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Cell cultures of *Ajuga reptans* L. to bioconvert emodin and aloe–emodin: an HPLC/ESI/MS investigation

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Abstract

In this paper, we describe the bioconversion of anthraquinones, emodin and aloe–emodin, carried out by cell cultures of *Ajuga reptans* L. The optimal biotransformation times ranged between 48 and 72 h of contact, and the % yields of bioconversion were near 35–38%. The HPLC/DAD and ESI/MS analyses were mainly applied to perform a preliminary characterisation of the bioconverted molecules. Five glycosides were obtained from emodin, the main one being 6-*O*-β-malonyl galactoside; eight different compounds were obtained from aloe–emodin with 11-*O*-β-malonyl galactoside as predominant derivative. Among the other minor derivatives, diglycosides as such as acyl glycosides, were also detected. Finally, the total content of the phenyl propanoidic compound, the main natural metabolites of these cell cultures, resulted unmodified by the addition of both the anthraquinones.

Keywords: Bioconversion; Cell culture; Emodin; Aloemodin; *Ajuga reptans*

1. Introduction

In vitro plant cell cultures are employed for secondary product biosynthesis for pharmaceutical and food applications and to biotransform appropriate precursors by specific enzymatic reactions such as glycosilation, oxidation and acylation [1]. The selective glycosilation of phenolic hydroxyl groups by chemical means involves complicated protection–deprotection procedures and it is rather difficult to obtain good yields from this procedure. Therefore, the possibility of performing this reaction by means of biological tools could be of interest for several applications.

Among the secondary metabolites of the *Ajuga reptans* L. cell cultures, monoterpenoids, diterpenoids and extensively glycosilated phenylpropanoids were found [2–4]. The presence of these structures suggested these plant cell cultures as a potential tool for the glycosilation of exogenous molecules. Previous investigations carried out on flavonoidic molecules [5,6] and on vanillin added as exogenous substances to Ajuga cell cultures, confirmed their ability to glycosidate exogenous substrates. With the aim of evaluating the capability of these cultures to carry out specific glycosilation on phenolic substrates, also 1,4 and 1,8-dihydroxy-anthraquinones were considered. The 1,8-anthraquinone glycosides are the active principles of herbal drugs with cathartic activity, belonging to the Rhamnus, Aloe and Rheum genera [7–9], and among these compounds emodin and aloe–emodin are the most common aglycones.

Moreover, several recent studies were focused on evaluating the anticancer activity of emodin and aloe–emodin that have shown interesting results with human cancer cell lines e.g. from liver and lung [10–12].

In this paper, we describe for the first time the bioconversion of emodin and aloe–emodin by cell cultures of *Ajuga reptans* L. with data relating to the optimal biotransformation

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time and bioconversion yields. The chemical structure of the principal compounds is also described mainly by HPLC/DAD analysis and ESI/MS investigations.

2. Experimental

2.1. Materials

All solvents used were HPLC grade; CH_3CN and MeOH for HPLC were from E. Merck (Darmstadt, Germany), while water was from Baker (J.T. Baker, Italy).

For the NMR analysis the solvents used were CD_3OD and DMSO from Merck (Darmstadt, Germany). All the anthraquinone molecules were purchased from Extrasynthèse (Lion-Geney, France).

2.2. Cell culture preparation

Callus cultures of *Ajuga reptans* L. were started from seeds of plants grown in the Botanical Garden of Casola Valsenio (Ravenna, Italy). Cells were grown on a modified Gamborg medium containing naphthalene acetic acid 1 mg/L, kinetin 1 mg/L and 2,4-dichloro-phenoxy acetic acid 0.2 mg/L (G5 medium). Solid medium contained 7 g/L of agar. Suspended cultures were prepared by transferring about 2 g of cells, grown on solid G5 medium, to 300 mL flasks containing 50 mL of G5 liquid medium and shaken on a rotator shaker at 120 rpm at 28 °C. After 1 week, 5 mL portions were distributed into other identical flasks. Five days later cultures were supplemented with 2 mL of a sterile 50% sucrose solution.

All the anthraquinones (100 μg/mL), previously dissolved in 0.5 mL of MeOH, were added to 11-day-old cultures.

2.3. Evaluation of the optimal biotransformation times

For time course determination of the biotransformations, samples for analysis were taken in the range 0–120 h, every 24 h. Then an equal volume of MeOH was added to the flasks to block enzymatic activities. In order to avoid browning effects, 1 mg/mL of ascorbic acid was also added. The samples were then homogenised and filtered and the hydro alcoholic solutions were directly analysed by HPLC/DAD. Determinations were carried out in duplicate and mean values were taken obtaining % CV values not over 5%.

2.4. Evaluation of the biotransformation yields

The optimal biotransformation yields were obtained measuring the area values from HPLC/DAD applying the following formula:

$$\%\,\text{yields} = \frac{\sum \text{Area of total bioconverted products}}{\text{Area of aglycone at time 0}} \times 100$$

The area values, expressed in mAu, both for emodin and aloe–emodin, were calculated at 430 nm corresponding to a relative maximum in the range 240–550 nm. Each datum is the average of two determinations performed in a parallel way during the same experiment; % CV within 5% were obtained.

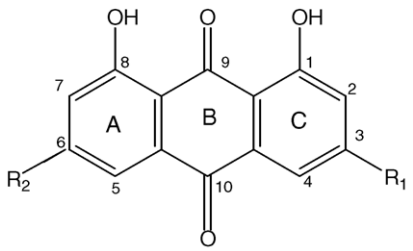
2.5. Extraction procedures

To concentrate the bioconverted products both for emodin and aloe–emodin, all supernatants from different times were combined and filtered obtaining about 1.2 L of hydro alcoholic solution. These were evaporated up to water (about 500 mL) and extracted with 4×50 mL of $CHCl_3$ to recover the residual free aglycone. The next step (developed to concentrate the anthraquinone molecules) was the extraction with 7×50 mL of ethyl acetate. The total intensely yellow-coloured extracts were then concentrated under vacuum (28–30 °C) and collected with H_2O/EtOH 1:9 (2 and 4 mL) and used for semi-preparative HPLC.

2.6. HPLC analysis

The analyses were carried out using a HP 1090L liquid chromatograph equipped with a DAD detector (Hewlett & Packard, Palo Alto, USA). The column was a 4.6 mm × 250 mm, 5 μm, LiChrosorb RP18 (Merck, Darmstadt, Germany) maintained at 26 °C equipped with a 4 mm × 10 mm LiChrosorb RP18 pre column. The eluents were (A) Acid H_2O at pH 3.2 by H_3PO_4; (B) MeOH and (C) CH_3CN. The following linear solvent gradient was applied: 4 min from (A) 92% and (C) 8%, within 8 min to (A) 72%, (B) 20% and (C) 8%, with a plateau of 12 min, 16 min to (A) and (B) 40% and (C) 20%, with a plateau of 8 min, finally 4 min to reach (B) 60% and (C) 40%. Total time of analysis 52 min, flow 1 mL min^{-1}.

Semipreparative HPLC: Separation of single compounds was obtained by using a pump Series 250 LC equipped with an LC 95 UV–vis detector (all from Perkin-Elmer, Norwalk,



	R_1	R_2
Emodin	CH_3	OH
Aloemodin	H	CH_2OH
Frangulin	CH_3	O-Rhamn.

Fig. 1. Reference structures for the considered anthraquinone molecules.