Phytochemical screening, antioxidant, analgesic and acetylcholinesterase inhibitory effects of *Pittosporum tobira* leaves

Ilhem Rjeibi,1 Anouar Ben Saad,1 Noura Brahmi,2 Jazia Sdayria,1 Anouar Feriani,1 Mohamed Salah Allagui,2 and Najla Hfaiedh1

ABSTRACT

The objective of this study was to determine in the first time the phytoconstituents and the in vitro antioxidant and enzyme inhibitory properties of the ethanol extract of *P. tobira* leaves (PTL) and to assess its in vivo pharmacological action on pain.

Phytochemical screening was done using standard protocols. The antioxidant potential was investigated using two methods including hydrogen peroxide and DPPH radical-scavenging assays. An assessment of the anti-acetylcholinesterase activity of PTL was performed. The analgesic activity was also evaluated using a hot plate and acetic acid assays.

Alkaloid, saponins, cardiac glycoside, terpenoids, flavonoids and reducing sugars were found to be present in the leaf of *P. tobira*. PTL showed a significant antioxidant capacity by inhibition of DPPH radical (EC_{50} = 143.4 µg/mL) and H_{2}O_{2} (EC_{50} = 46.29± 2.1 µg/mL) scavenging activities. Moreover, acetylcholinesterase enzyme was strongly inhibited by PTL with the IC_{50} value of 100.79 µg/mL. The analgesic effect of PTL was evaluated in mice after intraperitoneal injection of acetic acid (1%, 10 mL/kg, ip). The results showed that PTL reduced significantly the abdominal writhing in mice with an inhibition percentage of 14.78%, comparable with standard paracetamol (10.31%). Furthermore, PTL (350 mg / kg) increased remarkably the reaction time of mice with relatively extended duration of stimulation in the hot-plate test when compared to the control groups.

We conclude that the leaf extract of *P. tobira* possesses potential antioxidant, anti-nociceptive, and anti-acetylcholinesterase properties which could be assigned to the presence of bioactive compounds in its composition.

Keywords: *Pittosporum tobira*; analgesic; antioxidant; anti-acetylcholinesterase; bioactive compounds.

INTRODUCTION

Various illnesses including carcinogenesis, arthritis, mutagenesis, heart disease, and cardiovascular diseases have been reported to be related to oxidative damage induced by reactive oxygen species.1 Antioxidant drugs have been given to patients to treat the above diseases and escape the oxidative stress. Unfortunately, their long-term therapeutic can induces adverse effects. For these reasons, the use of medicinal plants has become the new way for several researchers to develop new drugs with greater safety and efficiency.

The plant antioxidant potential was demonstrated to be correlated with the oxidative stress defense. The beneficial health effect of the intake of the dietary plant has been essentially accredited to their bioactive compounds, mainly antioxidant polyphenolic compounds that can be found in seeds, roots, fruits, vegetables, cereals and herbs.2

Pain is a distasteful sensation coupled with several pathologic disorders, including inflammation and tissue damage. Hence, the treatment of these problems involves analgesics, notably anti-inflammatory drugs. Unfortunately, the prolonged use of synthetic drugs is usually accompanied by numerous side effects, like the neuropathy, nephrotoxicity, and irritation. Plant products, with great efficiency and weak side effects, are desirable as a source for new chemical therapeutics.

The phytopharmaceutical studies have demonstrated the antioxidant, anti-hyperalgesic, antimicrobial, inflammatory and cytoprotective activities of *Pittosporum species*.3,4

*Pittosporum tobira* (*Pittosporaceae*) have long been used as folk medicines in many countries. Modern pharmacological studies revealed that *P. tobira* possess various biological activities such as antimicrobial5 antitumor6 and neuroprotective.7 The active components from *P. tobira* were reported to be saponins, carotenoids, essential oil and triterpenoids.7 In our previous studies, we have demonstrated that *P. tobira* seed extracts have

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anti-hemolytic and antioxidant activities in vitro. However, to our knowledge, there are as yet no published studies concerning the analgesic activity of P. tobira in vivo.

The objectives of the present study were to determine the phytochemical contents, antioxidant and acetylcholinesterase inhibitory activities in vitro, as well as, to investigate the antinociceptive effect of P. tobira leaves (PTL) in mice.

MATERIALS AND METHODS

Chemicals and reagents
The chemicals and reagents used in the work were purchased from Sigma-Sigma Chemical (St. Louis, MO).

Plant material and preparation of extract
Pittosporum tobira leaves were collected in November 2015 from the southwestern Tunisia and stored in the herbarium of the Faculty of Sciences, University of Gafsa, Tunisia. Leaves (120 g) were dried and extracted for three times with ethanol (1200 L) with agitation for 24 hours. The solution were filtered using Whatman No. 1 filter paper, concentrated under reduced pressure to dryness and stored for further use.

Phytochemical Screening
To study the chemical composition of the leaf from P. tobira qualitative tests were done to screen the presence or absence of metabolites including tannins, flavonoids, terpenoids, alkaloids, reducing sugars, cardiac glycosides and saponins.

Determination of total phenolics
This test was assayed using the protocol of Dewanto et al. Briefly, 125 µL of leaves were mixed with 125 µL of Folin-Giocalteu reagent (10%). After 1 min of incubation, 1500 µL of sodium carbonate solution (7%) were added to the mixture and incubated in the dark of 90 min. The absorbance of the solution was performed at 760 nm in a UV-Vis spectrophotometer (Shimadzu, 1240 model, Tokyo, Japan).

Determination of total flavonoids
This test was assayed using the protocol of Dewanto et al. Briefly, 1000 µL of samples were mixed with 5% sodium nitrite solution (750 µL). After 5 min, 10% aluminium chloride solution (500 µL) was added and the mixture was allowed to stand for a further 5 min, and then sodium hydroxide (500 µL) was added to the solution. The absorbance was measured at 510 nm.

Antioxidant activity in vitro
DPPH radical scavenging assay
This test was monitored using the stable free radical DPPH as described by Tlili et al. In brief, 1 mL of extract solutions (100-500 µg/mL) was mixed with 2 mL of a freshly prepared DPPH solution (0.1 mM in ethanol) and kept at room temperature for 30 min in the dark. The absorbance was measured at 515 nm. DPPH scavenging effect was calculated using equation (i):

\[
\% \text{ scavenging effect} = \frac{A_0 - A_1}{A_0} \times 100
\]

Hydrogen peroxide scavenging assay
The H$_2$O$_2$ scavenging activity of leaf extract was determined according to the method reported by Liu et al. with some modifications. One milliliter of sample with different concentration (10-250 µg/mL) was mixed with 2.4 mL of phosphate buffer (0.1 M, pH 7.4) and 0.6 mL of H$_2$O$_2$ solution (40 mM). The mixture was shaken vigorously and incubated at room temperature for 10 min. Then, the absorbance of the reaction mixture was determined at 230 nm. The H$_2$O$_2$ scavenging activity was calculated using equation (ii) $A_1$ is the absorbance in the presence of the tested extract, $A_2$ is the absorbance of the control (water instead of the sample) and $A_3$ is the absorbance of the sample only (water instead of H$_2$O$_2$ solution). The Vitamin C was used as positive control.

\[
\% \text{ scavenging activity} = \frac{A_0 - A_1}{A_0} \times 100
\]

Acetylcholinesterase inhibition
Acetylcholinesterase (AChE) inhibitory activity was evaluated by the modified procedure of Ellman et al. using acetylcholinesterase (AChE) as the enzyme and acetylthiocholine iodide (ATCI) as the substrate. Briefly, 400 µL of Ellman’s reagent DTNB (5,5-dithio-bis(2-nitrobenzoic) acid), 50 µL of extract (25-200 µg/mL) and 25 µL of AChE prepared in sodium phosphate buffer (pH 8.0) were incubated for 15 min at 25°C. Then, 125 µL of ATCI was added to the mixture and kept under incubation for 10 min at 25°C. At last, the absorbance was taken at 412 nm. Galanthamine (10-50 µg/mL) was the positive inhibitor in this procedure.

Anti-nociceptive activities in vivo
Animals and treatments
The anti-nociceptive test was performed on Swiss albino white mice of about 24 ± 4 g body weight (BW). Animals were cared according to the Tunisian code of practice for the Care and Use of Animals for Scientific Purposes and the European Code of Ethic.
convention for the protection of vertebrate animals used for experimental and other scientific purposes (Council of Europe No 123, Strasbourg, 1985).

**Acute toxicity study**
Mice were divided into 5 groups, with six animals in each group. The control group received distilled water orally while the other groups received different doses of PTL (250, 500, 750 and 1000 mg/kg of BW) and observed for toxic symptoms and death rate within 12 and 24 hours.

**Acetic acid test**
The writhing phenomenon was induced by intraperitoneal injection of acetic acid 1%. Mice (n=18) were divided into three groups:
Group 1 (control), mice received distilled water.
Group 2 (PTL) mice were given PTL at 350 mg/kg BW for 60 min and then acetic acid solution was injected intraperitoneally (1%, 10 mL/kg, ip).
Group 3 (positive control) mice received paracetamol (100 mg/kg, po) and then acetic acid solution was injected intraperitoneally (1%, 10 mL/kg, ip).

The number of writhing movements was counted for 15 min and the percentage of protection was expressed using the equation of Dongmo et al.

\[
\text{Number of writhing in control} - \text{Number of writhing in treated} \times 100
\]

**Statistical analysis**
Statistical analysis was performed using the SPSS version 18.0 software. All data were analyzed using Analysis of Variance technique followed by Student’s t test. All values are expressed as mean ± SD.

**RESULTS**

**Phytochemical Properties**
The preliminary phytochemical screening carried at *P. tobira* extract proved the presence of alkaloid, saponins, cardiac glycoside, terpenoids, flavonoids and reducing sugars (Table 1).

The total phenolic content expressed as gallic acid equivalent in milligrams per gram of extract was 75.04 mg GAE/g DW. Total flavonoid content expressed as milligrams of quercetin equivalent per gram of extract was estimated to be equivalent to 11.23 mg QE/g DW.

**Antioxidant activity of PTL in vitro**
As shown in Figure 1A, the DPPH scavenging activity of PTL increased in a manner dependent of the concentration. At the concentration of 450 µg/mL, PTL showed the highest radical scavenging activity value (83.21%) but lower than vitamin C (97.13%). The effective concentrations at which DPPH were scavenged by 50% (EC50) was found to be 143.4 µg/mL. Antioxidant activity determined using the H2O2 scavenging assay showed results comparable to those obtained with DPPH scavenging activity (Figure 1B). EC50 values were found to be 46.29± 2.1 µg/mL but 2.77-fold lower than that of vitamin C (16.66± 0.32 µg/mL).

**Acetylcholinesterase inhibitory activity of PTL**
AChE inhibitory activity of the ethanol extract from *P. tobira* leaves in a dose-dependent manner was illustrated in Figure 2. Results revealed that PTL exhibited strong anti-acetylcholinesterase activity with IC50 value of 100.79 µg/mL. However, this value was ten time lower than that of Galanthamine (the reference drug, IC50 = 20.63 µg/mL).

### Table 1
**Phytochemical screening of different extract of the ethanol extract of *Pittosporum tobira* (PTL).**

<table>
<thead>
<tr>
<th>Name of phytochemical</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tannins</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>++</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>+</td>
</tr>
<tr>
<td>Reducing sugars</td>
<td>+</td>
</tr>
<tr>
<td>Cardiac glycosides</td>
<td>++</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
</tr>
</tbody>
</table>

(+) = presence of constituents; (-) = absence of constituents; (++) = presence of constituents extensively.

### Table 2
**Effect of the ethanol extract of *Pittosporum tobira* (PTL) and paracetamol on acetic acid induced writhing in mice**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Number of writhing</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>61.21 ± 4.12</td>
<td>-</td>
</tr>
<tr>
<td>PTL</td>
<td>350</td>
<td>14.78 ± 2.08**</td>
<td>75.85</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>100</td>
<td>10.31 ± 2.09**</td>
<td>83.15</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM (n = 6); percentage of inhibition of number of abdominal writhes in 20 min for each experimental group.

**p < 0.001 significantly different as compared to the control.**
ANALGESIC ACTIVITY

Acute toxicity
Animals did not show any clinical signs of toxicity up to a dose of 1000 mg/kg BW. At this selected dose of PTL, all animals survived and no mortality was observed until the end of the experiment and the lethal dose was considered to be > 1000 mg/kg BW. In this study, a dose of 350 mg/kg BW was chosen to investigate the anti-nociceptive effect of P. tobira.

Acetic Acid-Induced Writhing
The effect of PTL on writhing response in mice is shown in Table 2. PTL produced an inhibition of acetic acid-induced abdominal constrictions in mice by 75.85% at 350 mg/kg. Paracetamol used as reference drug, decreased the number of writhes by 83.15%.

Hot plate
The results of the hot plate assay (Table 3) indicated that the reaction time for the mice was significantly increased with PTL (82.53%), at 350 mg/kg. Paracetamol showed a significant protective effect (65.07% inhibition), at a dose of 100 mg/kg.

DISCUSSION

Natural antioxidants have been considered to play an important role in the prevention of many diseases. In this study, the phytochemical, anti-acetylcholinesterase and antioxidant properties of P. tobira leaf extract, as well as its capability to reduce pain were investigated.

The phytochemical screening identified the presence of numerous important medicinal compounds, such as flavonoids, saponins, cardiac glycosides, alkaloids, terpenoids and reducing sugars. These compounds are recognized to be biologically active by means of various mechanisms. Saponins play an important role in the inflamed tissue and also used for the prevention of cancer and hemolysis. Terpenoids have generally been recognized to possess anti-cancer, antinociceptive and anti-inflammatory potential. Alkaloids have numerous biological benefits, like antitussive, antinociceptive and neuroprotective properties. In addition, the phytochemical screening revealed that P. tobira is a good source polyphenols and flavonoids which are known for their potent antioxidant activities.

PTL was found to have strong DPPH radical scavenging activity which indicates the scavenging capacity of PTL bio-antioxidant compounds by their hydrogen donating ability. Furthermore, leaf exhibited important antioxidant activities on H₂O₂ scavenging activity. Indeed, antioxidants can eliminate free radical (H₂O₂) by transforming them into water. In this study, the potent antioxidant properties of PTL may be due to their richness in antioxidant components as revealed by Oh et al.²

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Table 3  Effect of the ethanol extract of *Pittosporum tobira* (PTL) and paracetamol on the hot plate test in mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Reaction time (s)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>4.81 ± 0.42</td>
<td>-</td>
</tr>
<tr>
<td>PTL</td>
<td>350</td>
<td>8.78 ± 0.68</td>
<td>82.53</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>100</td>
<td>7.94 ± 0.29</td>
<td>65.07</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM (n = 6).

"p < 0.01 significantly different from the negative control group.
Alzheimer’s disease (AD) is recognized as a neurodegenerative disorder and reducing the activity of acetylcholinesterase is the most common opinion for the treatment of patients suffering from this disease. A wide variety of plant secondary metabolites has been reported for lowering the pathogenesis of AD through various mechanisms like the modulation or inhibition of enzyme activities and the management of oxidative stress. This study, revealed that PTL exhibited interesting anti-acetylcholinesterase activity with is may be due to the important amount of phenolic compounds.

Thermal stimulation by the hot plate and chemical irritation by acetic acid are the two widely used pain models employed with the objective of identifying possible peripheral and central effects of the P. tobira leaf extract.

Acetic acid produces pain affords rapid evaluation of the peripheral analgesic action. This effect is essentially related to the stimulation of chemo-sensitive nociceptors and increase in the level of prostaglandins and histamine in peritoneal fluids. These chemical mediators stimulate peripheral nociceptive neurons and induce dilatation of arterioles and venules with contraction and separation of endothelial cells, resulting in increased vascular permeability. Results showed that the plant extract of P. tobira inhibited acetic acid-induced writhing in mice similar to paracetamol, suggesting that the analgesic effect of this plant might be linked to inhibition of the function of the above mechanisms. To confirm the anti-analgesic effect of PTL, the extracts were tested in the heat-induced pain model. The advantage of the hot plate test is the strong sensitivity to pain and little tissue damages.

PTL showed significant effect on the latency time of response to the hot plate. This analgesic property was similar to that of paracetamol. Therefore, pre-treatment of mice with PTL inhibited neurogenic-induced pain is a sign of supra-spinal analgesia of the plant extract.

In addition, the mechanism of analgesic activity of P. tobira could be due to its bioactive substances that raised pain threshold by depressing pain receptors centrally in the brain. This antinociceptive activity may be related to the reduction on the liberation of the inflammatory mediators or to the blockage of receptors resulting on peripheral antinociceptive effect.

Moreover in our recent studies, we have demonstrated that the seeds from P. tobira were sources phenolic acids including caffeic acid, p-coumaric acid, ferulic acid, and gallic acid. Such compounds have been proved to exhibit numerous pharmacutical properties especially anti-nociceptive effects.

CONCLUSION
This study has shown that the leaf ethanol extract of P. tobira is endowed with significant antioxidant and anti-acetylcholinesterase activities in vitro, associated with an antinociceptive effect in mice. However, further works are necessary in order to characterize the mechanism(s) responsible for these biological activities.

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DISCLOSURE STATEMENT
The authors declare that there is no conflict of interest.

REFERENCES
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