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Phytochemical study guided by the myorelaxant activity of the crude extract, fractions and constituent from stem bark of *Hymenaea courbaril* L.

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ABSTRACT

Ethnopharmacological relevance: *Hymenaea courbaril* L. (Caesalpinoideae) is used in Brazilian folk medicine to treat anemia, kidney problems, sore throat and other dysfunctions of the respiratory system, such as bronchitis and asthma, although such properties are yet to be scientifically validated.
Aim of the study: In order to give a scientific basis to support the traditional use of *Hymenaea courbaril*, this study was designed to evaluate antioxidant, myorelaxant and anti-inflammatory properties of the ethanol extract from stem bark and its fractions. The myorelaxant effect of astilbin, a flavonoid isolated from the bioactive ethyl acetate fraction (EAF), has also been evaluated.
Material and methods: In the present study ethanol extract from stem bark (EEHC) and fractions were analyzed using bioassay-guided fractionation. The following activities were investigated: antioxidant by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, myorelaxant on rat tracheal smooth muscle, and anti-inflammatory using ovalbumin-induced leukocytosis and airway hyperresponsiveness in rats.
Results: The results of the present investigation show that the whole extract of *Hymenaea courbaril* and some of its fractions strongly scavenged DPPH radical. The extract showed myorelaxant activity on rat trachea, being EAF its highest efficient fraction. Bio-guided study allowed the isolation of astilbin, a well-known flavonoid. The activity induced by this compound indicates that it may be partly responsible for the myorelaxant effect of EAF. EAF reduced contractions that depended on divalent cation inflow through voltage-operated Ca^{2+} channels (VOCCs) or receptor-operated Ca^{2+} channels (ROCCs), but it was more potent to inhibit VOCC- than ROCC-dependent contraction induced by Ca^{2+} addition in ACh-enriched Ca^{2+}-free medium. Oral pretreatment of antigen-challenged animals with EAF prevented airway hyperresponsiveness on KCl-induced contraction and reduced the number of total white cells, particularly eosinophils and neutrophils in bronchoalveolar lavage.
Conclusions: This study provided scientific basis that *Hymenaea courbaril* presents potential antioxidant, myorelaxant and anti-inflammatory actions, which support its use in folk medicine to treat inflammatory airway diseases.

1. Introduction

The genus *Hymenaea* (Fabaceae, Caesalpinioideae) includes fourteen species, nine of them found in several regions in Brazil including the lowland tropical ecosystems that follow uniform distribution in the Amazon forest (Lee and Langenheim, 1975; Campos and Uchida, 2002). Most species of this genus has economic value by providing high quality wood, resins, fruits and edible barks rich in tannins, a fact which justifies their use in folk medicine. *Hymenaea courbaril* L., popularly known in Brazil

Abbreviations: ACh, acetylcholine; AcOEt, ethyl acetate; BALF, bronchoalveolar lavage fluid; CCh, carbachol; CH_2Cl_2, dichloromethane; DEAF, dichloromethane: ethyl acetate fraction; DF, dichloromethane fraction; DPPH, 1,1-diphenyl-1-picryl-hydrazyl; EAF, ethyl acetate fraction; EEHC, *Hymenaea courbaril* stem bark ethanol extract; HDF, hexane:dichloromethane fraction; Hex, hexane; HF, hexane fraction; MeOH, methanol; MF, methanol fraction; NMR, nuclear magnetic resonance; OVA, ovalbumin; ROCCs, receptor-operated Ca^{2+} channels; TLC, thin layer chromatography; VOCCs, voltage-operated Ca^{2+} channels

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as "jatobá", is a tree whose leaves, roots, fruits, and especially the stem bark are traditionally employed in folk medicine by means of infusions and decoctions to treat anemia, kidney problems, sore throat and other airway diseases such as bronchitis and asthma (Cartaxo et al., 2010).

Beyond the presence of polyphenolic constituents, several other compounds - mainly *enantio*-labdanoic and *enantio*-halimane type diterpenes and sesquiterpenes - have been isolated from the seed pods (Nogueira et al., 2001; Jayaprakasam et al., 2007), stem bark (Nogueira et al., 2002), trunk resin (Cunningham et al., 1974; Marsaioli et al., 1975), and the peel of the ripe fruits (Aguiar et al., 2010) of *Hymenaea courbaril*. Chemical analysis of the yellowish sweet powder obtained from its fruits yielded sucrose and linolenic acid (Jayaprakasam et al., 2007). Furthermore, the sesquiterpenes α-copaene, spathulenol and β-selinene were identified in the essential oil from the peel of the ripe fruits, while germacrene-D, β-caryophyllene and bicyclogermacrene were the major compounds in the oil from unripe fruits (Aguiar et al., 2010).

So diverse chemical composition may provide the known antioxidant, anti-inflammatory (Jayaprakasam et al., 2007), antiviral (Cecílio et al., 2012) and anticancer (Keiji et al., 1999) properties already reported to extracts, fractions or compounds isolated from *Hymenaea courbaril*. The essential oil obtained from the peel of the fruits also possesses strong larvicidal activity against *Aedes aegypti* (Aguiar et al., 2010).

Considering the importance of *Hymenaea courbaril* L. to Brazilian folk medicine, the present work was carried out to establish the antioxidant, anti-inflammatory and myorelaxant effects of this species through bioassay-guided fractionation of the ethanol extract of the stem bark, describing the isolation and identification of a known flavonoid astilbin, in order to scientifically support its properties and medicinal use.

2. Material and methods

2.1. Plant material

The stem bark of *Hymenaea courbaril* L. was collected in April 2011 in Crato, State of Ceará, Brazil. The botanical identification was obtained by comparison with a voucher specimen (#EAC 49901) deposited at the Prisco Bezerra Herbarium, Departamento de Biologia, Universidade Federal do Ceará, Ceará, Brazil.

2.2. Animals

Male wistar rats (200–300 g) were housed under standard conditions with free access to food and water at the vivarium of the Universidade Federal do Ceará, being the study protocol submitted to and approved by its local Animal Ethics Committee (protocol no. #37/12).

2.3. Solutions and drugs

The physiological salt solution was a modified Krebs–Henseleit solution of the following composition: 118.0 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25.0 mM $NaHCO_3$, 1.2 mM KH_2PO_4, and 10.0 mM glucose. Solutions with a high KCl content were prepared by adding KCl to the bath from a 3 M KCl solution in distilled water.

Carbachol (CCh), acetylcholine (ACh), verapamil, glycol ether diamine tetraacetic acid (EGTA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ovalbumin, pentobarbital were purchased from Sigma (St. Louis, USA), and deuterated methanol from Tedia (Ohio, USA). Salts, reagents and solvents (all of analytical grade) were purchased from Sigma or Merck (Darmstadt, Germany).

2.4. Preliminary phytochemical screening

The phytochemical profile was determined following the procedures described by Matos (2009), in which the identification reactions were based on the presence of chemical groups or revealed by thin layer chromatography (TLC).

2.5. Preparation of the extract, bioassay-guided fractionation and astilbin isolation

Air-dried stem barks of *Hymenaea courbaril* (500 g) were triturated and subjected to exhaustive extraction by conventional maceration with ethanol (13 × 1 L) at room temperature for a period of 7 days. After filtration, the solvent was removed at 40 °C under reduced pressure, to yield 86 g of an ethanol extract of *Hymenaea courbaril* (EEHC). After evaluation of the myorelaxant activity, EEHC was submitted to bioassay-guided fractionation in column chromatography over silica gel (Merck 60–120 mesh) using hexane (Hex), dichloromethane (CH_2Cl_2), ethyl acetate (AcOEt) and methanol (MeOH) employed pure, or in binary mixtures (1:1) according to the polarity profile of the eluent, which resulted in 6 fractions (yield values are shown as percentage w/w of the whole extract weight): hexane (HF; 0.094%), hexane:dichloromethane (HDF; 0.25%), dichloromethane (DF; 1.62%), dichloromethane:ethyl acetate (DEAF; 1.25%), ethyl acetate (EAF; 2.41%) and methanol (MF; 76.68%) fractions. HF and HDF were not submitted to bioassays due to their low yield on chromatographic treatment.

Except for those showing low yield (HF and HDF), fractions were then pharmacologically tested in isolated preparations of rat trachea contracted with either CCh or with a high K^+ concentration (60 mM). Once identified a given bioactive fraction (EAF), 1.5 g of EAF were subjected to column chromatography using silica gel (as the stationary phase), eluted initially with hexane followed by more polar eluents, such as dichloromethane, ethyl acetate and methanol. In total, 230 fractions (5 mL each) were collected and analyzed by TLC. Those showing a similar result were combined. The fraction 180–189 (49.6 mg), by revealing apparent low chemical complexity in TLC, was solubilized in acetone and their soluble part (37.3 mg) was submitted to chromatography on Sephadex LH 20 with MeOH, yielding 26 fractions (2 mL each), that were analyzed by TLC. The subfraction 11–15 (30.3 mg, yield 2.02% w/w; percentage of the EAF weight) named HCC-4 was submitted to NMR analysis, which allowed the identification of astilbin.

2.6. Evaluation of the antioxidant properties of Hymenaea courbaril extract and fractions

The antioxidant activity of the extract and fractions were evaluated by measuring the reduction of the free radical 1, 1-diphenyl-1-picrylhydrazyl (DPPH). The samples (1.0 to 1000.0 μg/mL) were dissolved in methanol and then added to a methanol solution of DPPH (60 μM). After 30 min., the UV absorbance of the resulting solutions was recorded at λ 517 nm (Brandy-Williams et al., 1995). The experiment was performed in triplicate and the average absorption was noted for each concentration. Trolox was used as the positive control. The free radical scavenging activity was calculated as a percentage inhibition of the DPPH radical by the sample or positive control. The IC_{50} value is the concentration required to scavenge 50% DPPH.

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