Antinociceptive Effect of Crude Extract, Fractions and Three Alkaloids Obtained from Fruits of *Piper tuberculatum*

Rosely Valéria Rodrigues, Débora Lanznaster, Daniela Tagliari Longhi Balbinot, Vinicius de Maria Gadotti, Valdir Alves Facundo, and Adair Roberto Soares Santos

This study investigated the possible antinociceptive effect of the crude extract, fractions and pure compounds (three alkaloids) obtained from fruits of *Piper tuberculatum* (Piperaceae) in acetic acid-induced visceral pain in mice. Oral administration of crude extract and fractions (CH$_2$Cl$_2$, EtOAc, methanol and hexane) (3—300 mg kg$^{-1}$) caused a dose-related and significant inhibition of the acetic acid-induced visceral nociceptive response. The crude extract, dichloromethane (CH$_2$Cl$_2$) and ethyl acetate (EtOAc) fractions were more potent than methanol and hexane fractions. The isolated alkaloids dihydro-piplartine, piplartine and 3,4,5-trimethoxy-dihydricinnamic acid (0.0001—30 mg kg$^{-1}$) exhibited significant and dose-related antinociceptive effects against acetic acid-induced visceral pain. The results show, for the first time, that crude extract, fractions and pure compounds obtained from *P. tuberculatum* produce marked antinociception against the acetic acid-induced visceral nociceptive response, supporting the ethnomedical use of *P. tuberculatum*.

Key words *Piper tuberculatum*; Piperaceae; alkaloid; pain; acetic acid

In recent years, the pharmaceutical industry has been giving considerable attention to the study of new drugs originated from natural sources. In this regard, it has been widely shown that many plant-derived compounds present significant analgesic effects. Of note, plants of the Piperaceae family are highly commercially, economically and medicinally important. In addition, chemical studies carried out on some of the species belonging to the genus *Piper* have demonstrated the presence of a great diversity of secondary metabolites with biological activities. Among these metabolites are lignans, neolignans, terpenes, chalcones, flavones, alkaloids, amides and propenylphenoles.

The genus *Piper* is widely distributed in tropical and subtropical regions of the world, and in some Northeast Brazilian communities there is widespread use of the species *Piper tuberculatum*, popularly known as “Jaborandi falso” or “pimenta darta,” used in folk medicine as an analgesic, sedative and antidote for snake bite.

It has been demonstrated that some of the extracts or compounds obtained from *P. tuberculatum* have larvicidal and insecticidal activities. Recently, Cicero Bezerra Felipe et al. have shown that piplartine, an amide alkaloid from *Piper tuberculatum*, exhibits anxiolytic and antidepressant effects in mice. Moreover, Bezerra et al. showed its inhibition of leukemia cell proliferation, while antitumor activity has also been demonstrated, in which *Piper* alkaloid-amides exhibited a potent cytotoxic activity towards tumor cell lines in culture. Additionally, the antitumor activity of piplartine was related to inhibition of the tumor proliferation rate, as observed by reduction of staining with Ki67, a monoclonal antibody that identifies a nuclear antigen associated with G1, S, G2, and M cell cycle phases, in tumors from treated animals.

Taking into account the biological activities of *P. tuberculatum*, it is surprising that no pharmacological study has been carried out on the possible antinociceptive effects of *P. tuberculatum* up to now. Here, we have therefore examined the possible antinociceptive action of the crude extract, fractions (dichloromethane—CH$_2$Cl$_2$, ethyl acetate—EtOAc, methanol and hexane) and isolated compounds (dihydro-piplartine, piplartine and 3,4,5-trimethoxydihydricinnamic acid) in the acetic acid-induced visceral nociceptive response, a classical chemical model of nociception, in mice.

MATERIALS AND METHODS

**Plant Material** *P. tuberculatum* fruits were collected in September 2005, at Porto Velho, Rondônia, Brazil. They were identified by the herbarium of Instituto de Pesquisa da Amazonia (INPA), where a voucher specimen was deposited under the number 211724.

**Extracts Preparation and Isolation of Active Compounds** The dried fruits of *P. tuberculatum* (1.1 kg) were powdered and extracted with ethanol (3 l) at room temperature. The solvent was fully evaporated under reduced pressure to yield a brown solid (36.0 g). Part of the extract (30.0 g) was chromatographed on a silica gel column and eluted with hexane, CH$_2$Cl$_2$, EtOAc and methanol, giving the respective fractions: 22.9% of hexane fraction (6.9 g), 21.3% of CH$_2$Cl$_2$ fraction (6.4 g), 22.3% of EtOAc fraction (6.70 g) and 30.0% of methanol fraction (8.9 g).

Part of the hexane fraction (4.5 g) was subjected to column chromatography over silica gel, and then eluted with mixtures of hexane and CH$_2$Cl$_2$ of increasing polarity, producing a total of 83 fractions. Fractions 10—19, after comparison by thin layer chromatography (TLC), were combined and purified by recrystallization with chloroform, resulting in 205.6 mg of a white amorphous solid (4.57%); 1. As with the hexane fraction, the CH$_2$Cl$_2$ fraction (5.1 g) was subjected to column chromatography over silica gel and eluted in the same way, resulting in 15 fractions. Fraction 9 was resubmitted to column chromatography over silica gel and eluted at the same gradient mentioned before, giving 7.1 mg of a white amorphous solid (14%); 2. The EtOAc fraction (5.1 g) was...
submitted to column chromatography and eluted with hexane, hexane/chloroform, chloroform, chloroform/methanol and methanol, with increasing polarity, resulting in 37.0 mg of compound (0.72%; 2) and 230.2 mg of a white amorphous solid (4.49%; 3). Through spectra analysis of \(^1\)H- and \(^{13}\)C-NMR unid and bidimensional, mass spectra and comparison with \(^1\)H- and \(^{13}\)C-NMR literature data \(^{11,12}\) it was possible to elucidate the structures of dihydro-piplartine \(\{N(3',4',5')-\)trimethoxydihydrocinnamoyl-\(\Delta^1\)-pyridin-2-one\} (1, Dih-Pip), pipartine \(\{N(3',4',5')-\)trimethoxyxycinnamoyl-\(\Delta^1\)-pyridin-2one\} (2, Pip), and 3,4,5-trimethoxydihydrocinnamic acid (3, TMDC) (Fig. 1). The chemical characterization of extract from \(P.\) tuberculatum was first provided by Facundo et al. \(^{15}\) and is possible that compound TMDC might be the biosynthetic precursor of Dih-Pip and Pip compounds.

Abdominal Constriction Response Induced by Intraperitoneal Injection of Acetic Acid: Experiments were conducted using Swiss mice (25—40 g) of both sexes, housed in single-sex cages under a 12 h light/12 h dark cycle (lights on at 6:00 a.m.) at a controlled temperature (22 ± 2°C) with access to food and water ad libitum. Animals (males and females were homogeneously distributed among the groups) were acclimatized at the laboratory for at least 1 h before testing and were used only once throughout the experiments. All experiments were approved by the Institutional Ethics Committee and were carried out in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals as previously specified. \(^{16}\) The numbers of animals and intensities of noxious stimuli used were the minimum necessary to demonstrate consistent effects of the drug treatments.

The procedure used for acetic acid (0.6%)-induced abdominal constriction was essentially similar to that described previously. \(^{14,15}\) Animals were pretreated orally via gavage per os (p.o.) with crude extract or fractions (3—300 mg kg\(^{-1}\)) 1 h before testing, or intraperitoneally (i.p.) with compounds namely dihydro-pipartine, pipartine and 3,4,5-trimethoxydihydrocinnamic acid (0.0001—30 mg kg\(^{-1}\)) 30 min before testing. Due to the limited availability of these compounds, it was not possible to test them orally. Control animals received the same volume of vehicle (10 ml kg\(^{-1}\)) by i.p. or p.o. route. After challenge, mice were placed in separate boxes and the number of abdominal constrictions was cumulatively counted over 20 min.

**Drugs**  
The drugs used were acetic acid and Tween 80 (Merck AG, Darmstadt, Germany). All other reagents used were of a high grade of purity. The degree of purity of the compounds obtained from \(P.\) tuberculatum was >98%. The crude extract, fractions and pure compounds from \(P.\) tuberculatum were dissolved in Tween 80 and diluted just before use in 0.9% NaCl. The final concentration of Tween 80 did not exceed 5% and did not have any effect itself.

**Statistical Analysis**  
The results were presented as means±S.E.M. of six to nine animals. The significance of differences between groups was analyzed by means of analysis of variance followed by Newman–Keuls’ multiple comparison test, with \(p<0.05\) being considered as indicative of significance. The ID\(_{50}\) values (i.e., the dose of the extract, fractions or compounds necessary to reduce the pain response by 50% in relation to the control value) were reported as geometric means accompanied by their respective 95% confidence limits. The ID\(_{50}\) values were determined by linear regression from individual experiments with linear regression GraphPad Software.

**RESULTS**  
\(P.\) tuberculatum fruits were extracted with ethanol (EtOH) at room temperature and the crude extract obtained was partitioned into hexane-, dichloromethane–CH\(_2\)Cl\(_2\)-, ethyl acetate–EtOAc- and methanol fractions. Repeated fractionation over silica gel columns of hexane-, CH\(_2\)Cl\(_2\)- and EtOAc-fractions yielded compounds Dih-Pip, Pip and TMDC that had been previously described \(^{11}\) (Fig. 1). The structures of Dih-Pip, Pip and TMDC were elucidated by comparison of their spectroscopic data \((\text{H-}, \text{C-NMR})\) with data in the literature.

Oral treatment of animals with crude extract and fractions (CH\(_2\)Cl\(_2\), EtOAc, methanol and hexane fractions) alone did not produce any irritation (data not shown), but did cause a dose-related and significant inhibition of the acetic acid-induced visceral nociceptive response in mice (Fig. 2). The calculated mean ID\(_{50}\) value (95% confidence limits) and inhibition values are shown in Table 1. At the ID\(_{50}\) level, crude extract, CH\(_2\)Cl\(_2\) and EtOAc fractions were approximately 1.2- to 3.3-fold more potent than methanol and hexane fractions and a well-known anti-inflammatory and analgesic drug (as-

**Table 1. Inhibition by Crude Extract, Fractions and Isolated Compounds from \(P.\) tuberculatum of Acetic Acid-Induced Abdominal Constriction**

<table>
<thead>
<tr>
<th>Drugs</th>
<th>ID(_{50}) (mg kg(^{-1}))(^a)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane fraction</td>
<td>154.1 (135.3—175.4)</td>
<td>75±8</td>
</tr>
<tr>
<td>Dichloromethane (CH(_2)Cl(_2)) fraction</td>
<td>46.6 (32.6—66.7)</td>
<td>77±8</td>
</tr>
<tr>
<td>Ethyl Acetate (EtOAc) fraction</td>
<td>49.8 (35.6—69.7)</td>
<td>71±7</td>
</tr>
<tr>
<td>Methanol fraction</td>
<td>61.5 (48.0—77.4)</td>
<td>77±7</td>
</tr>
<tr>
<td>Crude extract</td>
<td>46.2 (38.0—56.1)</td>
<td>89±5</td>
</tr>
<tr>
<td>Dihydro-pipartine</td>
<td>0.07 (0.04—0.1)</td>
<td>56±7</td>
</tr>
<tr>
<td>Pipartine</td>
<td>5.4 (4.2—6.9)</td>
<td>82±7</td>
</tr>
<tr>
<td>3,4,5-Trimethoxydihydrocinnamic acid</td>
<td>0.0031 (0.0018—0.0053)</td>
<td>81±5</td>
</tr>
<tr>
<td>Aspirin(^b)</td>
<td>23.9 (13.1—43.6) (i.p.)</td>
<td>83±2</td>
</tr>
<tr>
<td></td>
<td>108.7 (92.7—126.6) (p.o.)</td>
<td>82±5</td>
</tr>
</tbody>
</table>

\(^a\) 95% confidence limits. \(^b\) Data from De Campos and colleagues. \(^{23}\)
pirin) (Table 1). Thereafter, the CH$_2$Cl$_2$, EtOAc and hexane fractions were selected for the isolation of compounds.

In the next stage of this study, the effect of isolated compounds was examined in the acetic acid-induced abdominal constriction test. Dih-Pip, Pip and TMDC exhibited significant and dose-related antinociceptive actions in this model of visceral pain (Fig. 3). The calculated mean ID$_{50}$ values (and 95% confidence limits) and inhibition values are shown in Table 1. At the ID$_{50}$ level, compounds Dih-Pip, Pip and TMDC were approximately 8.5- to 49700-fold more potent than the crude extract and fractions obtained from _P. tuberculatum_. Dih-Pip was 28.4 times more potent than the hexane fraction (original fraction), while Pip and TMDC were 666- and 16080-fold more potent than CH$_2$Cl$_2$ and EtOAc, respectively.

Furthermore, TMDC was 1748.4- and 22.6-fold more potent than Dih-Pip and Pip, respectively.

**DISCUSSION**

This study showed for the first time that crude extract, fractions and compounds isolated from _P. tuberculatum_ possess an antinociceptive effect against visceral pain evoked by the intraperitoneal injection of acetic acid in mice. It is well known that _P. tuberculatum_ is widely used in folk medicine as analgesic and sedative, as well as antidote for snake bite.

The results reported here indicate that the crude extract and EtOAc, CH$_2$Cl$_2$, hexane and methanol fractions obtained from _P. tuberculatum_ inhibited, in a dose-dependent manner, the nociceptive response elicited by acetic acid. To our knowledge this is the first report of this kind in the literature. When compared with aspirin, a well-known non-steroidal anti-inflammatory drug (NSAID), crude extract and EtOAc, CH$_2$Cl$_2$ and methanol fractions were 1.8- to 2.4-fold more potent in inhibiting the acetic acid-induced nociceptive response. However, the hexane fraction was 0.7-times less potent than aspirin in this model of visceral pain.

Furthermore, the isolated compounds were more potent, when compared with aspirin, in inhibiting the writhing response. TMDC, which exhibited the lowest ID$_{50}$, was up to 7709.7-fold more potent than aspirin, while Dih-Pip and Pip were 341.4-fold and 4.4-fold more potent, respectively, than the NSAID mentioned before. Moreover, it could be suggested that these compounds contributed, at least in part, to the antinociceptive effect of CH$_2$Cl$_2$, EtOAc and hexane fractions obtained from _Piper tuberculatum_ fruits, once they demonstrate more potency in inhibiting the nociceptive response even at lower doses. The isolated compound that obtained major yield, Pip, was more effective in inhibiting the nociceptive response, but was not the most potent among the...
compounds tested. Otherwise, TMDC, the most potent treatment tested here, was the isolated compound with lower yield. Therefore, it could be suggested that isolated compounds might synergically contribute to the action of crude extract, once crude extract was the most potent between the oral treatments.

The abdominal writhing induced by acetic acid in mice, described as a typical model of visceral inflammatory nociception, has long been used as a screening tool for the assessment of analgesic and anti-inflammatory properties of synthetic and natural compounds. It has been accepted that acetic acid acts by releasing endogenous substances that excite primary sensory neurons, such as endogenous inflammatory mediators (i.e. kinins, prostanooids, NO and substance P), and cytokines (i.e. TNF- α, IL-1 β and IL-8), modulated by mast cells and macrophages resident in the peritoneal cavity. These substances will stimulate primary afferent neurons, enhancing aspartate and glutamate release at cerebrospinal fluid. Taken together, those previous findings and the results presented here might indicate that the antinociceptive action of crude extract, fractions and compounds isolated from P. tuberculatum in the acetic acid abdominal constriction test could be due to inhibition of the release of TNF- α, interleukin-1 β and interleukin-8 by resident peritoneal cells. Another possibility is that the crude extract, fractions and compounds isolated from P. tuberculatum inhibits the further activation of glutamate systems caused by acid. However, these possibility remains to be tested in future studies.

Recently, Fontenele et al. showed that Pip significantly inhibited the human platelet aggregation induced by arachidonic acid, collagen or ADP. The authors suggest that the Pip-antiplatelet action could be related to the inhibition of cyclooxygenase activity. Thus, it could be suggested that the isolated compound with lower yield, TMDC, the most potent between the oral treatments, was the isolated compound with lower yield. Therefore, it could be suggested that isolated compounds might synergically contribute to the action of crude extract, once crude extract was the most potent between the oral treatments.

In conclusion, the present results showed, for the first time, that crude extract, fractions and the isolated alkaloids dihydro-pipilartine, pipilartine and 3,4,5-trimethoxydihydrocinamic acid, obtained from P. tuberculatum, had significant antinociceptive actions in the visceral pain model in mice. Furthermore, these data support, at least in part, the ethnomedical use of P. tuberculatum.

Acknowledgments This study was supported by grants from CAPES and CNPq, Brazil. RV Rodrigues is a PhD student in experimental biology, D Lanznaster is an undergraduate biology student and DT Longhi-Balbinot is a PhD student in Neuroscience. They thank CNPq and CAPES for financial support.

REFERENCES