Biochemical Systematics and Ecology 78 (2018) 21–30

Contents lists available at ScienceDirect

Biochemical Systematics and Ecology

journal homepage: www.elsevier.com/locate/biochemsyseco

Characterization and optimization of phenolics extracts from *Acacia* species in relevance to their anti-inflammatory activity

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ARTICLE INFO

Article history:
Received 5 October 2017
Received in revised form
3 March 2018
Accepted 5 March 2018

Keywords:
Acacia tortilis
Acacia farnesiana
Acacia longifolia
Phenolics
Anti-inflammatory activity
COX-1
COX-2

ABSTRACT

Three *Acacia* species: *A. farnesiana, A. tortilis* and *A. longifolia,* were investigated for seasonal variations of the phytochemical constituents and their influence on in-vitro anti-inflammatory activities. Samples of leaves and bark of the three species were collected in two different seasons and extracted using 50% and 70% ethanol. LC-MS analysis revealed the presence of rutin in the leaves of all three species, while catechin was commonly present in the bark. Other major components included vicenin 2, isoquercitrin, kaempferol 3-O-rutinoside and isorhamnetin 3-O-rutinoside in *A. farensiana* leaves, while *A. longifolia* leaves contained myricetin 3-*O*-rhamnoside, quercitrin and luteolin. The level of rutin varied according to the flowering period being highest in winter in *A. tortilis* (245.4 μg/mg) and *A. longifolia* (3.0 μg/mg). The content of total phenolics (TPC) showed a marked variation among the three species, which was reflected to some extent in the COX-1 and COX-2 enzymes inhibition. Optimization modeling was performed for better visualization and utilization of the data showing a strong inverse correlation between TPC and the mean inhibition of COX-2 in case of the leaves ($r = 0.783$). Finally, cluster analysis of the data obtained from the leaf samples reflected on the taxonomical classification of the species.

1. Introduction

Plants have been widely used since ancient times as a primary remedy for various ailments. Their folk uses have encouraged various phytochemical and pharmacological screenings and provided an unlimited source for drug discovery endeavors due to the unmatched chemical diversity (Sasidharan et al., 2011; Pacifico et al., 2016). Medicinal plants constitute a broad array of bioactive secondary metabolites, among which are phenolics, having several beneficial effects to humans especially in the prevention of chronic degenerative diseases, including cancer, cardiovascular and neurodegenerative disorders (Bhullar and Rupasinghe, 2013; Fernández-Arroyo et al., 2015; Mocanu et al., 2015; Ramon et al., 2014).

The genus *Acacia* is the second largest genus of the family Fabaceae, comprising more than 1350 species worldwide, with members found in almost all habitats, of which, approximately 800 are found in Australia, 130 in Africa and 20 in India (Jelassi et al., 2014). Plants of genus *Acacia* are widely used in traditional medicine for diarrhea, urinary tract infections, headaches, sore throat, gastritis, tuberculosis and bronchial asthma (Stohs and Bagchi, 2015). Phytochemical investigation of different *Acacia* species showed the presence of phenolic acids, flavonoids, tannins, alkaloids and terpenes, which were responsible for numerous pharmacological effects, showing hypoglycemic, anti-inflammatory, anti-bacterial, anti-platelet aggregation, anti-hypertensive, analgesic, anticancer and anti-atherosclerotic activities (Jelassi et al., 2016).

Acacia farnesiana (L.) Willd., *Acacia tortilis* Hayne and *Acacia*

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https://doi.org/10.1016/j.bse.2018.03.001
0305-1978/

longifolia (Andrews) Willd. are three prominent *Acacia* species commonly grown for ornamental purposes and widely distributed in gardens of Egypt. Recently, these species were re-classified under two different taxa out of five of the Fabaceae family; namely *Vachellia* and *Acacia*, and the species under study renamed: *Vachellia farnesiana* Wight & Arn. (*Acacia farnesiana*), *Vachellia tortilis* (Forssk.) Galasso & Banfi (*Acacia tortilis*), and *Acacia longifolia* (Kyalangalilwa et al., 2013; Murphy et al., 2010). The phytochemical literature on these species is quite limited, but flavonoid galloyl-glycosides and flavonoid glycosides were reported in *A. farnesiana* (Ramli et al., 2011). Apigenin 6,8-bis-*C*-*β*-D-glucopyranoside (vicenin) and rutin were identified in *A. tortilis* (Yadav and Kothiyal, 2013), and naringenin and isorhamnetin 3-*O*-rutinoside in *A. longifolia* (Andrade et al., 2010; Thieme and Khogali, 1975). Reports of folk medicine on these species revealed that stems of *A. tortilis* have been used in Somalia to treat asthma, and dried powdered bark has been used as a disinfectant in wound healing (Verma, 2016). *A. farnesiana* has been used for the treatment of inflammation in Mexico, as well as for lowering abdominal pain and headache, and for arthritis in China (Hossan et al., 2010). *A. longifolia* has been used in folk medicine for asthma (Güler et al., 2015). From the biological point of view, studies on *A. tortilis* showed marked inhibition of mammalian α-glucosidase significantly reducing postprandial blood glucose level (Bisht et al., 2013). For *A. farnesiana,* it was reported that it possessed potent anti-inflammatory and analgesic activity (Huey-Jiun et al., 2014; Ramli et al., 2011). To our knowledge, no pharmacological testing has been reported regarding *A. longifolia* so far.

In this study, a phytochemical investigation on the *Acacia* species in question regarding their phenolic content and anti-inflammatory activity is presented. It is worth noting that although there are reports of studies regarding seasonal variation of secondary metabolites, yet no compiled representation of the optimum collection season of pharmaceutically important constituents could be traced (Soni et al., 2015). Hence, we were prompted to determine whether the collecting season affects the chemical composition and consequently the anti-inflammatory activity of the three *Acacia* species. It also seemed interesting to determine whether closely related species show resemblance in terms of phenolic content.

2. Materials and methods

2.1. Plant material

Leaves and bark of *A. farnesiana*, *A. tortilis*, and *A. longifolia* were collected in December 2015 (winter) and May 2016 (spring) from Sekem farms, located 60 km north of Cairo, Egypt (30°25′02.9″N 31°38′11.5″E). The plants were identified by taxonomists at the botanical garden of Sekem farms, Belbis, Egypt (Fig. 1) as *Acacia farnesiana* (L.) Willd., *Acacia tortilis* Hayne and *Acacia longifolia* (Andrews) Willd. (Allen and Allen, 1981). Voucher specimens (PHG-P-AT189), (PHG-P-AT190) and (PHG-P-AT191) (Supplementary Material Fig. S4) are kept in the herbarium of the Faculty of Pharmacy, Ain Shams University. Five samples for each organ in each species were collected at random (different branches; different leaf size) and all samples were treated as one during extraction procedures. Plant materials were air-dried, coarsely cut (2 cm^2) and extracted twice by maceration at room temperature (24 °C). Solvents of 50% ethanol and 70% ethanol were used for extraction for each sample. Both extracts were evaporated to dryness under reduced pressure at 40 °C using a Heidolph rotary vacuum evaporator, and lyophilized using a LyoQuest freeze dryer. Extraction resulted in 12 extracts from leaves and 12 extracts from bark.

2.2. LC-MS analysis

UHPLC-HR-MS/MS and UHPLC-ESI-MS were used for the qualitative and quantitative analysis respectively. In qualitative analysis, the assay was performed using an ultimate 3000 hyperbaric liquid chromatography system coupled to a Q Exactive hybrid quadropole orbitrap mass spectrometer via an ESI ion source. Chromatography system consisted of an Ultimate 3000 autosampler, an Ultimate 3000 diode array detector, UltiMate 3000 RS Column Compartment. Xcalibur software was used for data collection and data analysis. A Phenomenex® column Kinetex 2.6 μm C18 100 Å, LC Column 100 × 2.1 mm (Agilent technologies, Waldbronn, Germany) was used for chromatographic separation. The mobile phase consisted of A: 0.2% formic acid in water and B: 0.2% formic acid in acetonitrile (VWR, U.S.A). The elution profile was 0–30min 2–20% B in A, 30–40 min, 20–60% B in A. The flow rate was 0.350 ml/min and the injection volume was 5.0 μL. The DAD acquisition range was 190–400 nm. ESI ion source was operated in negative mode with the following parameters: dry gas flow (N2) 8.0 L/min with a capillary temperature set at 330 °C; source heater temperature set at 300 °C; sheath gas flow set at 50 arbitrary units; auxiliary gas flow set at 10 arbitrary units; source voltage set at 3.00 kV; capillary voltage set at −16 V. Mass spectra were recorded between m/z 166–2000 with a mass resolution of 70,000. Collision-induced fragmentation experiments were performed in the ion trap using helium as a collision gas, with voltage cycles from 0.3 up to 2 V and collision energy set at 35 eV. The constituents were tentatively identified by comparison of their specific chromatographic data including UV spectra, monoisotropic mass and MS/MS fragmentation patterns to literature data or by comparison with authentic reference compounds.

Quantitative analyses were carried out on a Dionex UltiMate 3000 system equipped with UltiMate 3000 RS pump, degasser, UltiMate 3000 RS Autosampler, UltiMate 3000 RS Column Compartment and Ultimate 3000 RS diode array detector (DAD). A Phenomenex® column Kinetex 2.6 μm C18 100 Å, LC Column 100 × 2.1 mm (Agilent technologies, Waldbronn, Germany) was used for chromatographic separation. The mobile phase consisted of A: 0.2% formic acid in water and B: 0.2% formic acid in acetonitrile (VWR, U.S.A). The elution profile was 0–30min 2–20% B in A, 30–40 min, 20–60% B in A. The flow rate was 0.350 ml/min and the injection volume was 5.0 μL. The LC system was coupled to a LTQ-XL mass spectrometer (Thermo Fisher Scientific, USA) with an ESI ion source and controlled by Thermo Tune Plus 2.7.0 software.

2.3. Quantitative analysis of extracts

Leaf extracts were quantified relative to rutin as an external standard (Roth, Karlsruhe, Germany). For this purpose, rutin calibration curve was prepared using seven different concentrations (2.5, 5, 10, 15, 30, 50, 60 μg/ml) injected at the same conditions as the samples. Bark extracts were quantified using catechin as external standard (Roth, Karlsruhe, Germany). For this purpose catechin calibration curve was prepared using eight different concentrations (3.75, 7.5, 15, 30, 60, 120, 150, 200 μg/ml) injected in the same conditions of the samples. Samples were injected in duplicates. From the resulting chromatograms, quantitation by normalization was attained where the peak area % of each peak in the chromatogram is calculated as a percentage of the total area of all peaks.

2.4. Assay on total phenolics

The content of total phenolics was determined according to a modified Folin–Ciocalteau procedure reported by Zhang et al.