Uncovering the biosynthetic pathway of bioactive compounds from Eclipta prostrata (L.) L. hairy roots

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Research Article

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Abstract

Eclipta prostrata (L.) L. is a medicinal plant of the Asteraceae family, and several extracts and isolated compounds of *Eclipta prostrata* (L.) L. showed a wide range of biological activities such as antimicrobial, anticancer, hepatoprotective, neuroprotective, hair growth promoting activities, and more recently against covid-19. *Eclipta prostrata* (L.) L. hairy roots produce wedelolactone (WL), demethylwedelolactone (DWL) and 3,5-di-O-caffeoylquinic acid (3,5-diCQA), and there is no data in literature regarding biosynthetic pathways are involved. To verify the biosynthetic route, feeding experiments were carried out using sodium [2-^{13}C]acetate, [3-^{13}C]DL-phenylalanine, and ^{13}C-labeled compounds (WL, DWL and 3,5-diCQA) were detected by ultra-high-performance liquid chromatography-quadrupole time of flight mass spectrometry (HPLC-QTOF-MS). Analysis showed that the metabolic pathways operative of coumestans (WL and DWL) are derived from acetate and shikimate pathways, while that the phenylpropanoid (3,5-CQA) biosynthesis is exclusively from shikimate pathway.

Introduction

Eclipta prostrata (L.) L., native to Brazil, is a medicinal species from the Asteraceae family (Souza et al., 2003). The biological efficacy of *E. prostrata* (L.) L. has been correlated to chemical constituents present in its extracts such as wedelolactone (WL), demethylwedelolactone (DWL). More recent activity data showed that WL displays anti-glycation and anti-diabetic activities and could be promisor therapeutic agent against obesity and related metabolic diseases (Shahab et al., 2018; Yao et al., 2022). WL reduces pulmonary fibrosis (Yang et al., 2019), attenuates doxorubicin-induced inflammation (Zhu et al., 2019), is promising agent in the mantle cell lymphoma treatment (Romanchikova et al., 2019), exhibits strong antioxidant activity (Li et al., 2020), may be a promising anti-inflammatory candidate to combat *Pseudomonas aeruginosa* keratitis (Xu et al., 2021) and reduces acute pancreatitis and associated lung injury (Fan et al., 2021). In previous work was investigated the suitability of *E. prostrata* (L.) L. hairy root cultures biosynthesize the 3,5-di-O-caffeoylquinic acid (3,5-diCQA) (Maciel et al., 2021), a promising biological compound against SARS-CoV-2, the virus that causes COVID-19 (Joshi et al. 2020; Shah et al. 2021; Sumon et al. 2021; Kadioglu et al. 2021). Although WL, DWL and 3,5-diCQA are known, their biosynthesis of compounds from *E. prostrata* (L.) L. are not related in the literature. Coumestans, such as coumestrol, are derived from shikimate and acetate pathways (Dewick, 2009), while the phenylpropanoid, such as chlorogenic acid, are synthesized by shikimate pathway (Dewick, 2009; Mahesh et al., 2007). Hence, the main aim of this article was to fed *Eclipta prostrata* (L.) L. hairy roots with sodium [2-^{13}C]acetate and [3-^{13}C]DL-phenylalanine. After time incubation the crude extracts were prepared and analyzed by HPLC-QTOF-MS in order to find ^{13}C-labeled WL, DWL and 3,5-diCQA.

Materials And Methods

Chemicals

The sodium [2-^{13}C]acetate and [3-^{13}C]DL-phenylalanine (^{13}C-precursors) were purchased from Sigma-Aldrich®.

Maintenance of Eclipta prostrata **(L.) L. hairy roots**

E. prostrata (L.) L. hairy roots C19 clone (Maciel et al., 2021) have been cultivated in MS liquid culture medium under agitation (100 rpm) at 25 ± 1ºC in the dark.

^{13} C-tracer experiments with sodium [2- ^{13} C]acetate and [3- ^{13} C]DL-phenylalanine in Eclipta prostrata (L.) L. hairy roots

Eclipta prostrata (L.) L. hairy roots C19 clone (one month old; 2 g each; n = 6) were inoculated in liquid MS medium with 30 g.L^{-1} of sucrose, pH 6.0 (± 0,05) supplemented with sodium [2-^{13}C]acetate (0.25%) or [3-^{13}C]DL-phenylalanine (10 mM). Additionally, a control group of roots that did not receive ^{13}C-precursors was also prepared (one month old; 2 g each; n = 6). Hairy root cultures were kept at 25°C ± 1°C, in the dark and under agitation at 100 rpm for 10 days (55–60% relative humidity). After 10 days of cultivation, the roots were harvested and subjected to extraction using methanol.

Equipment And Hplc-qtof-ms Conditions From Feeding Experiments

Methanolic extracts were performed in a Shimadzu HPLC System (pump LC-20AD, auto-injector SIL-20A HT, column oven CTO-20A, system controller CBM-20A and degasser DGU-20A3), coupled to diode array detector (SPD-M20A) and a micrOTOF-QII (Bruker) mass spectrometer, operating with an electrospray ionization source (ESI-MS) in negative analysis mode. A volume of 5 µL at a concentration of 50 µg.mL^{-1} of the samples was injected into a Luna C18 column (250 mm x 4.6 mm, 5 µ, Phenomenex). The chromatographic condition used was: (A:B; A-H_2O ultrapure + 0.1% formic acid; B-MeOH + 0.1% formic acid), in an exploratory gradient elution; from 0–40 min starting at (95:5) until (0: 100). The flow of the mobile phase was 1.0 mL.min^{-1} and the compounds were monitored at λ = 210–600 nm. The operating parameters used in the Z-spray source were: capillary voltage = 3000 V, end plate offset 500 V, Z-spray source temperature = 200°C, nebulizer gas pressure 4 bar and desolvation gas flow = 8–10 L.min^{-1}. The mass range used in the full-scan analysis mode was 100 to 600 Da.

Results And Discussion

Isotopic labeling studies were carried out in order to elucidate the WL, DWL and 3,5-diCQA biosynthesis. *Eclipta prostrata* (L.) L. hairy roots were inoculated in liquid medium supplemented with sodium [2-^{13}C]acetate or [3-^{13}C]DL-phenylalanine, and after 10 days of culture, a methanolic extract from fresh hairy roots was prepared and analyzed by HPLC-QTOF-MS (see supplementary data). Table 1 shows ^{13}C-incorporation data from WL, DWL and 3,5-diCQA compounds after isotopic labeling experiments using

sodium [2-^{13}C]acetate and [3-^{13}C]DL-phenylalanine. During incorporation, for each ^{13}C added, one mass unit was expected to be added. The calculation is based on the ratio between the molecular ion peak area, and its isotopes peak area, for both the control and ^{13}C-precursor experiments. The smaller value of the ratio (between M-H peak area and its corresponding isotope $[M\text{-}H + 1]^-$, $[M\text{-}H + 2]^-$ or $[M\text{-}H + 3]^-$ peak area), means ^{13}C incorporation during biosynthetic process, thus confirming the incorporation of the ^{13}C-labeled precursor (Musquiari et al., 2021). WL (m/z = 314.246) and DWL (m/z = 300.219) coumestans incorporated three acetate units from sodium [2-^{13}C]acetate and, therefore, the ion M + 3 was selected, showing that the acetate pathway is one of the metabolic pathway in the biosynthesis of these compounds (Fig. 1). In the experiment using [3-^{13}C]DL-phenylalanine only one ^{13}C unit was incorporated and, therefore, the ion M + 1 was selected (see supplementary data). Thus, WL and DWL are biosynthesized by both the acetate and shikimate pathways. It is likely that DWL is the precursor of WL, because the chemical structures differ by just one methyl group. The S-Adenosyl methionine enzyme (SAM) probably mediates an addition of one methyl group into WL. The isotopic labeling of 3,5-diCQA (m/z = 515.443) led to the incorporation of two units of [3-^{13}C]DL-phenylalanine confirming the shikimate pathway is the exclusive route for the phenylpropanoid biosynthesis (Fig. 1). WL, DWL and 3,5-diCQA are present in *Eclipta prostrata* (L.) L. hairy roots derived from a common precursor, the shikimic acid, and whose pathway is formed from the regenerative process of the carbohydrate erythrose-4-phosphate, synthesized during the photosynthesis, further of the phosphoenolpyruvate, an intermediate from glucose metabolism. Thereby, the isotopic experiments allowed us to establish the biosynthetic pathway of bioactive compounds from *E. prostrata* (L.) L. hairy roots.

Table 1

Mass spectrometric data of isotopic experiments using sodium [2-^{13}C]acetate and [3-^{13}C]DL-phenylalanine in *Eclipta prostrata* (L.) L. hairy roots.

compound	retention time	ratio	control	sodium [2-^{13}C]acetate	[3-^{13}C]DL-phenylalanine
3,5-diCQA	**23'**	**RM + 2**	12.5	-	1.22
DWL	**25'**	**RM + 1**	5.2	-	3.6
		RM + 3	219.6	4.19	-
WL	**30'**	**RM + 1**	5.2	-	4.7
		RM + 3	113.2	39.4	-

(RM + 1: value of the ratio between $[M\text{-}H]^-$ peak area and its corresponding isotope $[M\text{-}H + 1]^-$ peak area; RM + 2: value of the ratio between $[M\text{-}H]^-$ peak area and its corresponding isotope $[M\text{-}H + 2]^-$ peak area; RM + 3: value of the ratio between $[M\text{-}H]^-$ peak area and its corresponding isotope $[M\text{-}H + 3]^-$ peak area).

Conclusion

Our isotopic experiments allowed us to establish the biosynthetic pathway of bioactive compounds from *E. prostrata* (L.) L. hairy roots; WL and DWL are from acetate and shikimate pathways while 3,5-diCQA is from shikimate pathway exclusively.

Declarations

Authors' contributions

A.A.L. and M.V.L. designed research; G.R.S.S. and A.A.L. performed research; A.A.L., M.V.L. and S. C. F. analyzed data; A.A.L. wrote the manuscript and all authors reviewed the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Supplementary Data

Supplementary Data not available with this version

Figures

Figure 1

Biosynthesis of WL, DWL and 3,5-diCQA after incorporation of sodium [2-^{13}C]acetate (in red dots) and [3-^{13}C]DL-phenylalanine (in blue dots) by *Eclipta prostrata* (L.) L. hairy roots.