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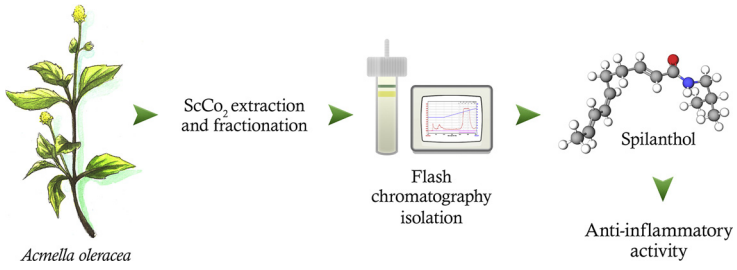
Isolation of spilanthol from *Acmella oleracea* based on Green Chemistry and evaluation of its *in vitro* anti-inflammatory activity

Veronica Santana de Freitas Blanco[a,b], Barbara Michalak[c], Ícaro Augusto Maccari Zelioli[d], Adriana da Silva Santos de Oliveira[e], Marili Villa Nova Rodrigues[e], Antônio Gilberto Ferreira[f], Vera Lucia Garcia[e], Fernando Antônio Cabral[d], Anna Karolina Kiss[c], Rodney Alexandre Ferreira Rodrigues[a,b,*]

[a] *Pharmacology, Anesthesiology and Therapeutics, Department of Physiological Sciences, Piracicaba Dental School, University of Campinas, Piracicaba, SP, Brazil*
[b] *Chemical, Biological, and Agricultural Pluridisciplinary Research Center (CPQBA), Department of Chemistry of Natural Products, University of Campinas, Campinas, SP, Brazil*
[c] *Medical University of Warsaw, Department of Pharmacognosy and Molecular Basis of Phytotherapy, Warsaw, Poland*
[d] *Laboratory of Extraction, Applied Thermodynamics and Equilibrium, Department of Food Engineering, Food Engineering Faculty, University of Campinas, Campinas, SP, Brazil*
[e] *Chemical, Biological, and Agricultural Pluridisciplinary Research Center (CPQBA), Department of Organic and Pharmaceutical Chemistry, University of Campinas, Campinas, SP, Brazil*
[f] *Federal University of São Carlos, Chemistry Department, NMR Laboratory, São Carlos, SP, Brazil*

GRAPHICAL ABSTRACT



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ABSTRACT

Acmella oleracea (jambu) is a South American plant whose pharmacological properties include antimalarial, anesthetic, and anti-inflammatory activities. However, the compounds contributing to the anti-inflammatory effect have received little attention. In this study, the active compound spilanthol was isolated using a Green Chemistry approach and presented significant anti-inflammatory activity in a lipopolysaccharide-activated human neutrophil model. First, the aerial parts of jambu were extracted and fractionated using supercritical carbon dioxide. The spilanthol-enriched fractions obtained were then subjected to further purification by flash chromatography, yielding spilanthol with 97% purity. Use of enzyme-linked immunosorbent assay (ELISA) revealed a reduction in the release of Interleukin-8 and Tumor Necrosis Factor - alpha by leukocytes exposed to spilanthol. In conclusion, this new approach enabled spilanthol to be obtained at high purity, in a fairly rapid procedure, while the results of the *in vitro* anti-inflammatory activity study indicated that the compound could be a promising new therapeutic agent.

* Corresponding author.
E-mail address: rodney@cpqba.unicamp.br (R.A.F. Rodrigues).

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1. Introduction

Concerns about protection of the environment have increased in recent decades, leading to the emergence of new protocols to reduce chemical pollution in several countries. The aim is to encourage sustainable development, seeking alternatives that avoid or minimize toxic waste production [1].

The extraction of compounds from plants involves a number of operations that usually require large amounts of hazardous solvents, whose residues may remain present in the final crude extract [2]. For this reason, there is great interest in the use of processes that can eliminate or reduce the use of organic solvents, such as supercritical fluid extraction. The efficacy, user safety, and chemical purity of isolated bioactive compounds obtained using the supercritical extraction technique combined with flash chromatography have already been proven experimentally [3].

The unique density and viscosity properties of a supercritical fluid enable it to penetrate a solid material more effectively, compared to ordinary liquids, resulting in greater diffusion, faster extraction, and no toxic residues in the final extract [4,5]. Extraction followed by supercritical fluid fractionation can be useful for improving the selectivity of the extraction. This technique exploits the different solubilities of compounds to be extracted by the supercritical fluid, with the selectivity being manipulated by varying the temperature and pressure conditions of a series of separators coupled to the extraction system [3].

Acmella oleracea (L.) R. K. Jansen (syn. *Spilanthes acmella* (L.) Murray), belonging to the family Asteraceae, is a plant native to South America, where it is locally used in cooking and for the treatment of toothache. In Brazil, it is popularly known as “jambu” or “Brazilian watercress”. In some other countries, it is known as “toothache plant", "eyeball plant", "para cress", or "akarkara" [6,7]. Numerous pharmacological properties of *Acmella* have been described in the literature, including diuretic [8,9], antimalarial [10], acaricidal [11,12], anesthetic [13–15], antioxidant [16], vasorelaxant [17], immunomodulatory [18], and larvicidal [19,20] activities.

The anti-inflammatory and analgesic activities of *Acmella oleracea* have also been the subject of several studies. Ratnasooriya & Pieris [21] demonstrated anti-inflammatory and anti-hyperalgesic effects of the aqueous extract of fresh *Acmella oleracea* flowers at dosages ranging from 500 to 1500 mg/kg, administered orally to rats. Dose-dependent effects were observed in models of acute and persistent inflammatory pain induced by formalin, as well as hyperalgesia induced by carrageenan.

In a preliminary study, Chakraborty et al. [22] reported the anti-inflammatory and analgesic activities of the extract of aerial parts of *Acmella oleracea* in experimental models using Wistar rats and Swiss mice orally administered with dosages between 100 and 400 mg/kg. Significant dose-dependent anti-inflammatory and analgesic effects were observed in the models of carrageenan-induced paw edema and abdominal contortions induced by acetic acid, as well as in the tail flick test.

Gupta et al. [23] evaluated a topical gel formulation containing 1% petroleum ether extract of jambu flowers, using a carrageenan-induced paw edema model. It was found that the anti-inflammatory activity of jambu gel was similar to that of sodium diclofenac gel. In addition, the formulation did not cause any symptoms of irritation or erythema in a skin irritation model using rats.

Nomura et al. [24] described the orofacial antinociceptive activity of the ethanolic extract of fresh jambu flowers at dosages of 10, 30, and 100 mg/kg, administered intraperitoneally in mice. There were decreases of neurogenic and inflammatory phases in formalin and capsaicin models, as well as decreased hyperalgesia in the hot plate model. These effects were suggested to be linked to modulation or blockade of transient potential receptors TRPV1 and TRPA1.

Inflammation plays an important role in the defense system of humans, as a response to an infection. The inflammation is beneficial, stimulating the healing process and initiating neutrophil recruitment. Neutrophils are released into the circulation, where they are the most abundant type of leukocyte. Once released, the neutrophils begin to seek signs of inflammation and, in a series of events, undergo transmigration through the vessel wall towards the inflamed tissue. After infiltration of the inflamed site, they generate chemokines and cytokines (mainly IL-8 and TNF-α). Interleukin 8 is a cytokine that has selective chemotactic activity for neutrophils and lymphocytes, at nanomolar and picomolar concentrations, respectively. This cytokine may be involved in leukocyte-vascular endothelial interactions, such as the invasion of neutrophils through a vessel wall by means of integrin attachment. Tumor necrosis factor (TNF) is a multifunctional cytokine considered to be a central mediator of a broad range of biological activities. These activities provide beneficial effects to the host in terms of inflammation and protective immune responses against a variety of infectious pathogens [25,26].

When RAW 264.7 macrophages stimulated with lipopolysaccharide (LPS) were exposed to spilanthol (syn. Affinin, $C_{14}H_{23}NO$, 221.339 g/mol), an alkylamide found in abundance in *Acmella oleracea*, at a concentration of 180 μM, the compound had a marked inhibitory effect on the production of TNF and other pro-inflammatory mediators [27].

In studies involving *in vivo* models, spilanthol at concentrations ranging from 1 to 20 mg/kg showed marked anti-inflammatory and antinociceptive activities in arthritis [28] and in the hot plate and acetic acid-induced abdominal contortions models [29]. Spilanthol commands a high market price, ranging from $6000 to $10,000 per 100 mg (96% purity standard), and is considered safe by the European Food Safety Authority (EFSA) for industrial use as a flavoring agent in products such as soups, processed vegetables, condiments, chewing gum, and dentifrices [7,30,31]. For these reasons, it is important to obtain spilanthol not only with a high degree of purity, but also free from any toxic solvent or other component that could cause harm, illness, or injury to humans, making it suitable for consumption [32].

Several methodologies have been proposed for the isolation as well as the synthesis of spilanthol [33–36]. However, there have been no reports of its isolation employing Green Chemistry methods. The present work describes a methodology for the isolation of spilanthol using Green Chemistry techniques, involving an extraction step and fractionation with supercritical carbon dioxide, followed by purification using flash chromatography with water and ethanol as the eluent. In addition, significant anti-inflammatory activity of spilanthol was demonstrated using a lipopolysaccharide-activated human neutrophil model.

2. Materials and methods

2.1. Reagents

All reagents and solvents used were analytical or chromatographic grade: absolute ethyl alcohol (Synth, São Paulo, Brazil), carbon dioxide (99.9%, White Martins, São Paulo, Brazil), acetonitrile and methanol (HPLC purity, Tedia, OH, USA), and n-hexane (ACS grade, Synth, São Paulo, Brazil). Ultrapure water was obtained from a Milli-Q purification system (Millipore, MA, USA). Spilanthol standard (88.5%) was acquired from ChromaDex (CA, USA), quercetin (95.0%), was from Carl Roth (Germany), and clarithromycin (≥95.0%) was from Sigma-Aldrich (Germany).

2.2. Plant material

The plants were grown in the experimental field of CPQBA/UNICAMP, located in the municipality of Paulínia, São Paulo State, Brazil (22°47′49.2″S, 47°06′48.2″W). Dr. John F. Pruski, of the Missouri Botanical Garden, performed the botanical identification. A voucher specimen was deposited in the Herbarium of UNICAMP, under identification number #181,452. Permission to access genetic patrimony was granted by the Brazilian National Council for Scientific and