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# Aboriginal bush foods: A major phloroglucinol from Crimson Bottlebrush flowers (*Callistemon citrinus*, Myrtaceae) displays strong antinociceptive and anti-inflammatory activity

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ABSTRACT

*Callistemon citrinus* (Myrtaceae) is a shrub native to Australia. Its flowers have been used as indigenous food among the aboriginal Australians. Numerous diseases, such as bacterial, fungal, viral and parasite infections have traditionally been treated with this plant. Gas chromatography–mass spectrometry (GC/MS) analyses of the flower and leaf extracts of *C. citrinus* revealed the presence of a major constituent, a phloroglucinol, 1-(2,6-dihydroxy-4-methoxyphenyl)-3-methylbutan-1-one (**1**) (up to 382.2 mg per 1 g of the flower extracts). Compound **1** was for the first time identified in this genus. This phloroglucinol exhibited potent antinociceptive and anti-inflammatory activities in a dose-dependent manner. In addition, this compound displayed strong *in vitro* antioxidant activity which could be easily connected with both anti-inflammatory and antinociceptive effects. Thus, compound **1**, as a plant constituent present in the diet of Aboriginal people, that helps with inflammation and pain, could have given them a better chance of survival in harsh conditions of the environment.


## 1. Introduction

Edible plants possess not only nutritional value, but also have medicinal properties (Carrillo, Prado-Gascó, Fiszman, & Varela, 2013; Sarkar, Ankolekar, Pinto, & Shetty, 2015; Vadivel, Nandety, & Biesalski, 2011). Identification and characterization of bioactive compounds from locally available, indigenous foods has become a central topic for many food and natural product chemists (Oliveira, Yamada, Fagg, & Brandão, 2012; Radulović, Blagojević, Stojanović-Radić, & Stojanović, 2013). Australian Aboriginal people have used both native and introduced flora (bush foods) as medicine and food for thousands of years (Hegarty, Hegarty, & Wills, 2001). Among the plants used in traditional and modern Australian communities as both food and medicine, one finds species belonging to the genus *Callistemon*, family Myrtaceae (Cock, 2012; Nash, 2000). The flowers of these plants, commonly referred to as "bottlebrushes", were sucked for their nectar or used to make sweet drinks (Nash, 2000). In addition to being a food source, *Callistemon* species also had roles as traditional bush medicines for Australian Aborigines (Cock, 2012).

The most widely cultivated member of this genus is *Callistemon citrinus* (syn. *Callistemon lanceolatus*, commonly known as Crimson or Lemon Bottlebrush), native to Australia and widely distributed in subtropical and tropical regions such as South America and tropical Asia (Goyal, Jain, Jain, & Sharma, 2012). This species has long been used for ethnomedical purposes to treat numerous diseases often accompanied by inflammation: gastrointestinal disorders, pain and bacterial, fungal, viral and parasite infections (Goyal et al., 2012; Sudhakar et al., 2004 and references cited therein).

Phytochemical studies (dealing mostly with leaf extracts) revealed *C. citrinus* to be a rich source of bioactive compounds. Up to now many different polyphenols (phenolic acids, flavonol glycosides hydrolysable gallo- and ellagitannins and tannins) and terpenes (Goyal et al., 2012; Jeong et al., 2009; Kim, Byun, Bandi, Hyun, & Lee, 2009 and references cited therein) have been identified from this plant. A number of previous reports showed that *C. citrinus* leaf extracts possess a range

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of biological activities: wound healing, hepatoprotective, cardioprotective, anti-inflammatory, antidiabetic, hypolipidemic, antioxidant, antithrombotic, as well as inhibition of cholinesterase and elastase activities (Goyal et al., 2012; Kumar, Kumar, & Prakash, 2011a,b and references cited therein). On the other hand, in regard to biological investigations, other aerial parts (i.e. flowers) of this plant taxon were left neglected. Additionally, there is only one report about the antinociceptive and anti-inflammatory effects of crude leaf essential oil in experimental animals (Sudhakar et al., 2004).

Prompted by the lack of detailed chemical data on *C. citrinus* flowers, its known enthnopharmacological uses and, finally, the wish to find a link between human consumption and beneficial effects of these flowers, we decided to perform a study of the constituents of *C. citrinus* flowers. Where available, we also studied the composition of the leaf extract as well. Preliminary GC–MS analyses of *C. citrinus* extracts revealed that all samples (both flower and leaf extracts) in our hands had a notably different chemical composition compared to that published to date (Goyal et al., 2012; Petronilho, Rocha, Ramirez-Chavez, Molina-Torres, & Rios-Chavez, 2013). However, these analyses did not permit us to identify some of the major constituents detected, thus we decided to try to isolate them in a pure state and elucidate their structures by additional spectral analyses. Another goal of our work was to test the hypothesis of plant polypharmacology (Radulović, Blagojević, Randjelović, & Stojanović, 2013) in this case. It was previously demonstrated (Goyal et al., 2012) that secondary metabolites of this plant species (present in the leaves of the plant) possessed antimicrobial activity, but what if this was not the only modus operandi for this plant providing the wanted (relieving) effect? A dietary plant antibacterial agent that also deals with the concomitant inflammation and pain would have been a real advantage in the life of Aboriginal people and could have given them a better chance of survival in harsh conditions of the environment. The same goes even for modern man. Certain types of inflammatory tissue injury are mediated by reactive oxygen metabolites (Wang et al., 2004). Several anti-inflammatory agents were shown to protect against oxidant-mediated inflammation and tissue damage by virtue of their ability to scavenge free radicals (Chen & Kang, 2013; Huang et al., 2011). It is also believed that there is an association between the antinociceptive action and inflammatory pain (Rodrigues et al., 2012). For these reasons we investigated the biological activity of the mentioned flower extract of differing chemical composition and the individual major pure component present in that extract in terms of anti-inflammatory, antinociceptive and antioxidant activity, as well.

## 2. Materials and methods

### 2.1. Drugs and chemicals

All solvents (dichloromethane, hexane, ethyl acetate (EtOAc), diethyl ether, methanol, ethanol), as well as anhydrous magnesium sulphate, potassium persulphate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,6-bis(1,1-dimethylethyl)-4-methylphenol (BHT), butylated hydroxyanisole (BHA), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were obtained from commercial sources (Aldrich, USA; Merck, Germany; Fluka, Germany). Pure compounds used for GC co-injection with the samples of *C. citrinus* (see compositional table, column Identification), α-pinene, sabinene, β-pinene, myrcene, *p*-cymene, limonene, 1,8-cineole, γ-terpinene, linalool, terpinen-4-ol, α-terpineol, geraniol, undecanal, eugenol, geranyl acetate, β-caryophyllene, caryophyllene oxide, stigmasta-5-en-3β-ol were purchased from Sigma-Aldrich as analytical standards of the highest available purity. Two alkane standard mixtures ($C_8$–$C_{20}$ and $C_{10}$; $C_{20}$–$C_{40}$) were purchased from the same manufacturer (Fluka). Aspirin (ASA; Bayer, Germany), indomethacin (Alfa Aesar, USA), morphine sulfate pentahydrate (Sigma-Aldrich, USA) and kappa carrageenan (Carl Roth, Germany) were obtained from the mentioned companies.

### 2.2. Plant material

The aerial parts of *C. citrinus* were acquired from the botanical gardens of Barcelona (Spain), in August 2012, provided by the courtesy of the gardens' botanists. During 2014, additional samples of either leaves or flowers (up to the amount of several grams) were collected from three sites: the urban settings of the city of Niš, Serbia, and Stavros, Greece (cultivated garden plants), and Wollongong, Australia (wild-growing population). Voucher specimens were deposited at the Herbarium of the Faculty of Science and Mathematics, University of Niš, under the accession numbers NR0742012 and NR0232014–NR0252014. The authenticity of the plant material was verified by the Herbarium curator.

### 2.3. General experimental procedures

Silica gel 60 on Al plates, layer thickness 0.2 mm (Kieselgel 60 $F_{254}$, Merck) was used for thin layer chromatography (TLC) and silica–gel 60, particle size distribution 40–63 μm (Merck, Germany) for "dry flash" and column chromatography. Spots on TLC were visualized by UV light (254 nm) and by spraying with 50% (v/v) aqueous $H_2SO_4$ followed by heating. $^1H$ and $^{13}C$ NMR spectra were recorded on a Varian Gemini 200 spectrometer operating at 200 and 50 MHz, respectively, in $CDCl_3$, at ambient temperature, with solvent residual or TMS signals used as a reference. Moreover, $^1H$ and $^{13}C$ NMR as well as DEPT and 2D spectra ($^1H$–$^1H$ COSY, HMBC, HSQC and NOESY with usual pulse sequences) in dimethylsulphoxide-$d_6$ (DMSO-$d_6$) were also recorded on a Bruker Avance III 400 MHz NMR spectrometer ($^1H$ at 400 MHz, $^{13}C$ at 100 MHz). Chemical shifts are given in ppm, coupling constants "*J*" are expressed in Hertz (multiplicity: s = singlet, br s = broad singlet, d = doublet, t sept = triplet of septuplets).

### 2.4. Gas chromatography (GC) and Gas chromatography/mass spectrometry (GC/MS) analyses

Chemical composition of the extracts and their chromatographic fractions was investigated by GC and GC/MS (Radulović, Mladenović, & Blagojević, 2014; Radulović, Zlatković, et al., 2013b). The GC/MS analyses (three repetitions) were carried out with a Hewlett-Packard 6890 N gas chromatograph equipped with a fused silica capillary column DB-5MS (5% phenylmethylsiloxane, 30 m × 0.25 mm; film thickness, 0.25 mm, Agilent Technologies, USA) and coupled with a 5975B mass-selective detector from the same company. The injector and interface were operated at 250 °C and 320 °C, respectively. Oven temperature was raised from 70 °C to 310 °C at a heating rate of 5 °C/min and then isothermally held for 10 min. As a carrier gas, He at 1.0 mL/min was used. The samples (1 mL of the fraction solutions in $Et_2O$ (1 mg per 1 mL)) were injected in a pulsed-split mode (the flow was 1.5 mL/min for the first 0.5 min and then set to 1.0 mL/min throughout the rest of the analysis; split ratio, 40:1). MS Conditions: ionization voltage, 70 eV, acquisition mass range, 35–650 amu, scan time, 0.32 s. GC (FID) Analysis was carried out under the same experimental conditions using the same column as described for the GC/MS. The preliminary percentage composition was computed from the GC peak areas without the use of correction factors. Qualitative analyses were based on the comparison of experimental linear retention indices relative to retention times of $C_7$–$C_{35}$ n-alkanes on the DB-5MS (Van den Dool & Kratz, 1963) with those reported in the literature (Adams, 2007), and by comparison of their mass spectra with those of authentic standards, as well as those from Wiley 6, NIST11, MassFinder 2.3, and a homemade MS library with the spectra corresponding to pure substances, and wherever possible, by co-injection with an authentic sample (as indicated in the compositional table).

Quantification of volatile components was achieved by means of both FID peak-area internal normalization with the use of response factors (RF) and the use of internal standards (octane and octadecane). Relative response factors (RRFs) were calculated on the basis of a

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