ANGIOTENSIN-CONVERTING ENZYME INHIBITION BY
PHTHIRUSA PYRIFOLIA(KUNTH) EICHER

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ABSTRACT
Angiotensin-converting enzyme (ACE) is part of the renin-angiotensin system and is responsible for the synthesis of angiotensin II, a potent vasoconstrictor, which relates it with the development of cardiovascular risk factors such as hypertension. Phthirusa pyrifolia, as scarcely studied species in matters of its biological activity, was evaluated in vitro to demonstrate its ability to inhibit ACE by the method used for determining the activity of this enzyme in serum, based on the enzymatic hydrolysis of Furylacryloyl - L - phenylalanyl - glycy1 – glycine(FAPGG), for serum ACE, until Furylacryloyl - L - phenyl and glycy1 - glycine (Gly - Gly). Inhibition percentages of 75.9 ± 10.4 % and 33.4 ± 9.4 % were found for the ethanolic extract of this species working in a reaction mixture concentration of 0.05 and 0.025 mg/mL, respectively. In addition, its toxicity against Artemia salina was determined finding a LD₅₀of 38.6 µg/mL. These results allow to consider Phthirusa pyrifolia as an important source of ACE inhibitory metabolites and suggest that this species may have a high toxic potential, which raises the need for toxicological studies of greater depth.

Keywords: Hypertension, angiotensin-converting enzyme inhibition, Phthirusa pyrifolia.
INTRODUCTION

The renin angiotensin system (RAS) constitutes one of the main mechanisms for blood pressure control. It comprises a series of enzymatic reactions leading to angiotensin II (Ang II), a peptide synthesized from the sequential proteolytic cleavage of its precursors and the main reaction product, which is particularly characterized by its high vasoconstrictor power (1). This feature allows to suggest a strong relationship between Ang II and the development of cardiovascular risk factors such as hypertension (2).

Hypertension, or high blood pressure, is considered to be the first risk factor for cardiovascular disease (3), which has been recognized as the leading cause of death worldwide (4). In order to control it, it has been developed a variety of drugs among which we can highlight those that have effect on the RAS and particularly on angiotensin-converting enzyme (ACE), responsible for the synthesis of Ang II, commonly called angiotensin-converting enzyme inhibitors (ACEI). Captopril, Enalapril and Fosinopril are all examples of ACEI’s currently available on the market (5). Despite of being highly effective, complications due to its administration and side effects have caused a tendency to develop new safer and more effective drugs, or, to discover of potential sources of these in biological systems, mainly in plants that have a history as antihypertensive in traditional medicine in different regions around the world (6-9).

Phthirusa pyrifolia (P. pyrifolia) or “matapalo”, as it is commonly known, is a hemi-parasitic plant from the Loranthaceae family, which subsists in many gymnosperms and angiosperms. It is widely distributed in Latin America, inhabiting areas ranging from southern Mexico to the southern coasts of Ecuador and Brazil. In Colombia, this species is found in The Andes and in the Amazon and Orinoco regions. Its ethno-pharmacology includes various uses for medical purposes. In Colombia, it has been used to treat fever, malaria and the body pain caused by malaria (10). In Brazil, this species is used as an antihelmintic and in the treatment of dry cough (11). There is evidence that within the local traditional medicine in the Department of Quindío, Colombia, P. pyrifolia is used as antihypertensive, however, there are no reports to this respect.

Knowledge about the biological activity of this species is scarce. To date, it has only been reported antimicrobial activity of a lectin derived from its leaves against various pathogenic bacteria and fungi (12). ACE inhibition properties of some species belonging to the Loranthaceae family have been studied. The aqueous, ethanolic and acetonic extracts of
Viscumarti culatum showed low percentages of inhibition when tested in vitro (13). Moreover, the methanolic extract of the aerial parts of Loranthus parasiticus showed no activity to ACE inhibition (14). To date, no reports have been found on the antihypertensive properties of P. pyrifolia or its properties to inhibit angiotensin-converting enzyme. Therefore, the objective of this study is to determine the effect of the ethanolic extract of this species on ACE activity as well as determine its toxicity against Artemia salina.

MATERIAL AND METHODS

Plant material

Phthirusa pyrifolia was collected in the facilities of the company ARAL - THEL ® Natural Products, located at Km 2 between Armenia – Calarcá, between 6 am and 7am. The specimen was identified by the HUA Herbarium, University of Antioquia (Registration number 3516-016).

Preparation of the ethanolic extract

Approximately 100 g of dried and ground material were macerated with 1 liter of 96% ethanol (EtOH) and continuously stirred for 15 days. At the end of this period the solution was filtered. This procedure was performed by ARAL - THEL ®.

The ethanolic extract of P. pyrifolia provided by ARAL - THEL ® was subjected to rotary evaporation under reduced pressure with a temperature of 40 °C to evaporate the solvent. The part of the extract destined to be used in the enzyme assay was subjected to separation of chlorophylls by adding a 1:7 ethanol/water mixture, concentrated by rotary evaporation and dried until constant weight was achieved. The solid obtained was re-suspended in 40% ethanol to achieve the concentrations needed for the assay, which were determined with an absorbance versus concentration curve. Two concentrations within the range in which the extract showed linear behavior at 345 nm were chosen. The solutions were stored at 4 °C until use.

For the toxicity assay against Artemia salina, the crude extract with chlorophylls was used. This was dry until constant weight was achieved. The solid obtained was initially diluted in Twin 20 and the remaining volume was completed with a 3.8% sea salt solution up to a concentration of 500 µg/ mL. The concentration of Twin 20 in the final solution was 0.5%. Dilutions were prepared from this solution at concentrations of 250, 175, 125, 75, 50 and 25
µg/mL using Twin 20 at 0.5% prepared in 3.8% sea salt solution. All solutions were stored at 4 °C until use.

ACE inhibition assay

Human serum was used as the source for ACE. Samples were taken from two apparently healthy volunteer males, without any diagnosed conditions like diabetes, obesity, hypertension, renopathy or some form of cardiovascular disease, who agreed to participate in the study after signing the informed consent.

Determination of ACE activity in serum was performed in vitro using the method of Simonetta Ronca - Testoni (15) modified by the Biochemistry Laboratory at the University of Quindío, plus certain considerations proposed by Serra et al. (16) for determining inhibition of ACE by plant extracts. The method is based on the enzymatic hydrolysis of Furylacryloyl - L - phenylalanyl - glycyl - glycine (FAPGG), by serum ACE until Furylacriloi - L - phenyl (FAP) and glycyl - glycine (Gly - Gly). Briefly, two tubes with 25 µl of serum each were added with 225µL of distilled water, 250 µL of buffer (0.8 mM FAPGG, 400 mMNaCl, 50 mM HEPES, pH 8.2), and plant extract at final concentrations of 0.05 and 0.025 mg / mL in the reaction mixture. As a blank, another tube was used containing exactly the same, plus EDTA 3.3 mM as ACE inhibitor. Distilled water and Captopril 500 nM were used as negative and positive controls, respectively. The tubes were incubated at 37 °C for 30 min. and left to stand on ice in order to stop the enzymatic reaction. Finally, the absorbance of each tube was measured at 345 nm using a Milton Roy Genesis 5 spectrophotometer. 10 trials per concentration were performed, 5 with each serum sample. During each test, the ACE activity with plant extract was measured in triplicate. The activity was obtained using the following equation:

\[
ACE\ activity = \left( \frac{\Delta A \times V_f \times 1000}{t} \right) \times \left( \frac{1}{0.5 \times V_s} \right)
\]

Where \( \Delta A \) is the absorbance difference between the blank and the samples, \( V_f \) is the final assay volume, \( t \) is the incubation time, 0.5 is the hydrolysis absorbance of 1 mM of FAPGG under test conditions, and \( V_s \) is the volume of the serum sample (0.025 mL). The ACE activity is expressed in units per liter (U/L). An ACE Unit (1U) is the amount of enzyme that converts 1 µmol of FAPGG in FAP and Gly - Gly per minute at 37 °C.

2.3.1 Inhibition percentage. The ACE inhibition percentage(%) for each extract was determined using the equation:
% I = [(A_c - A_s) / A_c] x 100 % \hfill (2)

Where A_c is the ACE activity of the negative control and A_s is the ACE activity in the presence of the plant extract or Captopril. Values are expressed as the average of the inhibition obtained in the ten repetitions.

**Test Sensitivity: Captopril dose-response curve**

The sensitivity of the method was assessed by a Captopril dose – response curve, a known ACE inhibitor. Concentrations of 10, 15, 30, 50, 100, 150, 300, 500 and 1000 nmol/L were tested. Six trials were conducted, three per serum sample, where each concentration of Captopril was tested in duplicate. The IC50 of Captopril was determined as the mean ± standard deviation of the values reported for the 6 trials.

**Toxicity against Artemia salina**

Toxicity tests were performed with Artemia salina obtained from TODO PESCA, Cra 7 # 12-41, Pereira - Risaralda, Colombia, according to the methodology proposed by Meyer et al. (17), taking into account some considerations proposed by Rincón et al. (18). Some modifications to these methods were implemented.

*Artemia salina eggs hatching.*

Previously certificated eggs of Artemia salina were hatched in 3.8 % sea salt solution for 24 hours in a plastic, transparent compartment, divided into two parts where one was covered to prevent the passage of light so that the larvae migrate into the illuminated portion through a net dividing the container. Subsequently, the nauplii were transferred to another vessel where they were incubated for another 24 hours under artificial light. A solution of yeast prepared in sea salt was added as food.

**Toxicity test with the extract**

After incubation, 1 mL of sea salt solution containing 10 nauplii and a drop of yeast solution were added to each well of a 24 well plate. Then, 1 mL of sea salt solution was added to each well followed by 1 ml of extract solution reaching a 3 mL final volume. The final concentrations for each extract solution were 166.7, 83.3, 58.3, 41.7, 25, 16.7 and 8.3 µg/mL. Each concentration was tested in triplicate. A control of 0.5% Twin 20 prepared in sea salt was used. The dead nauplii were counted after 24 hours of exposure and mortality rates were calculated for each dose.
Statistical analysis
All statistical analyzes were performed with the statistical software SPSS Statistics 17.0. ACE activities and percentages of inhibition for the extract at each concentration as well as for controls were compared using a one-way ANOVA followed by a Post Hoc analysis (Duncan, Tukey) establishing a level of significance when $P < 0.05$. These values are expressed as mean ± standard deviation.
Toxicity parameters $LD_{10}$, $LD_{50}$ and $LD_{90}$ were calculated by Probit analysis using the Statistical Software SPSS 17.0 having a 95% confidence interval of.

RESULTS AND DISCUSSION
The in vitro effect of the Phthirusa pyrifolia ethanolic extract on ACE activity and its toxicity against Artemia salina were investigated in this study. This plant was selected based on its use as an antihypertensive in local traditional medicine in the Department of Quindío.

ACE Inhibition
Average ACE activity in presence of P. pyrifolia ethanolic extract at concentrations of 0.05 and 0.025 mg/mL and in presence of 500 nM Captopril is shown in Figure 1. A considerable decrease in ACE activity at both concentrations is observed, with significant differences from the activity reported for the negative control ($P < 0.05$). The decrease in the enzymatic activity is considerably more noticeable when the concentration of the extract is 0.05 mg/mL. A reduction in ACE activity is also observed when working with 500 nM Captopril, also exhibiting statistically significant differences compared to the negative control ($P < 0.05$).

![Figure 1. Average ACE activity in presence of ethanolic extract of P. pyrifolia at concentrations of 0.05 mg/mL (black) and 0.025 mg/mL (grey). Where C (-) is the activity for the negative control (white) and C (+) for positive control (inclined lines). Significant differences from negative control: * $P < 0.05$.](image-url)
The inhibition percentages for each extract concentration are shown in Table 1. To date, no reports have been found relating to the ACE-inhibiting properties of *P. pyrifolia*, so the present study constitutes the first report on the matter. High percentages of inhibition for both extract concentrations can be observed, being significantly higher when working at a concentration of 0.05 mg/mL, exceeding even a 50% inhibition. Other species belonging to the Loranthaceae family have been studied with respect to their properties to inhibit ACE. Nyman et al. (13) reported percentages of inhibition around 20% for the *Viscumartii culatum* aqueous, ethanolic and aceton extracts. According to the authors, the inhibition percentage observed is not enough to consider this species for future research aimed at the identification and isolation of its active compound. Moreover, the methanol extract of the aerial parts of *Loranthus parasiticus* showed inactivity as ACE inhibitor (14). It has been established that the presence of a particular class of secondary metabolite, is frequent in organisms belonging to the same taxon (19), however, in contrast with the findings for other species of the Loranthaceae family, *P. Pyrifolia* seems to be in fact, an important source of ACE inhibitory metabolites, although the type of metabolites responsible for this effect is still unknown.

Table 1. Inhibition Percentages (% I) of the *P. pyrifolia* ethanolic extract.

<table>
<thead>
<tr>
<th>Species</th>
<th>Part used</th>
<th>Extract</th>
<th>Concentration mg/mL</th>
<th>Inhibition % *</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Phtthirusa pyrifolia</em></td>
<td>Leaves</td>
<td>Ethanolic</td>
<td>0.05</td>
<td>75.9 ± 10.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.025</td>
<td>33.4 ± 9.4</td>
</tr>
</tbody>
</table>

* Results are presented as the mean ± standard deviation of 10 repetitions.

Several authors have proposed that plant species that have shown ACE inhibition higher than 50% deserve subsequent research aimed at the identification and isolation of its active constituents (8,13,20,21). Whilst there was no intention to apply this criterion in this study, it is interesting to note that *P. pyrifolia* properly fits within these parameters and the percentage of inhibition observed for this species (75.9 ± 10.4 % at 0.05 mg / mL) propose it for further studies in order to determine its active compounds. Additionally, in similar studies the concentrations at which the plant extracts are assessed generally range from 0.1 to 0.33 mg/ml in the reaction mixture, significantly higher than those evaluated in this study for *P. pyrifolia* (0.05 and 0.025 mg/mL), which in turn reinforces the importance of considering this species as an important source of ACE inhibitory metabolites.
To date there are no reports about the antihypertensive properties of *P. pyrifolia* or about its use in traditional medicine for the treatment of hypertension. Nevertheless, in the Department of Quindío, Colombia, there is evidence that *P. pyrifolia* is used for this purpose. Based on this, our results suggest that if there is any antihypertensive effect, this may be supported by ACE inhibition. Reciprocally, ACE inhibition found for *P. pyrifolia* suggests an antihypertensive potential for this species, which also allows to consider it as a therapeutic alternative for the treatment of this disease. Further studies are recommended in this regard.

**Test sensitivity: Captopril dose-response curve**

The method sensitivity was evaluated by determining the IC50 of a reference ACE inhibitor, Captopril, using a dose-response curve (data not shown). The IC50 value for Captopril under our assay conditions was 355.8 ± 58.4 nmol/L which corresponds to a final concentration in the reaction mixture of 1.78 ± 0.29 nmol/L. To date there are no reported IC50 values for the Captopril under the same assay conditions, however, considering the large variability between IC50 values reported for Captopril (Serra et al. 2005) this result corresponds to what is described in literature. It has been reported an IC50 of Captopril of 14.1 nmol/L, which corresponds to a final concentration in the reaction mixture of 1.28 nmol / L (16). The disparity between the reported values may be due to several factors, including the differences between the sources of the enzyme for each method, test conditions and calculation methods (22).

**Toxicity against Artemia salina**

The toxicity of *P. pyrifolia* against *Artemia salina* was determined. The test is an easy, economical and effective method for preliminary assessment of toxicity. In the test, shellfish were exposed to certain concentrations of the ethanolic extract of *P. pyrifolia* for 24 hours and the lethal dose 10, 50 and 90 (LD<sub>10</sub>, LD<sub>50</sub>, LD<sub>90</sub>) were calculated based on the mortality obtained in the assay. DL<sub>10</sub>, DL<sub>50</sub>, DL<sub>90</sub> are defined as the extract concentrations at which mortality reaches a rate of 10%, 50% and 90% respectively.

Table 2 shows the values for LD<sub>10</sub>, LD<sub>50</sub>, and LD<sub>90</sub> after 24 hours of exposure. As a parameter for assessing the toxicity of a substance, several authors have defined that a plant extract can be considered active when the LD<sub>50</sub> value is below 1000 µg/mL (17,23,24). Thus, a LD<sub>50</sub>of 38.6 µg/mL suggests that the species *P. pyrifolia* has a high toxicity.
Table 2. Toxicity of the ethanolic extract of *P. pyrifolia* against *Artemia salina*

<table>
<thead>
<tr>
<th>Species</th>
<th>Part used</th>
<th>Extract</th>
<th>Toxicity against <em>Artemia salina</em> (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Phthirusa pyrifolia</em></td>
<td>Leaves</td>
<td>Ethanolic</td>
<td>DL&lt;sub&gt;10&lt;/sub&gt; (CI&lt;sub&gt;95&lt;/sub&gt;) 14.1 [5.3 – 22.2]</td>
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<td></td>
<td></td>
<td></td>
<td>DL&lt;sub&gt;50&lt;/sub&gt; (CI&lt;sub&gt;95&lt;/sub&gt;) 38.6 [25.5 – 49.5]</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>DL&lt;sub&gt;90&lt;/sub&gt; (CI&lt;sub&gt;95&lt;/sub&gt;) 105.9 [81.7 – 165.7]</td>
</tr>
</tbody>
</table>

CI: Confidence interval.

A strong correlation between toxicity assay against *Artemia salina* and cytotoxic activity against tumor cells has been described (17,24,25). According to authors, high toxicity against *Artemia salina* may well suggest the presence of metabolites with anticancer potential. This assessment is based on the fact that plant extracts that showed high levels of toxicity against *Artemia salina* also showed high cytotoxic activity when tested against various tumor cell lines. This correlation was not found by dos Santos et al. (22) who reported that most of the extracts that showed cytotoxicity in a tumor cell line showed no toxicity against *Artemia salina*. In this case the authors suggest that low toxicity against *Artemia salina* suggests less general toxicity, which in turn may indicate a possible anticancer potential of the extract components. However, the general trend seems to favor the correlation between the two tests. Based on this, the high toxicity found for the ethanolic extract of *P. pyrifolia* against *Artemia salina* may suggest the presence of metabolites with a possible cytotoxic potential against cancer cells, for which further studies in this area are recommended.

Although this result also suggests a toxic potential of *P. pyrifolia*, it is important to point out that it is not enough to rule out this species, or phytotherapeutic products thereof, as a therapeutic alternative for the treatment of some diseases, such as hypertension. However, it does propose this species to be subject of more careful management. Given this, toxicological studies of greater depth in order to determine its viability as a phytotherapeutic product for human consumption are needed. It also should be noted that the biological activity of a plant species can be due to several compounds independently, so it can be assumed that the compound or compounds responsible for the ACE inhibition observed in this study are not necessarily the same to which we can attribute the observed toxicity effect against *Artemia salina*.

**CONCLUSIONS**

The present study showed a high percentage of ACE inhibition by the ethanolic extract of *Phthirusa pyrifolia* at relatively low concentrations suggesting that this species is an
important source of ACE inhibitors secondary metabolites for which further studies are recommended in order to identify active compounds. The high toxicity against *Artemia salina* found for the ethanolic extract of *Phthirusa pyrifolia* suggests a toxic potential for this species, however, conducting further toxicological studies to confirm or rule out this assessment is necessary.

According to the findings of this study, the possibility of using the ethanolic extract of *P. pyrifolia* as a therapeutic alternative for the treatment of hypertension remains dormant. However, in-depth studies are needed to confirm this assumption.

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**REFERENCES**


