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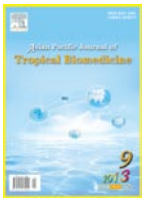

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# Pharmacological potential of *Populus nigra* extract as antioxidant, anti-inflammatory, cardiovascular and hepatoprotective agent

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PEER REVIEW

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**Comments**

The authors have presented their findings effectively. Endothelium independent vasorelaxant effect was found to be correlated with the antioxidant activity of the plant extract which is due to the presence of flavanoids. Moreover, histopathological examination revealed complete protection against $AlCl_3$ induced hepatic toxicity. *P. nigra* extract was identified as a potential anti-inflammatory, hepatoprotective and vasorelaxent agent.
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ABSTRACT

**Objective:** To evaluate antioxidant, anti-inflammatory, hepatoprotective and vasorelaxant activities of *Populus nigra* flower buds ethanolic extract.
**Methods:** Antioxidant and anti-inflammatory activities of the extract were assessed using respectively the ABTS test and the animal model of carrageenan-induced paw edema. Protection from hepatic toxicity caused by aluminum was examined by histopathologic analysis of liver sections. Vasorelaxant effect was estimated in endothelium-intact and -rubbed rings of porcine coronary arteries precontracted with high concentration of U46619.
**Results:** The results showed a moderate antioxidant activity (40%), but potent anti-inflammatory activity (49.9%) on carrageenan-induced mice paw edema, and also as revealed by histopathologic examination, complete protection against $AlCl_3$-induced hepatic toxicity. Relaxant effects of the same extract on vascular preparation from porcine aorta precontracted with high concentration of U46619 were considerable at $10^{-1}$ g/L, and comparable ($P>0.05$) between endothelium-intact (67.74%, $IC_{50}$=0.04 mg/mL) and -rubbed (72.72%, $IC_{50}$=0.075 mg/mL) aortic rings.
**Conclusions:** The extract exerted significant anti-inflammatory, hepatoprotective and vasorelaxant activities, the latter being endothelium-independent believed to be mediated mainly by the ability of components present in the extract to exert antioxidant properties, probably related to an inhibition of $Ca^{2+}$ influx.

KEYWORDS
*Populus nigra*, Polyphenols, Anti-inflammatory activity, Hepatoprotection, Vasorelaxation

## 1. Introduction

Endothelial dysfunction is common in several chronic disease conditions, such as hypertension, diabetes, and atherosclerosis[1]. Underlying causes of endothelial dysfunction is an impaired vasodilation, mediated mainly by a deficiency in nitric oxide (NO) and/or increased inactivation of NO by reactive oxygen species[2]. Therefore, an enhanced expression of endothelial nitric oxide synthase (eNOS) contributes to the recovery of normal endothelial mechanisms. NO is also implicated in many physiologic processes such as anti-inflammatory and antioxidant by preventing respectively leukocyte adhesion to vascular endothelium and oxidative modification of low-density lipoprotein (LDL) cholesterol[3]. In fact, inflammation and oxidation play a key role in

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coronary artery pathology and other manifestations of atherosclerosis[4]. Therefore, we now understand that at least some of the cardiovascular benefits attributable to medical treatment may result from reductions in inflammatory and oxidant processes. In particular, plant-derived compounds such as phenolic acids, flavonoids and tannins have been reported to possess potent antioxidant and anti-inflammatory activities that can contribute to the prevention of many pathologies, including cardiovascular disease[5]. Recent physiological, pharmacological and biochemical studies appear to support the wisdom of traditional medicinal practices owing to the fact that vegetable-based medication is well adapted to body's physiology and therefore causes only minor side effects. Consequently, the observed global trend to use this type of medication to prevent or treat several pathologies is justified.

In Algeria, traditional uses of *Populus nigra* (*P. nigra*) are wide in the treatment of many conditions related to endothelial dysfunction such as inflammations, arthritis, bronchitis and respiratory tract diseases. A scientific validation of pharmacological actions of this plant with respect to *in vitro* antioxidant and anti-inflammatory activities has been reported[6]. However, vasorelaxant, *in vivo* anti-inflammatory and hepato-protective activities, to the best of our knowledge, have never been investigated.

In order to better understand the protective effect of *P. nigra* buds ethanolic extract on the endothelial and liver functions, experiments were performed to determine anti-inflammatory, antioxidant and relaxant activities of extract.

## 2. Materials and methods

### 2.1. Drugs and chemicals

All the reagents and chemicals, unless otherwise stated, were purchased from Sigma.

### 2.2. Collection of plant material

Fresh flower buds of *P. nigra* were collected in March from a remote area in the forest of Tizi Neftah, Province of Amizour, Department of Bejaia, Algeria. The plant was identified by Dr. M.S Benabdelmoumène taxonomist, Department of Botany, University of Bejaia, Algeria.

### 2.3. Plant sample extraction

The fresh flower buds of *P. nigra* were air-dried in the shade and ground to a fine powder of 63 μm in diameter. A total of 300 mg of this powder were extracted with ethanol (1: 6, w:v) at room temperature for 24 h. The reunified extractive liquid was evaporated under vacuum.

### 2.4. Animals

Albino mice of either sex weighing around 20 g and purchased from Pasteur Institute (Algiers, Algeria) were used in these experiments. They were provided with standard food and water *ad libitum*, and were maintained at a constant temperature of (23±1) °C, relative humidity of (65±5)% and 12/12 h light/dark cycle. All the animals were acclimatized to the laboratory environment for two weeks before the beginning of the experiments which were conducted in strict compliance with internationally accepted principles for laboratory animals.

### 2.5. Quantification of total phenols, flavonoids and tannins

Determination of total phenols, flavonoids and tannins in the extract was achieved spectrophotometrically and as reported in a previous publication using the methods developed by Lowman and Box[7,8], Maksimovic *et al.*[9] and Hagerman and Butler[10], respectively. Total phenol content was based on the reduction of Folin-Ciocalteu reagent by phenol compounds. Flavonoid content was determined by incubation of extract solution and aluminum chloride reagent. Tannins were determined by precipitation using bovine serum albumin. Concentrations of total phenols, flavonoids and tannins were deduced from standard curves using catechin, quercetin and tannic acid, respectively.

### 2.6. ABTS scavenging activity

Antioxidant capacity was measured based on the scavenging of $ABTS^{\bullet+}$ [(3-ethyl benzothiazoline 6-sulfonic acid) diammonium salt] radical cation which was generated by mixing solutions of ABTS (7 mmol/L) and potassium persulfate (2.45 mmol/L) incubated in the dark at room temperature for 12-16 h[11]. The product was diluted in ethanol for optimal absorption of approximately 0.7 at 734 nm. The decolorization of the $ABTS^{\bullet+}$ solution by 100 μg/mL of the test sample or reference compound (quercetin) was monitored by a decrease in absorption at 734 nm during 30 min. The antioxidant activity was calculated as the percentage of $ABTS^{\bullet+}$ inhibition, according to the formula:

$\%\ \text{inhibition}=[(A_0-A)/A_0]\times 100$

where $A_0$ and A represented the absorptions of the control and the sample (extract or standard) respectively.

### 2.7. Acute toxicity studies

Acute toxicity studies were carried out with the ethanolic extract at a dose of 200 mg/kg which was administered for 4 weeks to separate groups of mice ($n$=7) after overnight fasting. Subsequently to administration of extract, the animals were observed for any toxic manifestation such as increased locomotor activity, diarrhea, coma and death.

### 2.8. Anti-inflammatory activity

Albino mice of either sex weighing around 20 g were fasted for 24 h prior to the experiment and deprived of water only during the experiment. According to the method of Winter *et al.*[12], animals were subjected to subplantar injection into the right hind paw of 0.1 mL λ-carrageenan (1%) and were divided randomly into allocated groups ($n$=7) and received the following solutions:

Group I: sterile saline (0.9% NaCl at 10 mL/kg); Group II: extract (200 mg/kg); Group III: sodium diclofenac (50 mg/kg), as reference drug.

Extract and diclofenac (both dissolved in 0.9% NaCl), were administered intraperitoneally 1 h prior to carrageenan injection, and the circumference of paw was measured at 1 h intervals thereafter for a period of 6 h. Increases in the linear circumference of the right hind paw were taken as an indicator of paw edema.

Percentage increase in edema (%IO) was estimated in terms of the difference in the zero time ($C_0$) linear circumference of the injected right hind paw, and its linear circumference at time t ($C_t$):

$$\%IO=(C_t-C_0)/C_0\times100$$

Percentage inhibition of the inflammatory reaction produced by carrageenan was calculated in the acute phase 5 h after its administration according to the following formula:

$$\%inhibition=(IO_c-IO_t)/IO_c\times100$$

Where $IO_c$ and $IO_t$ represented the mean increase in paw circumference in control and treated groups, respectively.

### 2.9. Hepato-protective activity

The protective effect of *P. nigra* buds ethanol extract against aluminum-induced hepatic toxicity was investigated using the modified method of Pan *et al*[13]. Aluminum chloride ($AlCl_3$) prepared in drinking water at a dose of 100 mg/(kg·d) was administered orally whereas extract 200 mg/(kg·d) and D-galactose 200 mg/(kg·d) (physiological solution) were given to mice by intraperitoneal injection 1 h prior to receiving $AlCl_3$. Animals ($n$=6) were treated daily and for 4 weeks during which period they had free access to standard laboratory diet and tap water and randomized into four groups: Group I: control received only the vehicle; Group II: received 200 mg/(kg·d) of extract; Group III: were treated with 100 mg/(kg·d) of $AlCl_3$ and 200 mg/(kg·d) of D-galactose; Group IV: (tested groups) received 100 mg/(kg·d) of $AlCl_3$, 200 mg/(kg·d) of D-galactose and 200 mg/(kg·d) of extract.

#### 2.9.1. Determination of hematological parameters

After anesthesia with diethyl ether, blood samples were collected. Blood hemoglobin and hematocrit were determined by standard laboratory procedures.

#### 2.9.2. Histopathological examination

All mice were sacrificed 24 h after the last treatment and overnight fasting. Their livers were harvested, washed in normal saline, blotted with filter paper, and weighed. Gross examination was conducted to detect any abnormalities and livers were subjected to histopathological analysis. Segments were fixed in formol solution and paraffin-embedded. For histological observation, deparaffinized sections (5 μm) were stained with Mayer hematoxylin for 2 min followed by 1% aqueous eosin during 40 seconds.

### 2.10. Vasorelaxant activity of P. nigra ethanolic buds extract

#### 2.10.1. Effect on eNOS expression

##### 2.10.1.1. Cell culture

Porcine coronary artery segments were washed with calcium-free phosphate buffer solution (PBS) to remove remaining blood. The endothelial cells were isolated by collagenase (1 mg/mL) (type I, Worthington) treatment, for 12 min at 37 °C and grown for 48-72 h in culture dishes containing medium MCDB 131 (Invitrogen) and 15% foetal calf serum supplemented with penicillin (100 U/mL), streptomycin (100 U/mL), fungizone (250 μg/mL) and L-glutamine (2 mmol/L) (all from Cambrex). All experiments were performed with confluent cultures of cells used at first passage. Cells were grown in serum-free culture medium in the presence of 0.1% bovine serum albumin (QBiogene) for 6 h prior to treatment.

##### 2.10.1.2. Western blot analysis

According to the methodology of Anselm *et al.*[14], after treatment by the extract ($10^{-4}$-$10^{-1}$ g/L) for 30 min at 37°C, the cells were washed twice with PBS and then lysed in extraction buffer [(Tris/HCl 20 mmol/L, pH 7.5, NaCl (150 mmol/L), $Na_3VO_4$ (1 mmol/L)], sodium pyrophosphate 10 mmol/L, NaF 20 mmol/L, okadaic acid 0.01 mmol/L, a tablet of protease inhibitor (Roche) and 1% Triton X-100 mmol/L (QBiogen)). Total proteins (20 μg) were separated on 8% SDS-polyacrylamide gels at 70 V for 2.5 h. Separated proteins were transferred onto polyvinylidine difluoride membranes (Amersham) at 100 V for 120 min. Membranes were placed in blocking buffer containing 3% bovine serum albumin (BSA), Tris-buffered saline solution (Biorad) and 0.1% Tween 20 (TBS-T) for 1 h. For detection of phosphorylated proteins, membranes were incubated with the respective primary antibody P-eNOS Ser-1177 (Cell Signaling Technology, dilution of 1:1 000) overnight at 4 °C. After washing, membranes were incubated with the secondary antibody (peroxidase-labeled anti-rabbit IgG, dilution of 1:5 000 (Cell Signalling Technology) at room temperature for 60 min. Pre-stained markers (Invitrogen) were used for molecular mass determinations. Immunoreactive bands were detected by enhanced chemiluminescence (Amersham).

#### 2.10.2. NO scavenging activity

The scavenging activity of NO radical was determined according to the method of Marcocci *et al*[15]. The reaction mixture (3 mL), containing 2 mL of sodium nitroprusside (10

mmol/L) and 0.5 mL of PBS (1 mol/L), was mixed with extract (100 μg/mL). After incubation (25 °C for 150 min), 0.5 mL of the reaction mixture was withdrawn and mixed with 2 mL of Griess reagent [prepared by mixing 1 mL of sulphanilic acid reagent (0.33%) with 1 mL of naphthyl ethylenediamine dihydrochloride (1% NEDA)]. The absorbance of the blue chromogen formed revealing the presence of nitrite ions was measured at 540 nm. The percentage of NO scavenging is calculated based on the following formula:

$\%NO\ scavenged=(A_{control}-A_{test})/A_{control}\times100$

where $A_{control}$ and $A_{test}$ were the absorbances in the absence and presence of extract, respectively.

#### 2.10.3. Vascular reactivity studies

Left anterior descending porcine coronary arteries were cleaned of connective tissue and cut into rings (4-5 mm in length). Endothelium was removed by rubbing the intimal surface of rings with a pair of forceps. Rings were suspended in organ baths containing oxygenated (95% $O_2$ and 5% $CO_2$) Krebs bicarbonate solution (composition in mmol/L: NaCl 119, KCl 4.7, $KH_2PO_4$ 1.18, $MgSO_4$ 1.18, $CaCl_2$ 1.25, $NaHCO_3$ 25, and D-glucose 11), pH 7.4, at 37 °C and the cyclooxygenase inhibitor indomethacin (10 μmol/L), for the determination of changes in isometric tension[14].

Prior to the start of the experiment, the functionality of the isolated rings was tested by subjecting them to contraction with KCl (80 mmol/L) after equilibration for 90 min under a resting tension of 5 g. Subsequently, after a 30 min washout period, rings were contracted with the thromboxane mimetic U46619 (1-60 nmol/L) to about 80% of the maximal contraction before addition of bradykinin (0.3 μmol/L) to check the presence of a functional endothelium. After washout and a 30 min equilibration period, rings were again contracted with U46619 before construction of a concentration-relaxation curve[14].

### 2.11. Statistical analysis

The experimental data obtained were expressed as mean ±SEM for a number of experiments. Significant differences between groups were determined using analysis of variance (ANOVA) followed by Student Newman-Keuls method for *post hoc* analysis. Differences were considered to be significant at $P<0.05$.

## 3. Results

### 3.1. Quantification of total phenols, flavonoids and tannins

Total phenols, flavonoids and tannins of *P. nigra* ethanolic extract were measured using the respective standards catechin, quercetin and tannic acid to obtain the following equations $y=0.0097x+0.0134$, $y=0.0597x+0.001$ and $y=0.3282x+0.0181$. Total phenols, flavonoids and tannins of *P. nigra* ethanolic extract were (51.78±4.56) mg catechin Eq/g of extract, (13.67±0.34) mg quercetin Eq/g of extract, and (228.72 ±6.90) mg tannic ac Eq/g of extract, respectively.

### 3.2. ABTS assay

The results of the ABTS assay indicated that the ethanolic extract of buds of *P. nigra* exhibited, at a concentration of 100 μg/mL, a percentage of (41.05±2.34) in the decolorization of ABTS, a moderate activity when compared to the reference quercetin which showed a percentage of (96.5±0.04).

### 3.3. Acute toxicity

Acute toxicity studies did not reveal any toxic symptoms or death in any of the animals at the dose of 200 mg/kg of ethanolic *P. nigra* buds extract.

### 3.4. Anti-inflammatory activity

Figure 1 indicates that in the control group (I), the onset of edema [(22.25±4.69)%] occurred as early as 1 h after carrageenan injection and was sustained through the 6 h of observation. On the other hand, the extract (200 mg/kg) caused a sharp decrease in paw edema from the 2nd [(21.99±5.58)%] until the 6th hour [(11.14±6.87)%] of treatment ($P<0.05$), in a similar manner as that of the reference drug diclofenac (50 mg/kg). In fact, with both extract and drug, the suppression of edema reached a maximal value 3 h following the administration of carrageenan, their respective inhibition percentages 49.93% and 62.56% being comparable ($P>0.05$) after 5-hour treatment.

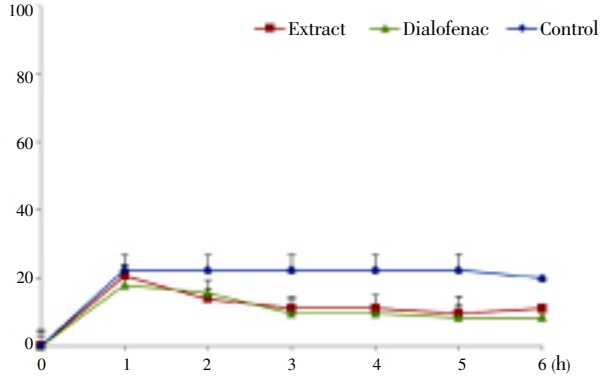


**Figure 1.** Changes in paw edema induced by carrageenan over time (h) in control and experimental groups.

### 3.5. Hepato-protective potential

#### 3.5.1. Hematological parameters

The results in Table 1 show hematological modifications associated with orally administered aluminum chloride in mice with and without pre-treatment with *P. nigra* extract. A significant decrease ($P<0.05$) in hemoglobin and hematocrit levels of the aluminum-treated group of mice (II), compared to the control group (I) was observed, demonstrating a

disequilibrium in these parameters and therefore signs of anemia. On the other hand, a recovery of the normal values was observed in Group III pre-treated with extract.

**Table 1**
Blood hemoglobin and hematocrit.

| Blood factors | Groupe I | Groupe II | Groupe III | Groupe IV |
|---|---|---|---|---|
| Hemoglobin (g/dL) | 12.40±0.77 | 11.40±0.96 | 10.10±1.12* | 13.60±0.71 |
| Hematocrit (%) | 37.45±2.95 | 35.0±1.2 | 28.15±3.85* | 38.50±1.55 |

Values are expressed as means±SD. Values are statistically significant at *$P$<0.05.

### 3.5.2. Histopathological examination

Data obtained from histological sections of mice livers of control groups receiving either the vehicle (Group I) or extract alone (200 mg/kg) (Group II) presented in Figure 2(A) and 2(B) respectively. It revealed normal hepatic architecture *i.e.* hepatocytes arranged as radiating plates around the central vein. There was no sinusoidal dilatation or bleeding foci, confirming the lack of toxicity of the extract.

On the other hand, we observed in both liver sections of mice (Group III) treated with $AlCl_3$ and D-galactose (Figure 2 $C_1$ and $C_2$), which were signs of hepatic damage such as a dilated (green arrow) and congested central hepatic vein (blue arrow) ($C_1$), the presence of some swollen cells, increased number of lipid vacuoles (yellow arrow) enlarged nuclei (white arrow), and infiltrating neutrophils (blue arrow) ($C_2$).

Most interesting, pre-treatment with extract (200 mg/kg) (Group IV) protected almost completely the liver against $AlCl_3$-induced hepatic damage and necrosis as observed in Figure 2(D). Specifically, histological examination of the liver of pre-treated animals with plant extract showed that fatty acid changes were less pronounced in comparison with $AlCl_3$-intoxicated mice that have not received extract.

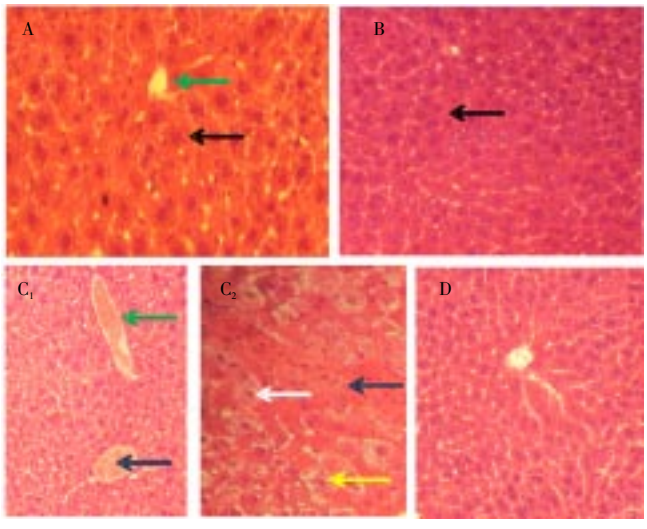


**Figure 2.** Photomicrographs of liver sections from mice stained with H&E (× 250).
A and B are the sections of the male mice liver of control group receiving only vehicle or extract [200 mg/(kg·d)], respectively. $C_1$ and $C_2$: Mice treated with $AlCl_3$ [100 mg/(kg·d), orally] and D-galactose [200 mg/(kg·d), *i.p*]. D: Pre-treatment with extract [200 mg/(kg·d), *i.p*].

## 3.6. Effect of *P. nigra* extract on the levels of eNOS and relaxant action

### 3.6.1. Effect on eNOS expression

Figure 3 shows that *P. nigra* extract did not change the level of phosphorylated eNOS which was the activated form of this enzyme after posttranslational modifications[1].

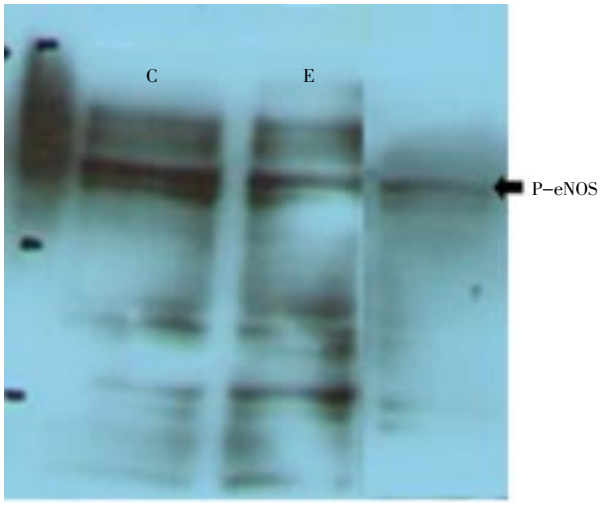


**Figure 3.** Western blotting representing the effect of *P. nigra* extract on phosphorylated eNOS expression.
C: control, E: ethanol extract.

### 3.6.2. NO scavenging activity

The ethanolic extract showed a negligible NO scavenging activity [(6±1)%], indicating that the tested extract did not have any effect on the level of this radical in the endothelium.

### 3.6.3. Vascular reactivity studies

The effects of the tested extract at various concentrations ($10^{-4}$-$10^{-1}$ g/L) on the vascular relaxing ability of the aortic rings pre-contracted with the thromboxane mimetic U46619 (1-60 nmol/L) were summarized in Figure 4. The results showed clearly a considerable ($P$<0.05) and dose-dependent increase in the percentage of relaxation which was comparable ($P$>0.05) in both endothelium-intact (67.74%, $IC_{50}$=0.04 mg/mL) and -denuded (72.72%, $IC_{50}$=0.075 mg/mL) rings of porcine coronary arteries at a concentration of $10^{-1}$ g/L.

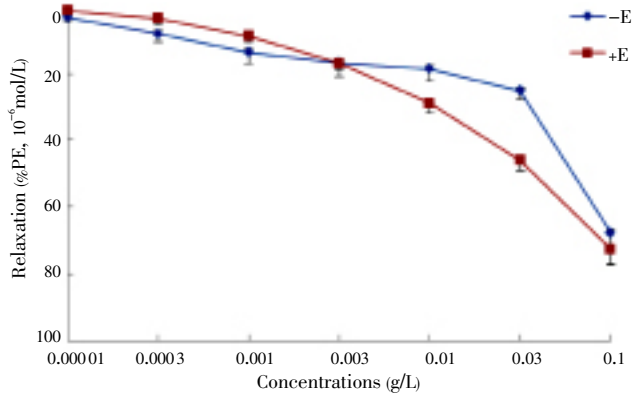


**Figure 4:** Relaxation effect of *P. nigra* extract on -intact (+E) and -denuded (-E) endothelium.

## 4. Discussion

The present findings indicate that *P. nigra* buds ethanolic extract is a powerful anti-inflammatory, hepato-protective and NO-independent vasodilator of coronary arteries, suggesting that it contains metabolites with such activities. Indeed, when compared to other plants such as *Fraxinus angustifolia*, *Pistacia lentiscus* and *Clematis flammula* whose antioxidant activities have been proven and whose total phenols, flavonoids and tannins have been determined in the same conditions[7], *P. nigra* buds ethanolic extract is considered to have important amounts of phenolic compounds.

In fact, the significant level of anti-inflammatory activity of the extract could be attributed to high amount of flavonoids such as quercetin, phenolic acids and aglycon flavonoids (pinocembrin) identified in previous investigations[6,16,17] in *P. nigra* buds extracts and revealed to possess high anti-inflammatory potential[6]. Other compounds like salicylic acid found in all *Populus* species are known for their antipyretic and analgesic activities[18,19]. Carrageenan-induced paw edema, a widely used model of acute inflammation, is a biphasic event, stimulating the release of histamine, serotonin and bradykinin in the first phase (30 min after carrageenan administration) and prostaglandin-like substances (PXs) in the second phase (0.5 to 5 h)[20]. Considering the fact that the peak of inhibition observed (3 h) coincides with maximum PXs synthesis[20], it is suggested that the significant anti-inflammatory activity of the tested extract may be due to inhibition of these mediators of inflammation. Therefore, the similar pattern of the anti-inflammatory response shared by the extract and the reference drug diclofenac reflects a common mechanism of action through inhibition of prostaglandin synthetase[21]. On the other hand, the antioxidant potential of the extract can participate in fighting inflammation through the scavenging of reactive oxygen species produced at the site of inflammation by migrating neutrophils (neutrophil infiltration).

Aluminum is a non-redox active metal with pro-oxidative effects *in vitro* and *in vivo* by potentiating pro-oxidant properties of transition metals such as iron and copper[22]. Hence, plant-derived compounds that possess antioxidant properties may be useful against the toxic effects of this active metal[23,24]. Specifically, the antioxidant potential of *P. nigra* buds extract, estimated to be moderate (ABTS test), in complete agreement with that measured by the ORAC test in a previous investigation[6], could contribute to the attenuation of the deleterious impact on the liver caused by Al exposure. In particular, phenolic acids, identified as the major contributors of the antioxidant potential of this plant[6], could be involved in the hepatoprotective activity displayed by the extract by counteracting the destructive effects of lipid peroxidation, accelerated by aluminum toxicity *in vitro* and *in vivo*[25]. The preservation of membrane integrity by *P. nigra* extract may therefore prevent the massive influx of $Ca^{2+}$ into the cell which can lead to necrosis. Therefore, it can be suggested that the hepatoprotective efficiency of the tested extract could be due to the inhibition of $Ca^{2+}$ influx, and/or radical scavenging activity. Decreased hemoglobin and hematocrit values are described by the researchers under different conditions of toxicity[25,26], as indicators of anemia caused by an interference with iron metabolism[26-28]. As anemia did not develop in extract-treated mice, it can be deduced that *P. nigra* extract prevents the dysfunction of iron metabolism caused by aluminum.

eNOS plays a central role in maintaining cardiovascular homeostasis by controlling NO bioavailability[29], the latter being a paracrine mediator that promotes vascular relaxation and interferes with pro-inflammatory pathways[1]. The activity of eNOS in vascular endothelial cells largely depends on posttranslational modifications, including phosphorylation[30]. It has been established that the vasorelaxant mechanisms triggered by polyphenols are related to their specific concentration and structure and can be either endothelium-dependent or -independent[31]. The mechanism elicited by the ethanol extract of *P. nigra* was not exerted via endothelium-derived NO and prostacyclin (PGI2), consistent with the absence of change in eNOS phosphorylation observed and the fact that inhibition of prostaglandin synthesis with indomethacin had no effect on extract-induced relaxation. Our findings are consolidated by those of other researchers who suggested that diet polyphenols are responsible for vasoprotective, anti-angiogenic, anti-atherogenic, vasorelaxant effects in animals and in patients[32], and in which some of them such as hydroxytyrosol present in olive oil and red wine could not induce a relaxant effect by a direct modulation of eNOS[33]. Additional investigations have reported that catechin, epicatechin, gallic acid and quercetin did not influence the maximal production of NO and did not stimulate eNOS expression in cells[34]. Consequently, it is suggested that the extract acts directly on smooth muscle, probably by impeding the calcium transmembrane transport inside the cell or its release from the sarcoplasmic reticulum[35,36]. This supposition is reinforced by previous reports which revealed that several flavonoids have been reported to promote vascular smooth muscle relaxation via different mechanisms, including changes in transmembrane calcium movement[37,38], phytoestrogens and 17β-estradiol caused endothelium-independent relaxation by inhibition of the extracellular calcium influx into vascular smooth muscle[39]. This study provides support for the traditional use of *P. nigra* buds in the treatment of inflammatory pathologies and evidence for other pharmacological effects that could be explored in the fields of hepato-protection and cardiovascular diseases.

Ongoing and future studies will explore the mechanism of action of *P. nigra* extract and will attempt to isolate and characterize its bioactive constituents.

## Conflict of interest statement

We declare that we have no conflict of interest.

## Acknowledgements

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## Comments

### *Background*

Endothelial dysfunction is a key physiopathological mechanism that contributes to several diseases. An impaired vasodilation mediated mainly by a deficiency in NO is the major reason of endothelial dysfunction. NO is also implicated in many physiologic processes such as anti-inflammatory and antioxidant. Therefore, search of potential NO synthase inhibitors is the need of time.

### *Research frontiers*

The present research work describes antioxidant and anti-inflammatory activities of the ethanolic extract of *P. nigra* flower buds estimated by *in vivo* and *in vitro* models. Hepatic toxicity caused by aluminum was examined by histopathologic analysis, while vasorelaxant effect was estimated in endothelium-intact and -rubbed rings of porcine coronary arteries.

### *Related reports*

Aluminum is a well known hepatotoxins to humans. Using natural antioxidants against metal-induced hepatotoxicity is a modern approach. It is reported that modulation of iNOS is important in the prevention of inflammation, and flavanoids are potent inhibitors of iNOS.

### *Innovations and breakthroughs*

Traditionally, *P. nigra* is used in the treatment of many conditions related to endothelial dysfunction. Some *in vitro* antioxidant and anti-inflammatory activities are also reported. In the present study, authors have demonstrated vasorelaxant, *in vivo* anti-inflammatory and hepato-protective activities.

### *Applications*

This study provides basis for the traditional use of *P. nigra* buds in the treatment of inflammatory pathologies. Other pharmacological effects such as hepato-protection and prevention of cardiovascular diseases could be explored.

### *Peer review*

The authors have presented their findings effectively. Endothelium independent vasorelaxent effect was found to be correlated with the antioxidant activity of the plant extract which is due to the presence of flavanoids. Moreover, histopathological examination revealed complete protection against $AlCl_3$ induced hepatic toxicity. *P. nigra* extract was identified as a potential anti-inflammatory, hepatoprotective and vasorelaxent agent.

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