

Original Research Article

Phenolic screening, antioxidant activity and diuretic effect of Moroccan *Pinus pinaster* Bark extract

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Abstract

Objective: This study aims to evaluate the chemical composition, antioxidant activity, and diuretic effects of Moroccan *Pinus pinaster* bark ethanolic extract (PPBE).

Materials and Methods: The phytochemical composition of PPBE was assessed using HPLC-DAD. Total polyphenols and flavonoids were quantified using the Folin-Ciocalteu and aluminum trichloride methods, respectively, while mineral content was determined by plasma mass spectrometry. Antioxidant activity was assessed using the reducing power assay, total antioxidant capacity, and anti-DPPH free radical assay. For the diuretic effect, sixteen male Wistar rats were divided into four groups: control (distilled water, 10 ml/kg of BW), furosemide (10 mg/kg of BW), and PPBE (200 and 400 mg/kg of BW) groups. After 15 days, plasma and urine were collected for creatinine, potassium, and sodium analysis, along with urine output measurement. Statistical analysis employed one-way ANOVA followed by Tukey multiple comparison test.

Results: The PPBE displayed high phenolic content and potent antioxidant properties. Besides, the PPBE phenolic screening showed nine phenolic compounds with ferulate glucoside, gallic acid, and catechin as the main compounds. The PPBE demonstrated a richness in essential minerals. Furthermore, at both doses (200 and 400 mg/kg) PPBE led to a notable elevation in urine flow, urinary sodium concentration, and creatinine clearance, without affecting plasma electrolytes. In contrast, furosemide caused a reduction in plasma potassium levels.

Conclusion: PPBE could serve as a bioactive component, antioxidant, or preservative in food formulation. Moreover, it exhibits a diuretic effect without altering plasma composition.

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Introduction

Forests are considered the primary source of medicinal plants and forest products (Ashmawy et al., 2020), producing large amounts of solid waste containing valuable substances. In this regard, there is a significant deficit in converting this waste into potentially innovative products that could generate more revenue compared to conventional commercial applications, considering sustainability and positive health effects (Klimánková, 2007). Currently, a wide range of illnesses can be linked to the intensified generation of free radicals and the resulting oxidative stress. In addition, it was revealed that oxidative stress and inflammation are contributors to kidney disease, potentially exacerbating further harm to the kidneys (Tucker et al., 2015). Consequently, there is a growing interest in discovering and promoting novel ingredients possessing antioxidant properties to avoid or prevent excessive oxidation (Willcox et al., 2004). Various compounds present in plants hold promise for use as nutraceuticals, notably phenolic substances, a diverse class of molecules exhibiting a broad spectrum of bioactive properties (Yesil Celiktaş et al., 2010). These include antioxidant, anti-inflammatory (Hajhashemi et al., 2021), anti-atherogenic, antibacterial (Ferreira-Santos et al., 2020), neuroprotective (Mansouri et al., 2021), and diuretic properties (Santos et al., 2021).

Diuretics, whether used independently or in conjunction with other medications, play a crucial role in treating conditions such as hypertension, congestive heart failure, ascites, and pulmonary edema (Lahlou et al., 2007; Roush et al., 2014). These diuretics enhance the excretion of water and salt (sodium) through urine, leading to a reduction in blood pressure and a decrease in blood volume (Ntchapda et al., 2016; Wile, 2012). Two commonly employed diuretics, thiazides and the high-ceiling loop diuretic furosemide, have been linked to various adverse effects and

cannot be administered in certain physiological cases. These include electrolyte imbalance, metabolic alterations, the onset of new diabetes, activation of the renin-angiotensin-neuroendocrine systems, and impairment of sexual function (Gupta and Neyses, 2005). The exploitation of plant by-products represents a fascinating potential for the discovery of new substances with a diuretic effect, if we consider that each of these plant by-products can contain hundreds or even thousands of secondary metabolites. The identification and purification of these molecules can bring interesting therapeutic interest to various pathologies.

In this sense, bark, or peel, is the outer layer of trees, bushes, plants, and some fruits. *Pinus pinaster* bark serves as a compelling reservoir of novel biomedical compounds and displays the highest content of bioactive compounds as compared to other commonly studied plant species, making it a promising candidate for therapeutic applications. (Mármol et al., 2019a). Until just a few years ago, it was commonly dismissed as a byproduct in the wood industry, with minimal applications such as fuel or as a substrate in gardening. Among its constituents are substances with diverse functions, including the ability to delay or prevent pathogen invasion storing reserve substances and plant hormones (Mármol et al., 2019b).

Pinus is recognized as the largest genus within the Pinaceae family, encompassing over 109 distinct species recognized by Farjon (2001) (Plomion et al., 2007). The *Pinus* genus has been extensively utilized in traditional medicine due to its various pharmacological attributes, including anti-aging and anti-inflammatory benefits. Additionally, there is potential for their use in addressing conditions such as liver diseases, skin disorders, and hypertension (Xie et al., 2015).

Pinus pinaster (*P. pinaster*), also known as *maritime pine*, is predominantly used

for nutritional and pharmaceutical applications. *P. pinaster* is found in regions such as Spain, the southern part of France, Italy, Portugal, and Morocco, with smaller populations in Malta and the northern part of Iran. *P. pinaster* subspecies differ in their cold and salt tolerance, as well as their terpenes and protein content (Rohdewald, 2002).

The bark of *P. pinaster* is a great source of phenols (Jerez et al., 2006). *P. pinaster* bark has been cited in the scientific literature for many possible applications. It has been reported that its extracts possess antioxidant, antiradical, and anti-inflammatory activities and are distributed as a nutritional supplement and an herbal-based medication, Pycnogenol® (Chupin et al., 2013).

The purpose of this study is to conduct a thorough chemical analysis of the phenolic compounds and mineral content present in extracts derived from the bark of *P. pinaster* utilizing a water-ethanol solvent. Additionally, the research aims to investigate the antioxidant potential and examine the diuretic effects of these extracts.

Materials and Methods

Bark origin and preparation

The *P. pinaster* bark was gathered in Sefrou (33°49'49.886''N, 4°50'7.134''W), Morocco. This plant was chosen for its many therapeutic properties. The bark was cleaned with water for 10 min, then washed twice with distilled water and left to air-dry in an oven at 40°C for two days (Ferreira-Santos et al., 2020). It was then crushed, reduced to powder, and stored in hermetically sealed bottles in a dry place away from humidity and light. The plant powder obtained was then used for extraction.

Pine bark extract

To do a traditional maceration at room temperature for 72 hr under agitation for 24 hr, a total of 20 g of *P. pinaster* bark

was contacted with 200 ml of ethanol/water (70-30% v/v) in 500 ml flasks. After extraction, the extract was vacuum-filtered using Whatman paper using a Buchner funnel. The filtrate was rotary-evaporated at 40°C under vacuum to remove ethanol. The filtrate was gathered and dried (Royer et al., 2013). The powder obtained was *P. pinaster* bark ethanolic crude extract (PPBE).

Animals

Sixteen male Wistar rats (172.62±14.62 g), were used to achieve this experiment. The breeding of these animals is done in the animal house of Sidi Mohamed Ben Abdallah University. Indeed, they were housed in cages where food and tap water were freely available, under ambient temperature conditions (25°C), with a natural cycle of 12 hr of light followed by 12 hr of darkness. The research was conducted by ethical guidelines for the use and care of laboratory animals, and approval from the Ethical Committee, Faculty of Sciences, Fez, Morocco, was obtained (USMBA-SNAMOPEQ 2017-03). All efforts were made to minimize animal suffering and the number of animals used.

Experimental design

The diuretic activity was evaluated following the protocol described by El Ghouizi et al. (El Ghouizi et al., 2020). Before beginning the experiment, each rat was put into a metabolic cage for 48 hr. Four groups (n=4) were employed and subjected to a 12-hour food deprivation period before testing, with access to water.

The control group (Group 1) received 10 ml/kg body weight of distilled water orally via gavage. Group 2 was administered with 10 mg/kg body weight of furosemide orally via gavage. Group 3 received 200 mg/kg body weight of PPBE, while Group 4 received a higher dose of 400 mg/kg body weight of PPBE.

The furosemide, a widely recognized loop diuretic, was employed as a standard

reference to evaluate and compare the diuretic effects of the PPBE extract, and the extract doses were chosen based on the study conducted by Hajhashemi et al. (2021). On treatment days 0 and 15, urinary samples were collected using graduated cylinders to quantify water excretion. Subsequently, the samples underwent filtration, centrifugation, and preservation for the analysis of sodium, potassium, and creatinine levels.

The animals were anesthetized using diethyl ether, and blood samples were collected from the retro-orbital plexus using heparinized capillary tubes at the end of the treatment (El Ghouizi et al., 2020). After that, the plasma was separated by centrifugation at $10,000 \times g$ for 10 min, and it was kept at -20°C so that creatinine, sodium, and potassium could be measured (Bakour et al., 2017). On day 15, creatinine clearance was also computed using the following formula:

$$\text{Creatinine clearance (ml/min)} = \left(\frac{U_{\text{crea}}}{P_{\text{crea}}} \right) * V$$

U_{crea} : Urinary creatinine concentration

P_{crea} : Plasma creatinine concentration

V : Urinary volume (ml/min)

Using the following formula, the osmolar clearance (Cosm) was also determined from urine volume (V),

plasma osmolarity (Posm), and urinary osmolarity (Uosm): $\text{Cosm} = (V \times \text{Uosm}) \times 1/\text{Posm}$.

Next, the below equation was used to calculate the free water clearance (CH_2O):

$$\text{CH}_2\text{O} = V - \text{Cosm}.$$

Free water reabsorption (TCH_2O) was assessed using the following formula:

$$\text{TCH}_2\text{O} = -(\text{CH}_2\text{O}).$$

High-Performance Liquid Chromatography with Diode-Array Detection Analysis

The analysis of phenolic compounds in PPBE was performed by a SHIMADZU PROMINANCE HPLC equipped with a degasser, a DAD detector, a quaternary pump type LC A20, and a thermostatically

controlled automatic injector. An Agilent Zorbax C18 column (4.6 mm x 150 mm, 5 μm 100A) was used for the HPLC separation of polyphenols. The column temperature was 30°C , the injection volume was 10 μl , and the flow rate of the mobile phase was 0.25 ml/min using a ternary gradient of acetonitrile, methanol, and water acidifier. To find the response factor, solutions of gallic acid and Tyrosol standards were administered under identical circumstances (Vivar-Quintana et al., 2018).

Quantification of total phenolic contents (TPC)

With certain modifications, the Folin-Ciocalteu reagent was used to calculate the total phenolic content (TPC) of PPBE. A volume of 500 μl of 10-fold diluted Folin-Ciocalteu reagent was mixed with 100 μl of the extract (Singleton and Rossi, 1965). Next, 400 μl of 7.5% sodium carbonate Na_2CO_3 was added to promote an alkaline medium for the redox reaction, then incubated at room temperature in the dark for 2 hr. The blue color produced was measured at 765 nm to estimate its absorbance at various intensities. Gallic acid equivalent (mg GAE/g DM) was used to express the data in milligrams per gram of dry matter weight.

Total flavonoid content (TFC)

Flavonoid levels were measured by a method using aluminum trichloride (AlCl_3) (Deghima et al., 2020). Here, 500 μl of the extract was combined with 500 μl of 20 mg/ml aluminum chloride (AlCl_3) solution and kept for fifteen minutes in the darkness. Under identical conditions, a standard calibration curve was also prepared with quercetin solutions at different concentrations (1 mg/ml; 0.5; 0.25; 0.125; 0.0625; 0.0312; 0.015; 0.007; 0.003; and 0.00), the absorbance was then measured at 430 nm. The total flavonoid concentration of the extract is expressed as milligrams of quercetin equivalents per gram of dry plant matter (mg QE/g DM).

Free radical scavenging activity (using 2,2-Diphenyl-1-picrylhydrazyl Assay)

This approach relies on assessing the capacity of antioxidants to capture the DPPH free radicals. The impact of PPBE on DPPH free radicals was evaluated using the method outlined by Jiménez-Moreno *et al.* (Jiménez-Moreno *et al.*, 2019). A series of dilutions of the extract with different concentrations (1mg/ml; 0.5; 0.25; 0.125; 0.0625; 0.0312; 0.015; 0.007; 0.003; and 0.00) was prepared. Then, 150 µl of each dilution was mixed with 2.85 ml of a freshly prepared methanolic solution of DPPH (0.025 g/L). In what concerns the negative control, the latter was prepared in parallel by mixing 150 µl of the solvent used with 2.85 ml of a methanolic solution of DPPH. After incubation in darkness for 30 min and at room temperature, the absorbance readings were carried out at 515 nm using a spectrophotometer. Using the following formula (1), the IC₅₀ was determined based on the graph produced by the percentage of inhibition.

IC₅₀ is equal to $[(A_0 - A_1/A_0) \times 100]$ ⁽¹⁾, where A₀ represents the absorbance of a blank sample with the same solvent and DPPH solution content as the negative control, and A₁ represents the sample's absorbance. The tests were conducted in triplicate, and the mean±SD of findings are shown.

Reducing power assay (RP)

The reducing power assay (RP) was done using the procedure outlined by El Ghouizi *et al.* (2021). A mixture of 200 µl of 0.2 M sodium phosphate buffer (pH 6.6) and 200 µl of 1% potassium ferricyanide was combined with 50 µl of PPBE. After 20 min of incubation at 50°C, 200 µl of 10% trichloroacetic acid, 200 µl of distilled water, and 120 µl of 0.1% ferric chloride were added to the mixture. 700 nm was used to measure the absorbance. Based on the graph of absorbance against extract concentration in the solution, the extract concentration yielding 0.5

absorbance (EC₅₀) was determined. As a positive control, ascorbic acid was employed. The tests were performed three times, and the mean ± SD of findings was reported.

Total antioxidant capacity assay (TAC)

The ammonium molybdate colorimetric technique, as described by El Ghouizi *et al.* (2021), was used to calculate the total antioxidant capacity (TAC). Briefly, 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) was mixed with 50 µl of PPBE. The mixture was capped and allowed to sit for 90 min at 95°C in a thermal block. The reaction mixture's absorbance was measured at 700 nm in comparison to a blank. The standard calibration used was ascorbic acid, and the findings are presented as mg of ascorbic acid equivalent/g of dry plant matter (mg EAA/g DM).

Mineral content

The mineral composition was determined using the method described by Zuluaga *et al.* (2014). Briefly, each sample was burned until only ash remained, and then, 5 ml of 0.1 M nitric acid was added to the ash of each sample. Dryness was achieved by heating the mixture. The solutions were increased to 25 ml with distilled water after 10 ml of the same acid was added. Mineral elements were determined using an air/acetylene flame, and the quantitative determination was carried out after calibrating the instrument using different concentrations of Ca, Na, K, Fe, P, Zn, Mn, B, Cu, Cr, Cd, and Ni dissolved in 0.1% lanthanum using inductively coupled plasma mass spectrometry (ICP-MS). The results were expressed in mg of mineral per kilogram of *P. pinaster* bark (mg/Kg). All samples were analyzed in triplicate.

Statistical analysis

The data is presented as mean±SEM. The statistical analysis was performed by

GraphPad Prism Software 8, using a one-way analysis of variance (ANOVA) followed by *post hoc* Tukey's multiple comparison test. A $p < 0.05$ denoted significant differences.

Results

HPLC-DAD analysis

The determination and characterization of phenolic compounds in the PPBE were conducted using High-Performance Liquid

Chromatography with Diode Array Detection (HPLC-DAD). The data is reported in Figure 1 and Table 1. According to the results, PPBE contained 9 polyphenol compounds, with the highest concentration registered for ferulate glucoside (31.07 ± 1.45 mg/g), gallic acid (24.52 ± 0.25 mg/g), catechin (21.02 ± 1.38 mg/g), proanthocyanidin (14.31 ± 0.97 mg/g), and a taxifoliol glucoside (9.1 ± 1.27 mg/g).

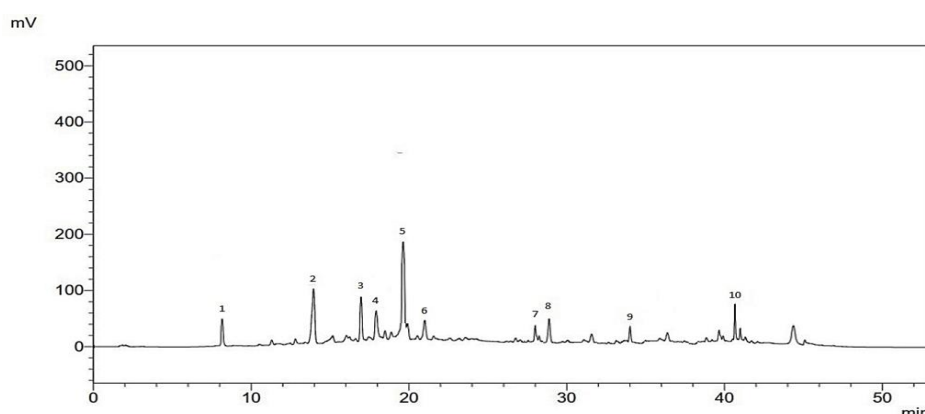


Figure 1. Chromatogram of ethanolic extract of *P. pinaster* bark (PPBE).

Table 1. Phenolic compounds identification and quantification in ethanolic extract of *P. pinaster* bark (PPBE).

	Phenolic compounds	Concentration in (mg/g)
1	Taxofoliol glucoside	9.1 ± 1.27
2	Gallic acid	24.52 ± 0.25
3	Catechin	21.02 ± 1.38
4	Epicatechin	8.9 ± 0.56
5	Ferulate glucoside	31.07 ± 1.45
6	Taxifoliol	5.13 ± 1.23
7	Procyanidin B2	7.9 ± 0.14
8	Procyanidin B3	8.33 ± 0.22
9	Procyanidin B1	7.87 ± 0.18
10	Proanthocyanidin	14.31 ± 0.97

Phenolic and flavonoid contents of PPBE

The PPBE was analyzed for total flavonoid and phenolic content through spectrophotometry. Table 2 illustrates the obtained results. The concentration of TPC in the extract was approximately 34.11 ± 0.02 mg GAE/g DM. Additionally, the extract exhibited a TFC level of around 24.96 ± 0.03 mg QE/g DM.

Antioxidant activities (DPPH, RP, and TAC)

As presented in Table 2, PPBE demonstrated noteworthy antiradical DPPH activity, showing an equivalent inhibition concentration (IC_{50} value of 9.76 ± 1.14 μ g/ml) compared to the standard ascorbic acid ($IC_{50} = 5.59 \pm 0.03$ μ g/ml). The RP assay further affirmed the potent reducing capability of PPBE, indicated by an EC_{50} of 0.037 ± 0.004 mg/ml. The overall antioxidant capacity

(TAC) was notably high, registering at 975.44 ± 24.59 mg EAA/g DM.

Mineral content assay

The analysis of the mineral composition of PPBE is summarized in Table 3. Notably, substantial quantities of macroelements, such as calcium (1250.42 ± 71.30 mg/kg), magnesium (148.97 ± 12.68 mg/kg), sodium ($58,90 \pm 1.55$ mg/kg), potassium (53.64 ± 6.16 mg/kg), and phosphorus (29.73 ± 0.38 mg/kg), were detected. The

bark also exhibited appropriate levels of microelements, including iron (30.96 ± 0.76 mg/kg), zinc (5.94 ± 0.28 mg/kg), manganese (2.76 ± 1.06 mg/kg), copper (0.22 ± 0.01 mg/kg), and chromium (0.11 ± 0.002 mg/kg). It is important to note that concentrations of toxic minerals, specifically, cadmium, and nickel, were determined to be below the established environmental safety limits and monthly levels of human intake.

Table 2. Total flavonoid content (TFC), total phenolic content (TPC), and antioxidant activity of *P. pinaster* bark extract (PPBE). The values are expressed in terms of means \pm SD of three experiments

Extract/ Standards	TPC (mg GAE/g DM)	TFC (mg QE/g DM)	DPPH (IC ₅₀ μ g/ ml)	FRAP (EC ₅₀ mg/ml)	TAC (mg EAA/g DM)
PPBE	34.11	24.96	9.76 ± 1.14	0.037 ± 0.004	975.44 ± 24.59
A. A	-	-	5.59 ± 0.03	0.031 ± 0.07	-

GAE: gallic acid equivalents; QE: Quercetin equivalents; DM: Dry plant matter; DPPH: 2,2-diphenyl-1-picrylhydrazyle; IC₅₀: Inhibition concentration 50; FRAP: Ferric Reducing Antioxidant Power; EC₅₀: Effective concentration 50; TAC: Total antioxidant activity; EAA: Equivalent acid gallic; AA: Ascorbic acid

Table 3. The mineral content of *P. pinaster* Bark expressed as mean \pm SD

Mineral content	Concentration (mg/kg)
	<i>P. pinaster</i> Bark
Calcium (Ca)	1250.42 ± 71.30
Magnesium (Mg)	148.97 ± 12.68
Sodium (Na)	$58,90 \pm 1.55$
Potassium (K)	53.64 ± 6.16
Phosphorus (P)	29.73 ± 0.38
Iron (Fe)	30.96 ± 0.76
Zinc (Zn)	5.94 ± 0.28
Manganese (Mn)	2.76 ± 1.06
Copper (Cu)	0.22 ± 0.01
Chromium (Cr)	0.11 ± 0.002
Cadmium (Cd)	< 0.01
Nickel (Ni)	< 0.01

Effect of daily oral administration of PPBE on urinary flow

The present study evaluated urinary flow on days 0 and 15 during the treatment period. Analysis of the data presented in Figure 2 revealed that the administration of distilled water did not lead to a significant alteration in urinary flow. However, oral administration of PPBE at both doses of 200 and 400 mg/kg B.W showed a highly significant increase in urinary output as compared to the control and furosemide groups ($p < 0.0001$). At the 200 mg/kg body weight dose, there was an elevation

in urine output from 4.5 ± 0.25 to 10 ± 0.5 ml/24 hr. Similarly, at the higher dose of 400 mg/kg body weight, urine output increased from 4.25 ± 0.37 to 9.5 ± 0.25 ml/24 hr.

In comparison, the administration of furosemide also induced a substantial increase in urine output compared to the distilled water group ($p < 0.0001$). These findings underscore the marked influence of PPBE on urinary flow, with both doses exhibiting significant effects.

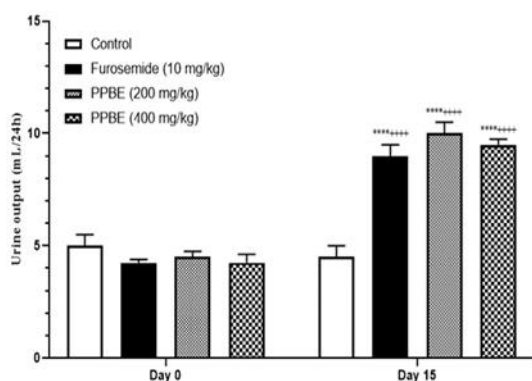


Figure 2. Urine output (ml/24 hr) with daily oral administration of PPBE, furosemide, and distilled water. Data are expressed as mean \pm SEM; PPBE D1= *P. pinaster* bark extract dose 1; PPBE D2= *P. pinaster* bark extract dose 2; *Comparison between day 0 and day 15 ****p<0.0001; +Comparison between control group and all groups in day 15++++p<0.001. # Comparison between furosemide group and PPBE ### p<0.001.

Effect of oral administration of PPBE on urinary electrolyte excretion

Table 4 outlines the effects of PPBE and furosemide on the urinary concentration of potassium and sodium. Furosemide and both PPBE doses

demonstrated a significant elevation in urinary sodium concentration compared to the control group (p<0.0001), with a dose-dependent increase. At the higher dose of 400 mg/kg body weight, a substantial value of 105 \pm 0.5 mmol/l was observed, while at the lower dose of 200 mg/kg body weight, the value was 103.5 \pm 1.75 mmol/L. Furosemide exhibited an increase of 124.8 \pm 0.2 mmol/L.

Regarding potassium, oral administration of PPBE resulted in a significant reduction in urinary potassium concentration compared to both the control and furosemide groups. As summarized in Table 4, results indicated a significant reduction in the urinary potassium elimination at both doses 200 and 400 mg/kg B.W (51 \pm 0.1 mmol/L, and 53.02 \pm 0.35 mmol/L respectively).

On the other hand, treatment with furosemide significantly increased kaliuresis to 72.1 \pm 0.1 mmol/L compared to all other groups (p<0.0001).

Table 4. Urinary excretion of sodium, potassium, and creatinine in normal rats after daily oral administration of distilled water, PPBE, and furosemide.

Groups	Urinary electrolytes (mmol/L)		Urinary excretion (mmol/24 hr)		Creatinine (mg/L)
	Sodium	Potassium	Sodium	Potassium	
Control	87.5 \pm 0.5	61.2 \pm 1.2	0.022 \pm 0.01	0.273 \pm 0.08	44.3 \pm 0.3
PPBE D1-200 mg	103.5 \pm 1.75 ****+###	51 \pm 0.1 ***+###	0.101 \pm 0.02***	0.511 \pm 0.61 ***+###	102.2 \pm 2.2 ****+###
PPBE D2-400 mg	105 \pm 0.5 ****+###	53.02 \pm 0.35 ****+###	0.090 \pm 0.009***	0.505 \pm 0.033**+	97.7 \pm 5.3 ****+###
Furosemide	124.8 \pm 0.2****	72.1 \pm 0.1***	0.082 \pm 0.01***	0.648 \pm 0.07***	53.4 \pm 1.6*

Data are expressed as mean \pm SEM; PPBE D1=*Pinus. Pinaster* bark extract dose 1 (200 mg/kg); PPBE D2= *Pinus pinaster* bark extract dose 2 (400 mg/kg); *Comparison between the control group and all groups. ***p<0.001; +Comparison between furosemide group and PPBE D1- 200 mg/kg. +++p<0.001; #Comparison between furosemide and PPE-D2 400mg group. #p<0.001

Effect of oral administration of PPBE on plasma electrolytes and creatinine levels

Table 5 describes the impact of PPBE and furosemide on serum electrolyte levels. Rats treated with two doses (200 and 400 mg/kg) did not show any significant changes in plasma concentrations of potassium and sodium compared to the control group. However,

the concentration of creatinine in the plasma was significantly reduced at the low dose of 200 mg/kg, with a value of around 3.11 \pm 0.01 g/l compared to the control (p<0.001). The high dose of 400 mg/kg did not significantly affect plasma creatinine. On the other hand, furosemide significantly decreased plasma potassium levels (p<0.001) compared to the control group, the lower and higher dose of PPBE.

Effect of oral administration of PPBE on creatinine clearance

On the final day of treatment, both PPBE and furosemide resulted in a significant elevation in creatinine clearance compared to the initial value and the control group. The increase was notably more pronounced in the PPBE-treated groups at different doses (200 and 400 mg/kg) than in the furosemide group, with the lower dose having the highest effect Figure 3).

Effect of oral administration of PPBE on osmolarity and free water clearance

The present study revealed that both PPBE and furosemide led to an increase in osmolar clearance and urine osmolarity, accompanied by a decrease in free water clearance compared to the control group ($p<0.001$). However, there was no significant impact of PPBE or furosemide on plasma osmolarity (Table 6).

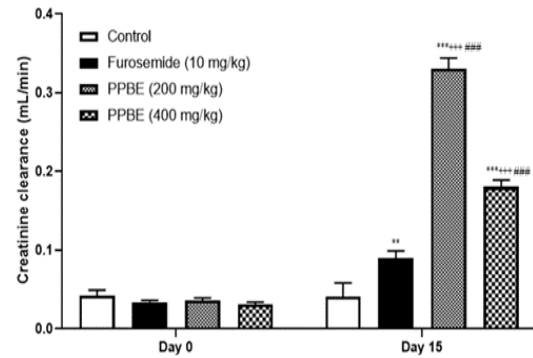


Figure 3. Creatinine clearance (mL/min) on plasma in normal rats after daily oral administration of PPBE, furosemide, and distilled water. Data are expressed as mean \pm SEM; PPBE D1= *P. pinaster* bark extract dose 1(200 mg/kg); PPBE D2= *Pinus pinaster* bark extract dose 2(400 mg/kg); *Comparison between the control group and all other groups *** $p<0.001$; +Comparison between furosemide group and PPBE D1- 200 mg/kg. +++ $p<0.001$. #Comparison between furosemide group and PPE D2- 400 mg/kg. # $p<0.001$

Table 5. Plasma concentration of creatinine, sodium, and potassium in normal rats after daily oral administration of PPBE, furosemide, and distilled water.

Groups	Plasma electrolytes (mmol/l)		Creatinine (g/l)
	Sodium	Potassium	
Control	136.03 \pm 2.03	5 \pm 0.5	5 \pm 0.5
PPBE D1-200 mg	138.33 \pm 0.67	4.8 \pm 0.02 ⁺⁺	3.11 \pm 0.01 ^{***++###}
PPBE D2-400 mg	141 \pm 1.01	4.5 \pm 0.25 ⁺	5.15 \pm 0.85 [#]
Furosemide	135.03 \pm 0.97	3.9 \pm 0.1 ^{***}	5.1 \pm 0.1

Data are expressed as mean \pm SEM; PPBE D1=*Pinus pinaster* bark extract dose 1(200 mg/kg); PPBE D2= *Pinus pinaster* bark extract dose 2 (400 mg/kg); *Comparison between the control group and all other groups. *** $p<0.001$; +Comparison between furosemide group and PPBE D1- 200 mg/kg. +++ $p<0.001$. #Comparison between furosemide group and PPE D2- 400 mg/kg. # $p<0.001$

Table 6. Impact of oral administration of PPBE, furosemide, and distilled water on urine osmolarity, plasma osmolarity, osmolar clearance, and clearance of free water on day 15.

Groups	Plasmatic osmolarity (mOsm/L)	Urinary osmolarity (mOsm/L)	Osmolar clearance (μ l/min)	Free water clearance (μ l/min)	Free water reabsorption (T_{CH2O}) (μ l/min)
Control	281.06 \pm 4.06	301.4 \pm 3.4	3.32 \pm 1.03	-0.20 \pm 0.01	0.20 \pm 0.01
PPBE D1-200 mg	285.66 \pm 1.34	313 \pm 3 ^{***++###}	7.62 \pm 0.86 ^{***}	-0.68 \pm 0.17 ^{***++###}	0.68 \pm 0.17 ^{***++###}
PPBE D2-400 mg	291 \pm 2.02	320.4 \pm 0.6 ^{***++###}	7.26 \pm 0.41 ^{***}	-0.65 \pm 0.07 ^{***++###}	0.65 \pm 0.07 ^{***++###}
Furosemide	279.06 \pm 1.94	397.8 \pm 0.6 ^{***}	8.91 \pm 1.03 ^{***}	-2.66 \pm 0.34 ^{***}	2.66 \pm 0.34 ^{***}

Data are expressed as mean \pm SEM; PPBE D1= *Pinus pinaster* bark extract dose 1 (200 mg/kg); PPBE D2= *Pinus pinaster* bark extract dose 2 (400 mg/kg) *Comparison between the control group and all other groups. *** $p<0.001$; +Comparison between furosemide group and PPBE D1- 200 mg/kg. +++ $p<0.001$;#Comparison between furosemide group and PPE D2- 400 mg/kg. # $p<0.001$

Discussion

The objective of this study was to examine the phytochemical constituents and assess the antioxidative and diuretic impacts of orally administered PPBE in Wistar rats. Pine, which has been a common plant in folk medicine remedies worldwide, has attracted considerable attention for its therapeutic potential. Different by-products from pine trees, particularly the bark, have been the focus of extensive exploration due to their abundant reservoir of phenolic compounds (Bhardwaj et al., 2021), which have proven important antioxidant activity. That plays a crucial role in protecting against oxidative stress (Toma et al., 2015). Polyphenols possess the potential to be utilized as a basis for developing new drugs.

The phytochemical screening of this research revealed that PPBE contained nine polyphenol compounds, including taxofoliol, gallic acid, catechin, epicatechin, ferulate glucoside, procyanidin B1, procyanidin B2, procyanidin B3, and proanthocyanidin. These results are aligned with those obtained by (Yesil-Celiktas et al., 2009), where four compounds are quantified using HPLC (epicatechin, catechin, taxifolin, and catechin gallate) in seven different pine species collected from both Germany (*P. ponderosa*, *P. nigra*, *P. parviXora*, and *P. sylvestris*) and Turkey (*P. sylvestris*, *P. nigra*, and *P. pinea*). In the same context, another investigation conducted by Vieito et al. (Vieito et al., 2019) identified gallic acid, taxifolin, ferulic acid, quercetin, and catechin in the phenolic profile of the hydroethanolic extract of *P. pinaster* bark collected from the northwest of Portugal.

The quantification assay of total polyphenol contents (TPC) and flavonoid contents (TFC) of PPBE showed a substantial quantity of phenolic compounds. The TPC concentration in our extract was around 34.11 ± 0.02 mg

GAE/g DM. This result is consistent with those reported by Ferreira-Santos et al. (2019), which demonstrated the high level of TPC in the bark extracts of *P. pinaster* from the north region of Portugal. Furthermore, Chupin et al. (2013) examined the bark of *P. pinaster* from the southwest of France, and they discovered that the TPC levels ranged from 22 to 62 mg GAE/g DM. Our data are superior to those found in the studies by Chupin et al. (2015), with a mean value of TPC 28.30 mg GAE/g bark of *maritime pine* from the Landes forest. Flavonoids are a subclass of polyphenols that are particularly abundant in PPBE. Flavonoids, known for their antioxidant qualities, have been linked to several health advantages. The total flavonoid contents (TFC) in our extract were around 24.96 ± 0.03 mg QE/g DM. The research conducted by Royer et al. (2013) showed that the hydroethanolic (95% ethanol) and aqueous extracts of Canadian pine species have TFC values between 6 and 39 mg quercetin equivalents/g extract. In addition, Sharma et al. (2015) derived hydroethanolic extracts (90% ethanol) with a high TFC concentration from the bark of three distinct pine species from India: *Pinus wallichiana*, *Pinus gerardiana*, and *Pinus roxburghii*. This rich and varied composition affects the antioxidant potential of PPBE and gives it a wide range of biological and pharmacologic properties.

Living systems continually produce free radicals, and an excess of these compounds can result in significant damage to tissues and biomolecules, potentially causing pathological conditions such as inflammation (Sharma et al., 2015) and impacting kidney function (Tucker et al., 2015). Utilizing antioxidants to scavenge free radicals may provide a defense against oxidative stress and help prevent cellular damage (Sharma et al., 2015). The antioxidant capacity of PPBE was measured by three different assays:

DPPH, PR, and TAC. Our result provided interesting antiradical activity. The IC_{50} value of DPPH was $9.76 \pm 1.14 \mu\text{g/ml}$, which is lower than those obtained by Ramos et al. (2022), with a mean value of $IC_{50} = 22.53 \pm 0.46 \mu\text{g/ml}$. The reducing power (RP) assay is also important, with an EC_{50} value of $0.037 \pm 0.004 \text{ mg/ml}$. This result is in line with those obtained by Apetrei et al. (2011) which show the strong reducing power of *Pinus cembra* L. bark value of $EC_{50} = 0.026 \pm 0.3 \text{ mg/ml}$. The total antioxidant capacity (TAC) of our PPBE was very significant, with a value of $975.44 \pm 24.59 \text{ mg EAA/g DM}$, and it was higher than those obtained by Jiménez-Moreno et al. (Jiménez-Moreno et al., 2019).

The originality of the current research consists of being the first to evaluate the diuretic effect of PPBE in Morocco. Diuretic medications are helpful for several illnesses, such as liver cirrhosis, hypertension, and hypercalciuria. It is important to demonstrate their effectiveness in controlling electrolytes and water balance, given their therapeutic application in managing edema (Hailu and Engidawork, 2014). The diuretic potential of PPBE was studied in the present work in normal rats. Data obtained proved that PPBE affects positive diuretic function during daily dose administration over 15 days.

PPBE is a source of essential minerals like potassium and magnesium, which are necessary for maintaining fluid balance and supporting kidney function. Our study indicated that PPBE is rich in these minerals and could potentially produce diuretic effects by influencing electrolyte balance. Both doses (200 and 400 mg/kg) of PPBE increased urinary output, urinary concentration of sodium, and decreased urinary potassium concentration compared with furosemide. This increase in sodium and water elimination could be beneficial in managing hypertension and edema diseases.

In general, diuresis is often defined by the following two phenomena: an increase in the excretion of water (urine output) and an increase in the elimination of electrolytes (Wile, 2012), similar to the action of furosemide. Furosemide enhances the rate of urine flow and promotes the excretion of sodium, potassium, and chloride. This is achieved through the inhibition of the $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ symporter in the thick ascending loop of Henle and the suppression of carbonic anhydrase (Aissaoui et al., 2008). The reason that our extract does not cause a significant increase in potassium excretion such as furosemide is probably due to various mechanisms of action. Maybe PPBE is not specifically designed to target the sodium-potassium-chloride co-transporter. Their diuretic effects are mediated through other pathways.

Moreover, the overall impact of PPBE on potassium excretion may depend on various factors such as polyphenol compounds, dosage, duration of exposure, and individual variations in renal function. It is important to note that while furosemide is a potent diuretic, it can cause excessive potassium loss, leading to hypokalemia. This is why patients taking furosemide often require monitoring of their potassium levels and may need potassium supplementation (Oh and Han, 2015). The safe use of our extract is a general assumption because it is natural and does not pose a risk of causing severe potassium depletion.

The two doses did not affect the plasma sodium, or potassium, although furosemide decreased serum potassium significantly ($p < 0.001$). As well the two doses caused an increase in creatinine clearance. These results show that our PPBE has a very strong effect on creatinine clearance, which reflects its ability to improve kidney function.

The administration of our PPBE increases water reabsorption and decreases free water clearance. It is important to consider the different mechanisms of renal

physiology. The diuretic effect may be mediated by inhibiting sodium and chloride reabsorption in the renal tubules, increasing the osmotic pressure of the renal tubular fluid, and promoting diuresis (Wile, 2012). The dual action reflects the body's need to maintain homeostasis, while diuresis increases urine output to eliminate excess fluid or solutes. Compensation mechanisms can conserve water, especially when hydration is a priority (Cadwallader et al., 2010). Furthermore, the reduction in free water clearance indicates a selective effect on dissolved water reabsorption, possibly regulating the water permeability of renal tubular cells (Natochin et al., 2021). To support these ideas, it is crucial to study the specific bioactive compounds in the extracts and their effects on the kidneys. The study by Dizaye et al. (2013) showed that certain compounds in the extract can modulate renal function and influence renal blood flow, tubular reabsorption rates, or hormonal responses (Jahromi and Jahromi, 2020).

It is still unknown what active ingredient in *P. pinaster*'s hydroethanolic extract causes the diuretic and natriuretic effects. Qualitative phytochemical screening of PPBE demonstrated the presence of flavonoids, proanthocyanidins, and phenolic acids, which possess antioxidant and anti-inflammatory properties (Jurikova et al., 2018), can contribute to improved endothelial function in blood vessels (Behl et al., 2020) which leads to improved blood flow to the kidneys and may affect renal function, increasing urine production. These compounds have been studied for their potential effects on various health conditions, including urinary tract infections, bladder dysfunction, and benign prostatic hyperplasia (BPH) (Csikós et al., 2021).

It is reasonable to suggest that these secondary metabolites may act individually or synergistically to produce the observed diuretic and natriuretic

activities of *P. pinaster* bark. Previous research has shown the diuretic and natriuretic effects of these phytochemicals through a variety of mechanisms. For instance, flavonoids, natural antagonists of A1 adenosine receptors, can trigger diuresis and sodium (Na⁺) excretion either by directly blocking Na⁺ reabsorption in the proximal tubules or by indirectly causing dilation of the afferent arterioles. The antagonistic action on these receptors is commonly linked with diuretic effects (Jacobson et al., 2002). Adenosine A1 receptors play a crucial role in the proximal convoluted tubule (PCT), where they mediate the reabsorption of 60–70% of the filtered sodium and water (Welch, 2015). Therefore, antagonists of adenosine A1 receptors preserve the glomerular filtration rate by dilating the renal afferent arterioles, enhancing renal blood flow, promoting sodium excretion (natriuresis), and increasing urine production (diuresis) (Modlinger and Welch, 2003). Additionally, some other studies have investigated the effects of pine bark extracts, such as Pycnogenol, which is derived from *P. pinaster*. Pycnogenol has been shown to improve symptoms associated with BPH, including increased urinary flow rate and reduced frequency of urination (Ledda et al., 2018).

In summary, the findings from this study indicate that the bark of *P. pinaster* of Morocco is rich in polyphenols and flavonoids, contributing to its notable antioxidant activity. The administration of two doses of PPBE resulted in considerable enhancement in urine volume, creatinine clearance, urine creatinine, and urine sodium without producing hypokalemia, in comparison to furosemide. The high diuretic activity observed in this study appears to be closely linked to the significant antioxidant components, such as the phenolic compounds, found abundantly in our extract. However, additional research is required to isolate, purify, and determine the structure of the specific diuretic

molecule responsible for this effect. It is also important to assess its safety, and long-term efficacy, and explore the potential mechanisms of action.

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Conflicts of interest

There is no conflict of interest as affirmed by the authors.

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Ethical Considerations

The animals used in this study were cared for following internationally accepted guidelines for the care and use of laboratory animals. Ethical approval was obtained from the University Sidi Mohamed Ben Abdallah in Fez, under the responsibility of the Natural Substances, Pharmacology, Environment, Modeling, Health and Quality of Life (SNAMEPEQ) laboratory. The experimental protocol was further reviewed and approved by the institutional committee on animal care at the Faculty of Science Dhar El Mahraz, University Sidi Mohamed Ben Abdallah, Fez, Morocco.

Code of Ethics

Ethical approval reference number: USMBA-SNAMEPEQ 23 FEB 2023

Authors' Contributions

Conceptualization, original draft writing, reviewing, and editing: Widad Tbatou. Abderrazak Aboulghazi: Designed the experimental protocols and

analysis test. Asmae El Ghouizi: Designed the experimental protocols, reviewing, and editing. Karima El-Yagoubi: Formal analysis, and editing. Najoua Soulo: Participated in the animal experimental work. Zineb Benziane Ouaritini, and Badiia Lyoussi: Methodology, resources, data validation, data curation, and supervision.

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