Investigation of in vitro and in vivo antioxidant and antidiabetic activities of *Pinus halepensis* extracts

Najoua Salhi[1] , Abdelhakim Bouyahya[2*] , Otman El Guourrami[3] , Meryem El Jemli[1] , Ilhame Bourais[2] , Amina Zellou[1], Yahia Cherrah[1] , My El Abbes Faouzi[1]

[1]Laboratory of Pharmacology and Toxicology, Faculty of Medicine and Pharmacy, Mohammed V University in Rabat, Morocco
[2]Laboratory of Human Pathologies Biology, Faculty of Sciences, Department of Biology, and Genomic Center of Human Pathologies, Faculty of Medicine and Pharmacy, Mohammed V University in Rabat, Morocco
[3]Laboratory of Analytical Chemistry and Bromatology, Faculty of Medicine and Pharmacy, Mohammed V University in Rabat, Morocco

A R T I C L E I N F O

A B S T R A C T

Introduction: *Pinus halepensis* is a medicinal plant used in traditional medicine for treatment of various pathologies including diabetes. The objective of this study is to perform a phytochemical study and to evaluate the antioxidant and antidiabetic activities of extracts of the bark of *P. halepensis*.
Methods: Total polyphenols, flavonoids and tannins were determined by the Folin Ciocalteu method, aluminum trichloride reagent (AlCl$_3$) and vanillin assay. Evaluation of the antioxidant activity was carried out using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and ferric reducing agent (ferric reducing). The antidiabetic activity was first revealed by enzymatic inhibition tests through measuring the residual activities of α-amylase and α-glucosidase, and then, by oral tolerance tests of glucose and starch in male Wistar rats. To verify the safety of plant extracts, acute oral toxicity was determined.
Results: The phytochemical analysis showed that the extracts of *P. halepensis* were rich in phenolic compounds. The anti-oxidation activity tests revealed a significant reducing power towards the radicals tested. In addition, *P. halepensis* inhibited the enzymes involved in diabetes (α-amylase and α-glucosidase) at very low concentrations. These effects were verified in the *in vivo* approach, in particular by using the starch tolerance test.
Conclusion: *P. halepensis* extracts showed remarkable antioxidant and antidiabetic effects. However, further investigations are necessary to identify the main compounds of *P. halepensis* and to evaluate their antioxidant and antidiabetic effects.

Introduction

Recently, several studies have shown the importance of the identification of natural compounds from medicinal plants with antioxidant and antidiabetic activities. In Morocco, the traditional medicine revealed numerous medicinal species; which could be an important source for the discovery of antioxidant, anticancer, and antidiabetic drugs. Among these medicinal plants, *Pinus halepensis* (belonging to Pinaceae family) is mainly found in the coastal areas of the western Mediterranean region including Morocco (1). This plant is used in Moroccan traditional medicine for the treatment of several diseases such as skin burn (2), against microbial infections and eczema (3), and against toothache (4). The chemical analysis of *P. halepensis* showed the presence of several chemical families such as terpenoids, polyphenols and flavonoids (5). These chemical compounds are known to exhibit numerous biological properties such as antimicrobial (6,7), anticancer (8,9), anti-inflammatory (9), antiparasitic, antioxidant and antidiabetic effects (10,11). The

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Corresponding author: Abdelhakim Bouyahya,
Email: boyahyaa-90@hotmail.fr

management of diabetes, as one the major complicated diseases, involves several therapeutic strategies such as intestinal inhibition of enzymes involved in carbohydrates degradation (12). These enzymes include α-amylase and α-glucosidase which degrade carbohydrates into simple sugars. Furthermore, the inhibition of these enzymes by natural products could be an alternative in diabetes treatment (13). Moreover, oxidative stress is implicated in the genesis of several diseases like diabetes. Thus, the combination between antioxidant and antidiabetic drugs is a promising strategy to prevent and treat the oxidative stress related diseases such as diabetes. Indeed, secondary metabolites of medicinal plants have shown several important antioxidant and antidiabetic effects (14,15). *P. halepensis* organic extracts and its volatile compounds have shown several pharmacological effects such as antimicrobial, antioxidant, anti-hemolytic, genoprotective and anti-inflammatory activities (16-20). However, at the best of our knowledge, the bark extracts of this plant have not been studied for its antidiabetic and antioxidant effects. The aim of this study was to determine the phytochemical contents and to evaluate the antioxidant and antidiabetic effects of *P. halepensis* bark extracts.

Materials and Methods
Standards and reagents
ρ-Nitrophenyl-α-D-glucopyranoside (pNPG), α-glucosidase from *Saccharomyces cerevisiae*, α-amylase from *Bacillus licheniformis*, acarbose, Folin-Ciocalteu reagent, rutin, catechin, 1,1-diphenyl-2-picrylhydrazyl (DPPH),2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid (Trolox), and ascorbic acid were purchased from Sigma-Aldrich (France). All other reagents and standards were of analytical reagent grade.

Plant collection and extraction
Barks of *P. halepensis* were collected from Khemisset Region (Morocco). The collected plant was authenticated at the Herbarium of Botany Department of the Scientific Institute of Rabat, Morocco (Voucher Specimen: RAB93519). The Barks (Figure 1) of the plant were dried at room temperature during one month; grounded into powder and then stored in glass brown bottle to preserve from light and moisture until use. For this study, two extracts (aqueous and ethanolic) were prepared. Briefly, for each extraction, 200 g of bark powder was macerated in one liter of the solvent (distilled water or absolute ethanol) under stirring in tightly closed bottles at room temperature for 24 hours. The extracts were filtered using a Buckner funnel and Whatman filter paper. Each filtrate was evaporated under vacuum using rotary evaporator at 50°C. Aqueous and ethanolic extracts were lyophilized and stored at 4°C for further use. The yield of extract recorded was 4.31% and 4.92% for the aqueous and the ethanolic extracts, respectively.

Figure 1. Bark of *Pinus halepensis*.

Animals
The animals used in this study were made up of rats and mice. For the acute oral toxicity test, nulliparous and non-pregnant Swiss mice, 2 to 2.5 months old and weighing between 24 and 36 g were used. For glucose and starch tolerance tests, male Wistar rats aged between 3 to 4 months and weighing between 150 and 200 g were used. The animals obtained from the pet store at the Faculty of Science in Kenitra were housed in cages and acclimated 2 weeks before the start of the experiment, at a temperature of 22°C (±3), at a relative humidity between 30% and 70%, and subjected to a light/dark cycle of 12/12 hours. During this period, the animals had free access to food and water. The used protocol was received the approval of a commission from the Animal Experimentation Ethics Committee of the Faculty of Medicine and Pharmacy of Rabat (FMPR). Moreover, the experimenters who practiced animal experimentation complied with the ethical rules applicable in this area.

Evaluation of acute oral toxicity study
Acute oral toxicity of *P. halepensis* extracts was carried out on Swiss female mice according to the limit test of the OECD 423 guideline at a dose level of 2000 mg/kg body weight using two steps (three rats in each step). After fasting overnight, three mice were received single oral dose of 2000 mg/kg body weight (BW). The animals were observed for gross behavioral, neurological, autonomic and toxic signs for 5 hours after extracts/vehicle administration and daily for 2 weeks. Food consumption and body weight were recorded daily for 14 days. Untreated control group receiving distilled water as vehicle was also assayed. As no toxic signs were observed, a second test was prepared containing a group of three rats treated at 48 hours intervals with the same dose for the confirmation.

Phytochemical study
Total phenolic content
Phenolic contents of *P. halepensis* extracts were determined by using the Folin-Ciocalteu reagent (21). A standard curve was evaluated using gallic acid concentrations ranging from 0.975 to 125 μg/mL. Firstly, 2.5 mL of 10% (v/v) of Folin-Ciocalteu reagent was mixed with 0.5 mL

of sample solution. The reaction was incubated at 45°C for 30 minutes after the addition of 4 mL of 7.5% (w/v) Na_2CO_3. The absorbance against blank was determined at 765 nm. TPCs were expressed as gallic acid equivalents per gram of dry weight of extract (mg GAE/g extract).

Total flavonoid content
TFC of *P. halepensis* extracts were determined as described by Dewanto et al (22). Briefly, 1 mL of the sample was added to 7.4 of distilled water and 0.3 mL of $NaNO_3$ (5%). Then, 0.3 mL of $AlCl_3$ (10% w/v) was added 5 minutes later. After 6 minutes of incubation, 2 mL of NaOH (1M) was added and this solution was mixed well and allowed to stand for 30 minutes at room temperature. The absorbance was measured against a blank at 510 nm. Calibration curve was established using Rutin as a standard in the range of 6.25-500 $\mu g/mL$. The flavonoid content was expressed as Rutin equivalent per gram of extract dry weight (mg RE/g edw).

Total tannin content
Tanin contents were determined by the method described by Julkunen-Tiitto et al (23). Briefly, an aliquot (50 μL) of each extract or standard solution was mixed with 1.5 mL of 4% vanillin (prepared with MeOH), then 750 μL HCl (12M) was added. The well-mixed solution was incubated at ambient temperature in the dark for 20 min. The absorbance was read at 500 nm. Catechin (12.5-1000 μg/mL) was used to make the standard curve and the results were expressed as mg of Catechin equivalents per gram of dry weight extract (mg CE/g edw).

Antioxidant activity
DPPH radical scavenging activity assay
Radical scavenging activity of the extracts was measured using the stable radical DPPH as described by Huang et al (24). A solution of DPPH (0.2 mM) was prepared, and 0.5 mL of this solution was mixed with 2.5 mL of the extracts (0.5–8 $\mu g/mL$). The reaction mixture was vortexed thoroughly and left in the dark for 30 minutes at room temperature. The absorbance of the mixture was measured at 517 nm in a spectrophotometer and the results were expressed as Trolox equivalent from the calibration curve, which ranged from 0.25 to 5 $\mu g/mL$. The percentage of scavenging activity was expressed as:

DPPH scavenging effect (%) = $((A0 − A1)/A0) \times 100$

Where: A_0: absorbance of the control and A_1: absorbance of the sample solution or standard.

Scavenging activity in this assay was expressed as IC_{50}, which represents the concentration of the extract required to inhibit 50% of the free radical scavenging activity.

ABTS radical scavenging assay
The ability of our extracts to scavenge the ABTS radical

was determined according to a previously described method (25). A solution of ABTS radical cation ($ABTS^{·+}$) was prepared by the reaction between 10 mL of 2mM ABTS in H_2O and 100 μL of 70mM potassium persulphate at room temperature in the dark for 24 hours. The $ABTS^{·+}$ solution was then diluted with methanol to obtain absorbance of 0.70 at 734 nm. Samples and standard were prepared at the concentration range of 2.5-50 $\mu g/mL$, 10-70 $\mu g/mL$ and 2.5-50 $\mu g/mL$ for ethanolic, aqueous extracts and Trolox, respectively. The tests were essayed in triplicate by diluting 200 μL of extracts (or standard) in 2 mL of the $ABTS^{·+}$ solution diluted with methanol and allowed to react for 1 minute. The absorbance was recorded on a spectrophotometer at 734 nm. The percentage of scavenging activity was determined by the following formula.

% of scavenging effect = $((A0 − A1)/A0) \times 100$

Where: $A0$: absorbance of the control, $A1$: absorbance of the sample solution or standard,
IC_{50} values were determined by modeling the concentration versus the % of inhibition.

Ferric reducing antioxidant power assay (FRAP)
Antioxidant activities of *P. halepensis* extracts were also tested by the FRAP method as previously described by Oyaizu et al (26) with some modifications. Ascorbic acid was used as standard to establish the calibration curve at the concentration range of 3.906–125 μg/mL. In briefly, 2.5 mL of 0.2M phosphate buffer (pH = 6.6) was added to 1 mL of sample and mixed with 2.5 mL of potassium ferricyanide solution (1%). Then, 2.5 mL of trichloroacetic acid (10%) was added to the mixture after an incubation time of 20 min at 50°C. Then, the mixture was centrifuged for 10 minutes (3000 rpm). Finally, mixture of 2.5 mL of distilled water and 0.5 mL $FeCl_3$ solution (0.1%, w/v) was added to 2.5 mL of the supernatant. Measurement was done using spectrophotometer. FARP antioxidant activities were expressed as ascorbic acid equivalent per gram of extract dry weight (mg AAE/g edw).

In vitro antidiabetic activity
α-Amylase inhibition assay
The α-amylase inhibitory of *P. halepensis* extracts was evaluated according to the protocol of Tadera et al (27) with some modifications. Briefly, a mixture of 50 μL of samples, 180 μL of phosphate buffer (pH 6.7; 0.02 M; 6 mM NaCl), and 20 μL of enzyme α-amylase (0.3 U/mL) was incubated at 37°C for 3 minutes. Then, 250 μL of 0.2% starch solution (enzyme substrate) prepared in phosphate buffer (pH = 6.7) was added to the reacting mixture. Therefore, the reaction mixture was incubated at 37°C for 15 minutes. Thereafter, 400 μL of HCl (1M) and 500 μL of Iodine (5mM) were added to the mixture and the absorbance was measured at 630 nm using UV-

Vis spectrophotometer. Acarbose was used as positive control. The percentage of inhibition was calculated by the followed formula.

% of inhibition = ((Abs $_{sub}$ – Abs $_{enz+sub}$) – (Abs $_{control}$– Abs $_{sample}$)) / (Abs $_{sub}$ – Abs $_{enz+sub}$)) *100

Where:

Abs $_{enz+sub}$: the absorption of positive control (substrate with enzyme)

Acb $_{sub}$: The absorption of negative control (substrate without enzyme)

As: The absorption of the samples (substrate with sample and enzyme)

Asb: The absorption of substrate and sample without enzyme.

Acarbose was used as a positive control. Moreover, the IC$_{50}$ values (inhibitor concentration necessary to inhibit 50% of the activity enzyme) were determined using the law equation by modulating the inhibition percentages versus the concentrations.

α-Glucosidase inhibition assay

The α-glucosidase inhibition activities of *P. halepensis* extracts were tested using the enzymatic activity measured at 405 nm by monitoring hydrolysis of *p*NPG and the results were expressed as IC$_{50}$ values. Briefly, 100 μL of sodium phosphate buffer (0.1 M, pH 6.7) containing the 0.1 U/mL α-glucosidase enzyme, were preincubated with 150 μL of the sample at 37°C for 10 minutes. 200 μL of 1mM *p*NPG solution was added to the enzymatic solution containing extract, and the reaction mixture was incubated at 37°C for 30 minutes. In this assay, acarbose was also used as positive control. The percentage of inhibition was calculated by the followed formula.

% of inhibition = ((Abs $_{sub}$ – Abs $_{enz+sub}$) – (Abs $_{control}$– Abs $_{sample}$)) / (Abs $_{sub}$ – Abs $_{enz+sub}$)) *100
Where:

Abs $_{enz+sub}$: the absorption of positive control (substrate with enzyme)

Acb $_{sub}$: The absorption of negative control (substrate without enzyme)

As: The absorption of the samples (substrate with sample and enzyme)

Asb: The absorption of substrate and sample without enzyme.

Acarbose was used as a positive control. Moreover, the IC$_{50}$ values (inhibitor concentration necessary to inhibit 50% of the activity enzyme) were determined using the law equation by modulating the inhibition percentages versus the concentrations.

In vivo antidiabetic activity
Oral glucose tolerance test (OGTT)

On six samples of non-diabetic rats, *P. halepensis* extracts were administered by gavage at two doses of 250 and 500 at mg/kg. A group of animals were given metformin (300 mg mg/kg). The animals of control group were given orally equal volume of water only. After 10 minutes, animals were administered orally with Glucose at a dose of 3 mg/kg. Blood samples were collected from the tail vein at 0, 30, 60, 90, 120, and 150 minutes. Total glycemic responses to OGTT were calculated from respective areas under the curves (AUC) of glycaemia during the 150 minutes observation period. The variation of glycaemia was defined as the difference between glycaemia at t_0 and a following time point.

Blood glucose concentrations were recorded and AUC determined. The maximum blood glucose concentration found during blood glucose determination was taken as the peak blood glucose. The formula used for AUC determination was as follows:

$$AUC\left(\frac{mg}{dL*H}\right) = \frac{(G0 + G30)}{2} \times (T30 - T0) + \frac{(G30 + G60)}{2} \times (T60 - T30)$$
$$+ \frac{(G60 + G90)}{2} \times (T90 - T60) + \frac{(G90 + G120)}{2} \times (T120 - T90)$$
$$+ \frac{(G120 + G150)}{2} \times (T150 - T120)$$

Oral starch tolerance test (OSTT)

On six samples of non-diabetic rats, *P. halepensis* extracts were administered by gavages at doses of 250 and 500 mg/kg. A group of animals were given acarbose (10 mg/kg). The animals of control group were given orally equal volume of water only. After 10 minutes, animals were administered orally with Starch at a dose of 3 mg/kg. Blood samples were collected from the tail vein at 0, 30, 60, 90, 120, and 150 minutes. Total glycemic responses to OSTT were also calculated from respective AUC of glycaemia during the 150 minutes observation period. Blood glucose concentrations were recorded and AUC determined. The maximum blood glucose concentration found during blood glucose determination was taken as the peak blood glucose. The formula for AUC determination was as follows:

$$AUC\left(\frac{mg}{dL*H}\right) = \frac{(G0 + G30)}{2} \times (T30 - T0) + \frac{(G30 + G60)}{2} \times (T60 - T30)$$
$$+ \frac{(G60 + G90)}{2} \times (T90 - T60) + \frac{(G90 + G120)}{2} \times (T120 - T90)$$
$$+ \frac{(G120 + G150)}{2} \times (T150 - T120)$$

Statistical analysis

Data were expressed as the mean values ± standard deviation (SD) for each measurement. The data were also analyzed by one-way analysis of variance (ANOVA). Post hoc procedure was used for significance of difference ($P < 0.05$).

Results
Total phenolic, flavonoid, and tannin contents

The bark of *P. halepensis* had an extraction rate of 4.31%

for the aqueous extract and 4.92% for the ethanolic extract. The contents of total polyphenols, flavonoids, and condensed tannins were determined from the calibration curves (y=0.0974x+0.0244; R^2=1), (y=0.0116x+0.0057; R^2=0.9999), and (y=0.0344x-0.0045; R^2=0.9963) plotted using standard gallic acid, rutin, and catechin, respectively. Total phenolic, flavonoid, and tannins contents of *P. halepensis* ethanol (PHEE) and aqueous (PHAE) extracts are summarized in Table 1.

Generally, it can be noted that the two aqueous and ethanolic extracts contained an important source of phenolic compounds. Indeed, the phenolic contents in PHEE and PHAE were respectively 423.51±1.14 and 401.85±3.26 mg GAE/g of extract. In addition, the flavonoid contents of the PHEE and the PHAE were respectively 865.40±12.10 and 738.97±1.39 mg RE/g of extract. Extracts of *P. halepensis* also contained significant tannin contents with 332.97±9.64 mg CE/g of extract for PHEE and 529.98±13.46 mg CE/g of extract for PHAE.

Antioxidant activity

The antioxidant activities of *P. halepensis* ethanol and aqueous extracts were evaluated using three complementary methods: DPPH and ABTS radical scavenging capacity, and FRAP. The results are summarized in Table 2. As listed, ethanol and aqueous extracts showed important antioxidant effects. Ethanolic and the aqueous of *P. halepensis* presented significant free radical neutralization capacities, with IC_{50} values of 3.28±0.37 and 3.1±0.42 μg/mL respectively for DPPH and 3.26±0.59 and 3.59±0.62 μg/mL for ABTS.

These results do not show any significant difference between the two extracts tested. By comparing the antioxidant capacity of the extracts with that of Trolox to reduce DPPH (2.42±0.18 μg/mL) and ABTS (2.61±0.34 μg/mL), it was noted that there was a significant difference between the two extracts and Trolox ($P<0.05$) used as a standard antioxidant for the two methods used. Moreover, PHEE exhibited an advantageous iron-reducing power, with an IC_{50} of 16.56±0.043 μg/mL and of 20.80±0.334 μg/mL, respectively. These results showed a significant difference ($P<0.05$) between the two tested extracts. The reducing power of the two extracts showed a lower effect compared with ascorbic acid which showed an IC_{50}=5.67±0.049 μg/mL. These results revealed a significant difference ($P<0.05$) between the extracts and the ascorbic acid used as standard.

In vitro antidiabetic effect

In vitro antidiabetic effect of *P. halepensis* ethanol and aqueous extracts was carried out by enzymatic inhibition of α-amylase and α-glucosidase. The resultants obtained was expressed as IC_{50} (Table 3).

As showed, ethanol extract exhibited more inhibitory effect on α-glucosidase and α-Amylase activities comparing to aqueous extract. Indeed, ethanol extract showed an important inhibition of α-amylase (IC_{50}=234.26±12.23 μg/mL) and α-glucosidase (IC_{50}=7.97±0.53 μg/mL). Moreover, aqueous extract inhibited α-amylase and α-glucosidase at IC_{50}=1196.38±11.80 and IC_{50}=9.37±0.41 μg/mL. IC_{50} values of PHEE and PHAE extracts were lower than those showed by acarbose (positive control) which inhibited α-amylase and α-glucosidase at IC_{50}=311.20±1.38 and IC_{50}=18.0±2.00 μg/mL, respectively.

In vivo antidiabetic effect
Oral glucose tolerance test
The results of the effect of PHAE and PHEE extracts at

Table 2. Antioxidant activity of the different parts of the plant determined by DPPH, ABTS and FRAP assays

Extracts	DPPH	ABTS	FRAP
PHAE	3.28±0.37[a]	3.59±0.62[a]	20.80±0.334[a]
PHEE	3.1±0.42[a]	3.26±0.59[a]	16.56±0.043[b]
Trolox	2.42±0.18[b]	2.61±0.34[b]	-
Ascorbic acid	-	-	5.67±0.049[c]

Different letters indicate the significant difference ($P < 0.05$) between aqueous extract, ethanolic extract and acarbose on the same column. Data are reported to mean (n = 3) ± SD. Abbreviations: PHAE, *P. halepensis* aqueous extract; PHEE, *P. halepensis* ethanolic extract.

Table 3. Enzymatic inhibition (IC_{50} values (μg/mL)) of *P. halepensis* extracts against α-amylase and α-glucosidase

Extracts	α-Glucosidase	α-Amylase
PHAE	9.37±0.41*	1196.38±11.80***
PHEE	7.97±0.53*	234.26±12.23*
Acarbose	18.01±2.00**	311.20±1.38**

Abbreviations: PHAE, *P. halepensis* aqueous extract; PHEE, *P. halepensis* ethanolic extract.
* Indicates the significant difference ($P < 0.05$) between aqueous extract, ethanolic extract and acarbose on the same column. Data are reported to mean (n = 3) ± SD.

Table 1. Total phenol and flavonoid contents of *P. halepensis* extracts

Extracts	Yield of extraction %	TPC (mg EAG/ g extrait)	TFC (mg ER/g extrait)	TTC (mg EC/g extrait)
PHAE	4.31	401.85±3.26	738.97±1.39	529.98±13.46
PHEE	4.92	423.51±1.14	865.40±12.10	332.97±9.64 mg

TPC: total phenolic content, TFC: total flavonoid content, TCC: total condensed tannins,
mg RE/g extract: mg of Rutin equivalent per gram of extract, mg GAE/g extract: mg Galic Acid equivalent per gram of extract, mg CE/g extract: mg Catechin equivalent per gram of extract.

doses of 250 and 500 mg/kg and of Metformin at the dose of 300 mg/kg, on the monitoring of blood glucose levels of normal rats subjected to an oral glucose tolerance test, are shown in Figure 2. After an oral glucose load, a statistically significant hyperglycemia was detected 30 min after all the animals were force-fed with glucose. This hyperglycemia tended to decrease over time for all treated groups. Analysis of the glucose monitoring curves (Figure 2) showed a reduction in blood sugar over time by the different products tested compared to normal saline (control batch). This reduction was lower for the batch treated with the aqueous extract and greater for the batches treated with the ethanolic extract at the dose of 500 mg/kg and with Metformin. The study of the effect of the various substances tested on the maximum blood sugar level (G Max) and on the AUC in the glucose tolerance test are shown in Table 4.

The batches of the rats treated with the aqueous extract at the doses of 250 and 500 mg/kg and with the ethanolic extract at the dose of 250 mg/kg did not show any significant reduction ($P < 0.05$) in G Max and AUC levels (Table 4) compared to the batch of control rats. The ethanolic extract at a dose of 500 mg/kg significantly ($P < 0.05$) lowered the two parameters studied compared to the control batch. Metformin also succeeded in significantly reducing ($P < 0.05$) G Max and AUC with the lowest values (Table 4).

The results of the evolution of starch tolerance in normoglycemic rats treated with acarbose and with the different extracts of the bark of *P. halepensis* are shown in Figure 3. After an oral starch load, a statistically significant hyperglycemia was detected in the control group 30 min after force-feeding the animals, tending to decrease over time. Acarbose was effective and significantly inhibited the hyperglycemia observed in control rats from the start of the experiment and throughout the experimental period. The different extracts of the bark of *P. halepensis* at

Table 4. Effect of aqueous and ethanolic extract of *P. halepensis* bark and Metformin on AUC and G Max after hyperglycemia caused by administration of glucose in normoglycemic rats

	AUC (mg/(dL*h)	G Max (mg/dL)
Control	295.29±5.17[c]	140,33±2,73[c]
Metformine (300 mg/kg)	232.88±11.65[a]	103,83±3,66[a]
PHAE (250 mg/kg)	291.63±12.74[c]	133,17±4,54[c]
PHAE (500 mg/kg)	280.71±6.76[c]	124,33±2,79[c]
PHEE (250 mg/kg)	274.88±8.58[c]	124,17±7,53[c]
PHEE (500 mg/kg)	261.92±24.72[b]	114,00±10,86[b]

Abbreviations: G Max, maximum blood sugar level; AUC, area under the curve; PHAE, *P. halepensis* aqueous extract; PHEE, *P. halepensis* ethanolic extract.
Different letters indicate the significant difference ($P < 0.05$) between aqueous extract, ethanolic extract and acarbose on the same column. Data are reported to mean (n = 3) ± SD.

different doses showed a remarkable anti-hyperglycemic effect compared to acarbose. Indeed, in addition to the total inhibition of hyperglycemia, the extracts produced a slight drop in blood sugar levels initially found. The importance of this decrease was greater in batches treated with ethanolic extract and in correlation with the concentration of extracts. The study of the effect of the different substances tested on the G Max and on the AUC in the starch tolerance test are shown in Table 5.

The batches of the rats treated with ethanolic extract and aqueous extract at different doses (250 and 500 mg/kg) and the batch treated with acarbose at a dose of 10 mg/kg showed G Max values significantly ($P < 0.05$) lower than that of the control rat group. For the AUC results, the two extracts at the different doses showed the lowest values with a significant difference ($P < 0.05$) compared to the control batch. Acarbose also showed AUC values significantly lower than that of the control batch but significantly higher than that of the batches treated with the two extracts at different doses.
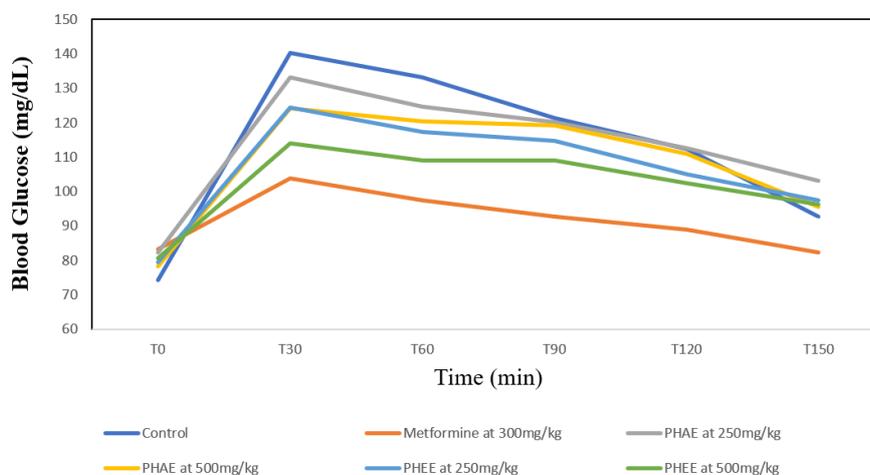
Figure 2. Blood glucose response during oral glucose tolerance test in normal rats treated with *P. halepensis* extracts and metformine. Abbreviations: PHAE, *P. halepensis* aqueous extract; PHEE, *P. halepensis* ethanolic extract.
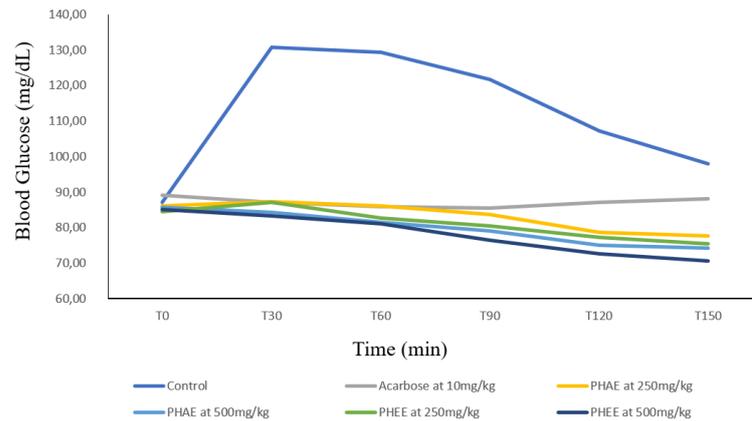
Figure 3. Blood glucose response during oral starch tolerance test in normal rats treated with *P. halepensis* extracts and Acarbose. Abbreviations: PHAE, *P. halepensis* aqueous extract; PHEE, *P. halepensis* ethanolic extract.

Toxicological investigation

Data obtained showed that 2000 mg/kg concentration of *P. halepensis* aqueous and ethanol extracts didn't induce any related signs of toxicity or mortality, during the 14 days of study, in all tested animals of each group. The treatment by each extract did not show a weight loss or changes in the behavioral pattern or any undesired pathologic changes of the animals. Therefore, the oral LD_{50} of *P. halepensis* is greater than 2000 mg/kg.

Discussion

The aim of the presented work was a phytochemical analysis and an evaluation of antioxidant and antidiabetic activities of the aqueous and ethanolic extracts of *P. halepensis*. Both extracts showed significant levels of polyphenols, flavonoids and tannins. In addition, the ethanolic extract was rich in bioactive compounds compared to the aqueous extract. Previous work reported that *P. halepensis* had several bioactive molecules belonging to secondary metabolites such as polyphenols, flavonoids, tannins, and terpenoids (5,17-19,28). Moreover, according

Table 5. Effect of aqueous and ethanolic extract of the bark of *P. halepensis* and acarbose on AUC and G Max after hyperglycemia caused by starch administration in normo-glycemic rats

	AUC (mg/(dL*h)	G Max (mg/dL)
Control	290.67±8.93[c]	130.67±3.27[c]
Acarbose (10 mg/kg)	217.08±23.81[b]	87.17±6.52[a]
PHAE (250 mg/kg)	208.83±9.13[a]	87.33±5.50[a]
PHAE (500 mg/kg)	199.88±10.28[a]	84.33±5.75[a]
PHEE (250 mg/kg)	203.79±7.32[a]	87.17±5.64[a]
PHEE (500 mg/kg)	195.46±10.51[a]	83.17±4.07[a]

Abbreviations: G Max, maximum blood sugar level; AUC, area under the curve; PHAE, *P. halepensis* aqueous extract; PHEE, *P. halepensis* ethanolic extract.
Different letters indicate the significant difference (*P* < 0.05) between aqueous extract, ethanolic extract and acarbose on the same column.
Data are reported to mean (n = 3) ± SD.

to the study by Kadri et al (29) made from the seeds of *P. halepensis* (Algerian pine), the total polyphenol contents found in the methanolic extract (50%) was 3.71 mg EAG/g of extract. This value is lower than that obtained in our study. However, Lantto et al (30) revealed a high content of total phenols (266 mg EAG/gE) in the acetone extract of the seeds of *P. halepensis*.

Pharmacological research on medicinal plants is always limited by the toxicological aspect of these plants. After the phytochemical study of the bark of *P. halepensis*, a toxicological study using acute oral toxicity in animals was performed to demonstrate the safety of *P. halepensis* extracts. The results of the acute oral toxicity limit test showed the total safety of the bark of *P. halepensis* with an estimated lethal dose 50 (LD_{50}) greater than 2 g/kg.

The bark of *P. halepensis* is used by herbalists and traditional healers in the treatment of some diseases including burns (2,31). The wide use of bark in traditional medicine confirms the results obtained in our toxicological study. This demonstrated that *P. halepensis* did not exhibit toxicity and should therefore be exploited as a promising source for identifying pharmacological bioactive compounds.

Oxidative stress is strongly implicated without the genesis of type 2 diabetes. Indeed, it plays an important role in the establishment of a state of imbalance in the regulation of blood glucose (32,33). The antioxidant effect was evaluated in vitro by three complementary tests, namely DPPH, FRAP and ABTS tests. Both extracts exhibited important antioxidant activities, in particular the ethanolic extract which reduced remarkably the tested radicals. Since we used other extract types of *P. halpensis*, our results were not completely similar to those indicated in the literature (5,9,18). Indeed, Bouyahya et al showed very important antioxidant effects of volatile substances extracted from *P. halepensis* from northern Morocco (5). In addition, Meziti et al (18) have also demonstrated that the methanolic extract of *P. halepensis* had significant

antiradical activity. The difference between the results found is certainly related to the composition of the extracts tested which are behind the antioxidant effects. Moreover, antioxidant molecules can help importantly the treatment of diabetes. Indeed, oxidative stress is involved in the genesis of diabetes and the use of natural antioxidant molecules constitutes and promising strategy for diabetes prevention (32,33).

In addition, the absorption of carbohydrates in the intestine plays an important role in the homeostasis of blood glucose. Indeed, α-amylase and α-glucosidase are two enzymes involved in the degradation of complex sugars into simple sugars. Inhibition of these enzymes decreases significantly the glucose level and may be an effective strategy for the treatment of hyperglycemia using natural products. In this way, the inhibitory activity of the extracts of *P. halepensis* against α-amylase and α-glucosidase was tested. The results showed that the ethanolic extract has an important inhibitory effect against both enzymes compared to the aqueous extract. These results exceed the effect shown by acarbose used as a positive control. To our knowledge, no study has been carried out for the antidiabetic properties of *P. halepensis*, and this is the first study that highlights the inhibitory effects of the ethanolic and aqueous extract of *P. halepensis*. In addition, several studies have shown that Moroccan plants possesses anti-enzymatic properties against the enzymes involved in the degradation of sugars in the intestine (34, 35). However, these studies remain *in vitro* investigations and do not accurately represent an *in vivo* drug effect. Thus, in our study, we also tested antidiabetic effects *in vivo* using the glucose tolerance test and the starch tolerance test. The results revealed that the extracts did not have a significant effect on the decrease in blood glucose concentration compared to Metformine (drug used in OGTT). On the contrary, the extracts, especially the ethanolic extract, showed remarkable effects in the starch tolerance test comparing to Acarbose. This shows that the antidiabetic activities of *P. halepensis* extracts are essentially dedicated by enzymatic inhibition. The results of the *in vivo* tests confirm those of *in vitro* enzymatic inhibition and show that *P. halepensis* contain bioactive substances exhibiting high antidiabetic power.

Conclusion
Both ethanolic and aqueous extracts of *P. halepensis* presented important effects with OSTT assay, while they showed significant antidiabetic effects in OGTT assay. These findings suggest that the antidiabetic activity is only mediated by the inhibition of digestive enzymes. The results of this study showed that *P. halepensis* extracts possessed important antioxidant and antidiabetic effects. The antidiabetic effects are related to the inhibition of enzymes implicated in sugar metabolism. Moreover, antioxidant effects of *P. halepensis* can also be useful to improve the management of people with diabetes. All

these results suggest that *P. halepensis* possesses several bioactive compounds which exhibit important antioxidant and antidiabetic effects. However, further investigations regarding the isolation of these main compounds and evaluation of their antioxidant and antidiabetic activities are needed.

Authors' contributions
NS conducted the experimentations, designed the analysis, and wrote a part of the manuscript. ME and OE contributed to animal experimentations. MEAF, YC and AZ conceived and supervised the study. AB analyzed the results, wrote the manuscript, and reviewed the final version. IB wrote a part of the manuscript and reviewed the final version. All read and confirmed the final version of manuscript for publication.

Conflict of interests
The authors have no conflicts of interest to declare.

Ethical considerations
Animal care and experiments were performed in accordance with the guidelines for the care and use of Laboratory of Pharmacology of the Faculty of Medicine and Pharmacy, Rabat, Morocco.

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