phenolics, flavonoids and anthocyanin of the methanol extract of *Pericarpium zanthoxyli* and their *in vitro* radical scavenging ability were investigated in this study. Furthermore, their effects on the expression of NO, iNOS and COX-2 in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells were evaluated to understand its mechanism of pharmacological actions.

EXPERIMENTAL

Preparation of plant extracts

Dried aerial parts of *Zanthoxylum bungeanum* Maxim collected in autumn were purchased from Kyung-Dong Oriental Market, Seoul, Republic of Korea. They were authenticated by Professor Emeritus Chang-SooYok, Department of Oriental Pharmacy, Kyung Hee University, Seoul, Republic of Korea. A quantity (100 g) was cut into small pieces and extracted three times with 70 % methanol (300 ml) for 3 h via sonication.

The resulting methanol extract was concentrated in a rotary evaporator and freeze-dried (FD5510 Freeze Dryer, Ilshin Lab., Republic of Korea).

Measurement of total phenolics

The total phenolic content (TPC) was determined using the Folin-Ciocalteu reagent with gallic acid as a standard [10]. Basically, 0.5 ml test sample was mixed with 0.5 ml Folin-Ciocalteu reagent and 1.5 ml 10 % sodium carbonate solution. After the mixture was reacted for 10 min at 75 °C, the absorbance was read spectrophotometrically (Ultrospec 2000, Pharmacia Biotech, England) at 760 nm. The results were expressed in milligram gallic acid equivalents per gram of dried sample.

Measurement of total flavonoids

Total flavonoids were measured using the method of Miliauskasa *et al* [11]. In brief, 200 µl test sample was mixed with 200 µl aluminum chloride in ethanol (20 mg/ml) and the volume was made up to 5 ml with 95 % ethanol. Following incubation for 40 min at 20 °C, the absorbance of the reaction mixtures was measured spectrophotometrically (Ultrospec 2000, Pharmacia Biotech, England) at a wavelength of 415 nm. Total flavonoid content of the extract was expressed in milligram rutin equivalents per gram of dried sample.

Measurement of total anthocyanins

The total anthocyanin was measured using a colorimetric assay [12]. Essentially, the extract (1.0 mg) was dissolved in acetate buffer (25 mM, pH 4.5, 1.0 ml). Sample absorbance was determined spectrophotometrically (Ultrospec 2000, Pharmacia Biotech, England) at 520 nm against de-ionized water. Anthocyanin content was expressed as milligrams of kouroumanin (cyanidin-3-O-glucoside; KO) equivalents per gram of dried sample.

Measurement of essential oil

Essential oil contained in the *Pericarpium zanthoxyli* extract was determined as described in Korean Pharmacopeia [13]. Briefly, the plant extract (5 g) was dissolved in 50 ml distilled water and subjected to reflux for 5 h; essential oil layer over xylene was measured.

Measurement of total saponin

The content of total saponin in the *Pericarpium zanthoxyli* extract was measured as described by Shibata *et al* [14]. The *Pericarpium zanthoxyli* extract dissolved in water was extracted with

ether, and the ether soluble layer was discarded. The resulting water layer was further extracted with saturated butanol. The butanol soluble fraction was collected, evaporated and the residue taken as saponin fraction.

DPPH radical scavenging activity

Briefly, 1.0 ml of aliquot of extract or control was mixed with 1.0 ml of methanol DPPH (0.1 mM) solution at 23 °C [15]. After a 30-min reaction, the absorbance was recorded spectrophotometrically (Ultrospec 2000, Pharmacia Biotech, England) at 515 nm. All the experiments were performed in triplicate.

Evaluation of nitric oxide (NO) radical scavenging activity

NO generated from sodium nitroprusside (SNP) was measured using Griess reagent method [16] whereby 0.5 ml of test sample was added to 0.2 ml of SNP (10 mM) and 1.8 ml of phosphate buffer (pH 7.4). The reaction mixture was allowed to incubate at 37 °C for 3 h. Thereafter, 1.0 ml of the reaction mixture containing nitrite was pipette and mixed with 1.0 ml of Griess reagent and allowed to stand for 30 min in diffused light. The absorbance of the pink colored chromospheres was measured spectrophotometrically (Ultrospec 2000, Pharmacia Biotech, England) at 540 nm against the corresponding blank solution.

Determination of lipid peroxidation (TBARS measurement)

The final products of polyunsaturated fatty acid oxidation reaction will react with thiobarbituric acid (TBA) to form red floaters. Thiobarbituric acid reactive substances (TBARS) were measured by Sroka's method [17]. TBA reagent was prepared as follows. Reagent A: 375 mg of thiobarbituric acid (Fluka Chemicals) and 30 mg of tannic acid were melted in 30 ml of hot water. Reagent B: 15 g of thiobarbituric acid was melted into 70 ml of 0.3 M hydrogen chloride solution. Thereafter, 30 ml of reagent A and 70 ml of reagent B were mixed up. Rapeseed oil (5.2 µl) was suspended in 2 ml of 0.2 M Tris-HCl buffer (pH 7.4) containing 15.6 mg of Tween-40. The resulting suspension was irradiated with UV light for 60 min at 25 °C and then 100 µl of *Pericarpium Zanthoxyli* extract mixed with 1 ml of the suspension. This mixture was irradiated with UV light for 30 min, 2 ml of TBA reagent placed in it, the mixture allowed to react for 15 min over boiling water, and centrifuged for 3 min at 100 x g. The supernatant was taken and its absorbance measured at 532 nm. Suppression of lipid and oxidation reactions was measured as

percentage, compared to the control group not containing *Pericarpium zanthoxyli* extracts.

Assessment of reducing power

The reducing power of the extract was measured according to the method of Oyaizu [18]. Test samples in phosphate buffer (2.5 ml, 0.2 M, pH 6.6) was added to postassium ferricyanide (2.5 ml, 10 mg/ml) solution and the mixture was incubated at 15 min at 30 °C. Trichloroacetic acid (TCA, 2.5 ml, 100 mg/ml) was added to the mixture, and 2.5 ml of the mixture was mixed with 2.5 ml of distilled water and 0.5 ml of ferric chloride (1.0 mg/ml). Absorbance was read spectrophotometrically (Ultrospec 2000, Pharmacia Biotech, England) at 700 nm.

Cell culture and measurement of nitric oxide

The murine macrophage, RAW 264.7 (ATCC[®]TIB-71) cells, were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified essential media (DMEM; Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated foetal bovine serum (FBS; Gibco, Invitrogen, Carlsbad, CA, USA), streptomycin (100 µg/ml) and penicillin (100 U/ml) at 37 °C in a 5% CO₂ atmosphere. Cells were seeded at a density of 2 × 10⁶ onto each well in a 6-well plate for 24 h before drug treatment. The cells were incubated with test sample at different concentrations (1.0, 0.1 and 0.01 mg/ml). Four hours later, the cells were stimulated with lipopolysaccharides (LPS, 1 µg/ml; Sigma Chemical Co., St. Louis, MO, USA) except for the control group for 20 h. The concentration of NO in culture supernatants was determined as nitrite, a major stable product of NO, by the Griess reagent assay. The absorbance of the pink colored chromospheres was measured spectrophotometrically (Ultrospec 2000, Pharmacia Biotech, England) at 540 nm against the corresponding blank solution and results were expressed as mM nitric oxide.

Western blot analysis

Cells were washed twice with 1x PBS, scraped off, and centrifuged at 5,000 rpm for 5 min at 4 °C. Cell pellets were resuspended in an appropriate volume of lysis buffer containing 10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM EDTA, 30 mM NaF, 0.1 mM Na₃VO₄, 1 % triton X-100, 0.5 % NP-40, 1 µg/ml leupeptin, 1 µg/ml aprotinin and incubated for 30 min on ice. Lysates were then centrifuged at 12,000 rpm for 10 min at 4°C and the supernatants were used as the total cell lysates. The protein concentration of each

sample was determined by the Bradford assay (Bio-Rad, CA, USA) [19]. 50 µg of protein were separated on 10 % sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA) as described by Towbin et al [20]. Each membrane was incubated for 1 h with 5 % skimmed milk in 0.1 M Tris-buffered saline-0.1 % Tween 20 (TBS-T) buffer (pH 7.4) to block non-specific binding and then incubated with primary antibodies that recognized iNOS (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), COX-2 (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and GAPDH (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Each protein was detected by using a chemiluminescence detection system according to the manufacturer's protocol (ECL, Amersham, UK). The band intensity was quantified by NIH ImageJ densitometric analysis.

Statistical analysis

Data analysis was carried out using Graphpad Prism 5.0 software, and the data are expressed as mean ± standard error of mean (SEM). The significance level of treatment effects was determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis; *p*-values less than 0.05 were considered statistically significant.

RESULTS

Phytochemical yield

Amount of total phenolic content in the *Pericarpium zanthoxyli* was 2.456 mg/g while the yield of total flavonoids and total anthocyanins was 0.127 and 20.34 mg/g, respectively. In addition, the extract contained 13.7 and 8.0 % of total saponin and essential oil, respectively.

Antioxidant activity

Fig 1A shows the dose-response results of the DPPH radical scavenging activities of the *Pericarpium Zanthoxyli*. DPPH radical scavenging ability of the extract at 0.01, 0.1 and 1.0 mg/ml was 46, 89 and 64 %, respectively, compared to control. In comparison, vitamin C, used as standard, decreased DPPH radicals by 23, 25 and 34 % at concentrations of 0.01 mg/ml, 0.1 mg/ml and 1.0 mg/ml, respectively. These results suggest that *Pericarpium Zanthoxyli* possesses hydrogen-donating abilities to act as an antioxidant. Fig 1B depicts that at 0.01, 0.1 and 1.0 mg/ml, the *Pericarpium Zanthoxyli* extract scavenged nitric oxide radicals by 28, 35

and 45 %, respectively, compared to control. Vitamin C at concentrations of 0.01, 0.1 and 1 mg/ml exerted 22, 26 and 52 % decrease of nitric oxide radicals, respectively.

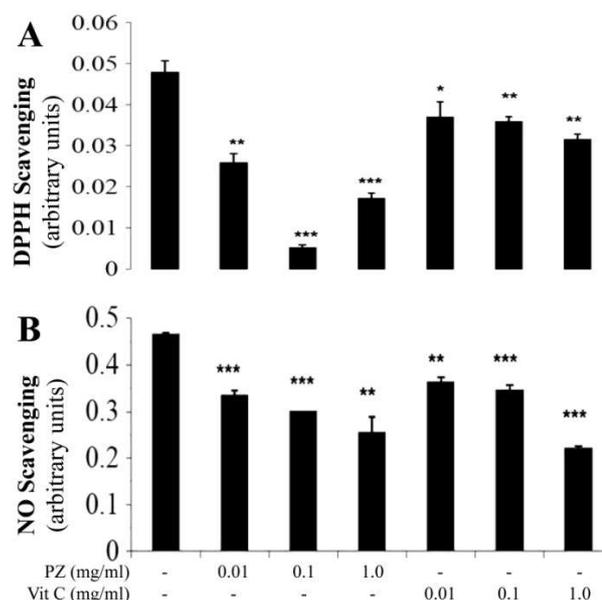


Fig 1: DPPH and NO scavenging activity by the *Pericarpium zanthoxyli* extract. Data are mean ± S.E.M (n = 3); **p* < 0.05 and ***p* < 0.01; ****p* < 0.001

Fig 2 shows the antioxidant data for the based on reducing power. The results indicate that the *Pericarpium Zanthoxyli* extract had reducing power of 2.0-, 6.1- and 28.9-fold at 0.01, 0.1 and 1 mg/ml, respectively, compared to control; for the reference standard, vitamin C, the corresponding values were 1.8-, 2.6- and 4.0-fold, respectively.

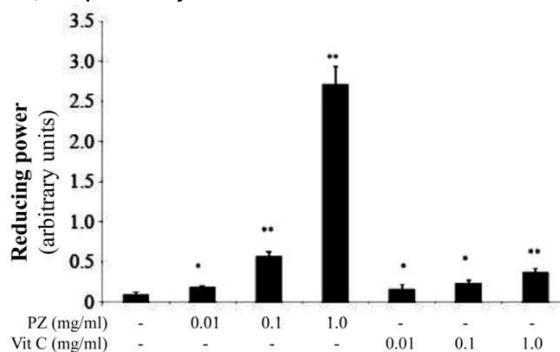


Fig 2: Reducing power by the *Pericarpium zanthoxyli* extract. Data are mean ± S.E.M (n = 3); **p* < 0.05 and ***p* < 0.01

The TBARS production was significantly suppressed by the *Pericarpium zanthoxyli* extract by 33, 34 and 35 % at concentrations of 0.01, 0.1 and 1 mg/ml, respectively, and was largely equivalent to Vitamin C in activity (Fig 3).

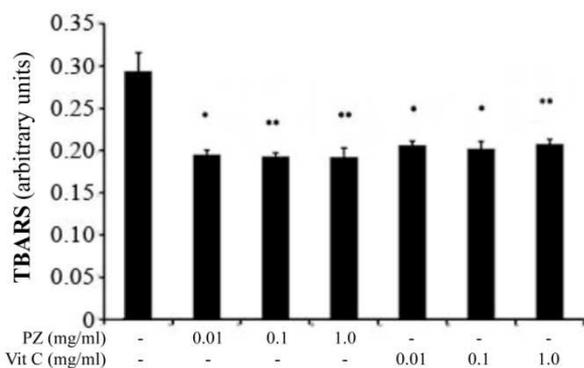


Fig 3: TBARS inhibition by the *Pericarpium zanthoxyli*. Data are mean ± S.E.M (n = 3); *p < 0.05 and **p < 0.01

Fig 4 shows that treatment with LPS induced a high release of NO to the culture medium but pretreatment with *Pericarpium zanthoxyli* extract resulted in inhibition of NO production by 41, 95 and 99 % at concentrations of 0.5, 0.1 and 1.0 mg/ml, compared to LPS-induced sample.

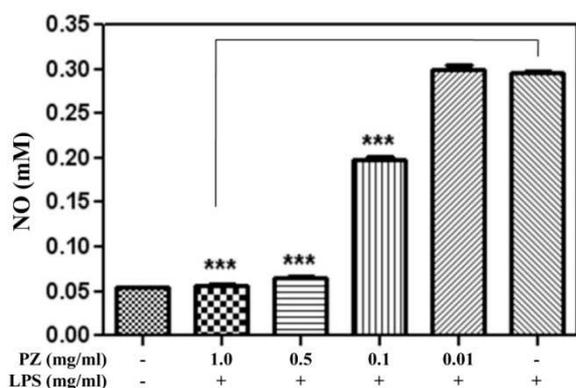


Fig. 4: Inhibition of NO production by the *Pericarpium zanthoxyli* extract. Data are mean ± S.E.M (n = 3); ***p < 0.001.

Fig 5 indicates that protein expressions of iNOS and COX-2 significantly increased (5.9- and 10.5-fold for iNOS and COX-2, respectively) upon LPS treatment in RAW 264.7 cells compared to control. The extract considerably inhibited the expression of LPS-induced iNOS (29, 38 and 100 %; Fig. 5A) and COX-2 (33, 19 and 84 %; Fig.5B) at 0.10 mg/ml, 0.1 mg/ml and 1 mg/ml, respectively, in RAW 264.7 cells.

DISCUSSION

Pericarpium zanthoxyli has long been used in oriental medicine for the treatment of various diseases such as pain, inflammation and bacterial infection. In the present study, the anti-oxidative mechanism of its extract has been explored. We propose that the extract inhibits

iNOS and COX-2 induced by LPS, thereby exerting its anti-oxidant actions.

The present study indicates that the extract contains significant amount of phenolics, including flavonoids and anthocyanins, which are known antioxidant components and have been recognized as having the potential to ameliorate diseases by helping to neutralize free radicals. Various ways are being developed to measure the potential of natural products with various antioxidant mechanisms. DPPH is a stable free radical and has been used extensively to test the ability of natural products to act as free radical scavengers or hydrogen donors [21]. Antioxidants either transfer an electron or a hydrogen atom to DPPH, thus neutralizing a number of DPPH molecules equal to that of hydroxyl groups [22]. In addition to scavenging DPPH, the extract also showed nitric oxide scavenging activity. The toxicity and damage caused by NO with superoxide anion is multiplied as they react to produce reactive peroxynitrite (ONOO-), leading to serious toxic reactions with protein, lipids and nucleic acids [23]. In this study, scavenging of free radicals increased with extract concentration and its activity was greater than that of vitamin C, the reference standard. The NO scavenging activity of the extract may help to arrest the chain reactions initiated by excess generation of ONOO- which is detrimental to human health.

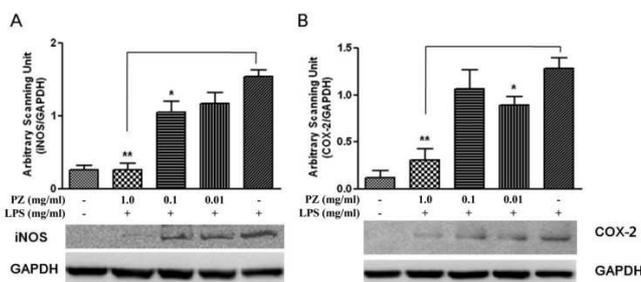


Fig 5: Effect of the *Pericarpium zanthoxyli* extract on LPS-induced iNOS and COX-2 induction. Data are mean ± S.E.M (n = 3); *p < 0.05 and **p < 0.01

Degree of lipid peroxidation is widely used as an index of oxidative stress and analyzed by measuring thiobarbituric acid reactive substances (TBARS) [24]. Lipid peroxides are unstable and are decomposed into various kinds of compounds including carbonyl compounds. As a group of small molecular weight substances, TBARS represents mainly malonaldehyde and some other compounds such as 4-hydroxy-2-nonenal (HNE) and acrolein. Its reducing ability generally depends on the presence of reductants [25]. The reductants causes the reduction of the Fe³⁺-ferricyanide complex to the ferrous form by

donating a hydrogen atom and exhibited anti-oxidative potential by breaking free radical chain reactions [26]. *Pericarpium zanthoxyli*'s remarkable reducing power could be of great benefit in the prevention and treatment of many diseases caused by oxidative stress. Thus, it cannot only remove various kinds of free radicals generated through oxidative stress but also inhibit the lipid peroxidation triggered by the generation of free radicals. These dual anti-oxidant actions could be of great value as a potential functional food candidate.

In macrophages, high concentrations of nitric oxide radical can be converted into peroxynitrites, which cause diverse chemical reactions in a biological system including nitration of tyrosine residue of protein, triggering lipid peroxidation, inactivation of aconites, inhibition of mitochondrial electron transport, and oxidation of biological thiol compounds [27]. The findings of the present study strongly suggest that *Pericarpium zanthoxyli* has the ability to reduce nitric oxide production by LPS in the RAW 264.7 macrophages. Results from Western blotting analysis further indicate that the enzyme, iNOS, is responsible for long-lasting nitric oxide production and it is strikingly induced by LPS as shown in this study. In addition to inhibition of NO release, the extract also significantly inhibited iNOS expression in LPS-stimulated RAW 264.7 macrophages in a dose-dependent manner. Interestingly, COX-2, which is responsible for proinflammatory prostaglandin formation, was increased by LPS and it was also inhibited by extract treatment. These results suggest that the anti-oxidative action of the extract is mediated by the inhibition of both iNOS-NO system and COX-2 enzyme.

Reactive oxygen and nitrogen species can be generated as by-products during oxidative processes of living organisms [28]. Many diseases, including aging, cancer, cardiovascular disease, periodontal disease, neurodegenerative disease and inflammation, are linked to excessive amounts of free radicals [29]. In the present study, we provide evidence that *Pericarpium zanthoxyli* extract exerts remarkable anti-oxidative actions via free radical scavenging activities, inhibition of NO production, iNOS and COX-2 expression and inhibition of lipid peroxidation. The results of the present study indicate that the plant has powerful free radical scavenging capacity which may be due to the presence of phenolics such as flavonoids and anthocyanins.

CONCLUSION

We propose that the immunomodulatory activity of the *Pericarpium zanthoxyli* extract depends on the inhibition of NO production and iNOS/COX-2 expression. The anti-oxidative actions of the extract could be useful in preventing and treating many diseases caused by oxidative stress and there can be exploited as an active component of functional foods.

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