

## Article

# Effect of Jasmonic Acid on the Elicitation of Phenolic Compounds and Naphthodianthrones in *Hypericum perforatum* L. Callus and Shoot Cultures

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## Abstract

*Hypericum perforatum* callus and shoot cultures were investigated for biomass production, phenolic compounds and naphthodianthrone biosynthesis, as well antioxidant activities upon elicitation with jasmonic acid (JA). Calli and shoots elicited with 50 and 100  $\mu\text{M}$  JA had comparable or better fresh biomass production, while application of 250  $\mu\text{M}$  JA displayed growth suppression effect. Total phenolics, flavonoids, and flavan-3-ols contents in JA-elicited calli were correlated to increased activities of phenylalanine ammonia lyase (PAL) and chalcone isomerase (CHI). In contrast, PAL and CHI activities in shoots were not significantly related to phenolic compound production, suggesting that JA up-regulated distinct phenylpropanoid/flavonoid pathways. Exogenous application of 250  $\mu\text{M}$  JA was selected as an efficient elicitor dose for enhanced production of hypericin and pseudohypericin in both cultures. Calli and shoots showed an early response to JA elicitation through significant enhancement of non-enzymatic antioxidant properties due to accumulation of phenolic compounds. Concerning antioxidant enzymes, it was shown that up-regulated peroxidase activity in calli and catalase activity in shoots had a pivotal importance in cells' protection against JA-mediated oxidative stress. Outgoing results indicated that elicitation of *H. perforatum* callus and shoot cultures with JA represents a promising biotechnological approach for production of bioactive metabolites with medicinal properties.

**Keywords:** antioxidant activity; elicitor; hypericins; phenylpropanoids; St. John's wort



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## 1. Introduction

*Hypericum perforatum* L. (St. John's wort) is a well-known medicinal plant, attracting enormous scientific interest worldwide due to its richness in biologically active compounds. This species exhibits various pharmacological activities, including antidepressant, antioxidant, antimicrobial, antiviral, antidiabetic, and anticancer properties that are related to the presence of a distinct group of secondary metabolites such as flavonoids, phenolic acids, acyl-phloroglucinols, naphthodianthrones, and xanthenes [1,2]. Considering this, *H. perforatum* products are among the best-selling phytopharmaceuticals and nutraceuticals that are commercially available on the market in the form of tablets, capsules, or extracts [3]. However, *H. perforatum* flowering shoots (*Hyperici herba*) used in the pharmaceutical and

cosmetic industry for the preparation of phytoproducts must fulfill some requirements related to the contents of bioactive compounds in the raw material [4]. Field-grown plants are the main source for supplying the pharmaceutical industry, but their uncontrolled exploitation can significantly decline natural *H. perforatum* populations [5]. Even cultivated *H. perforatum* plants can substitute wild-growing plants as a crude material for the preparation of numerous formulations; the production of fast-growing and high-yielding plants with phytochemical stability represents a great challenge in the agro-farming industry [5,6]. In addition, cultivated plants are exposed to seasonal, climatic, and ecological variations that may alter phenolic compound levels and associated biological activities [7]. Therefore, the implementation of innovative strategies for optimal growth of *H. perforatum* under controlled conditions may offer a new platform for the achievement of raw biomass with a consistent content of bioactive metabolites.

A large number of investigations have been focused on the optimization of protocols for the cultivation of *H. perforatum* under controlled and aseptic conditions to obtain different types of in vitro cultures with continuous and uniform production of target compounds [8,9]. These in vitro cultures have been recognized as prospective systems for evaluation of their capacity for overproduction of *H. perforatum*-specific metabolites by using various biotechnological approaches, including modification of nutrient medium composition, precursor feeding, genetic engineering, bioreactor cultivation, and elicitation [9–11]. To the best of our knowledge, elicitation is the most commonly exploited and appealing strategy for the enhancement of pharmacologically active compounds in *H. perforatum* cell, callus, shoot, and root cultures [9,12]. This approach is based on the application of various elicitors that are perceived as stress signals by plant cells and provoke signal transduction reactions resulting in up-regulation of secondary metabolite pathways. Elicitation of plant cells is also followed by overproduction of reactive oxygen species (ROS) that trigger defense mechanisms through the activation of non-enzymatic and enzymatic antioxidants to combat oxidative stress [13]. The elicitors can be classified as biological stressors or endogenous elicitors, primarily obtained from microbial or plant sources, or abiotic stressors, commonly known as exogenous elicitors, that comprise physical and chemical elicitors [13,14]. A particular group of elicitors are phytohormones such as salicylic acid (SA), jasmonic acid (JA), and other plant growth regulators that represent chemical messengers or signaling molecules in the modulation of plant responses to stressful conditions. Even though these signaling molecules are present in very low amounts in the plant cells, their exogenous application may cause increased secondary metabolite biosynthesis [15,16].

Jasmonates, including JA and methyl jasmonate (MeJA), have been used as promising elicitors for increased production of phenolic compounds in *H. perforatum* in vitro cultures. In this view, *H. perforatum* cell cultures have been widely used in elicitation experiments with JA and MeJA for modulation of the biosynthesis of flavonoids, xanthonenes, and hypericins [17–20]. It has been shown that *H. perforatum* cell suspensions elicited with JA are characterized by the activation of phenylpropanoid/flavonoid metabolism through phenylalanine ammonia lyase (PAL)- and chalcone isomerase (CHI)-mediated production of various phenolic compounds [19,20]. Despite these encouraging elicitation experiments, the main limitations of using JA-elicited cells are the absence of glandular structures as the main sites for storage of naphthodianthrones and phloroglucinols, as well as the lack of cell differentiation that is sometimes essential for metabolite productivity [8,19]. Taking this into account, *H. perforatum* in vitro shoots and plants with dark and translucent glands on the leaves have been subjected to elicitation with jasmonates to increase the production of hypericins and hyperforins [21–23]. Also, *H. perforatum* adventitious roots cultured in bioreactors have been elicited with MeJA for large-scale production of hypericin [24]. Even though jasmonates have been shown to up-regulate hypericin biosynthesis

in shoot and root cultures, the effect of jasmonic acid on the concomitant production of naphthodianthrones and phenylpropanoids in *H. perforatum* calli as partially differentiated cultures have never been explored. According to our hypothesis, elicitation of *H. perforatum* callus cultures with JA might trigger a series of defense responses through activation of phenylpropanoid/naphthodianthrone metabolic flux and considerable modulation of antioxidant state.

The central objective of this study was to determine how different concentrations of JA, applied over different post-elicitation periods, influence the accumulation of phenolic compounds and naphthodianthrones, as well as the activation of associated antioxidant mechanisms in *H. perforatum* callus cultures. For this purpose, investigations have been focused on the effects of elicitor treatments on the following:

- (1) Fresh biomass production;
- (2) Total phenolic, flavonoid, flavan-3-ol, and anthocyanin contents;
- (3) Chromatographic quantification of naphthodianthrones (hypericin and pseudohypericin);
- (4) Enzyme activities of phenylalanine ammonia lyase (PAL) and chalcone isomerase (CHI);
- (5) Non-enzymatic antioxidant properties (NEAOP) by the  $\beta$ -carotene bleaching method and enzymatic activities of peroxidase (POD) and catalase (CAT).

Additionally, to provide a comparative perspective, the effects of JA on callus cultures were evaluated alongside differentiated shoot cultures. By achieving these objectives, this study provides, for the first time, a comprehensive insight into the simultaneous modulation of phenolic and naphthodianthrone metabolism by JA in *H. perforatum* calli, thereby advancing our understanding of their biosynthetic potential under controlled elicitation conditions.

## 2. Materials and Methods

### 2.1. Chemical and Reagents

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The reference standards of hypericin (HYP) and pseudohypericin (PHYP) were HPLC grade with >99% and >98% purity, respectively (PLANTA Naturstoffe Vertriebs GmbH, Vienna, Austria). The solvents used for mobile phase were LC-MS grade purchased from Sigma-Aldrich (Steinheim, Germany).

### 2.2. Establishment of Shoot and Callus Cultures for Elicitation

In vitro seedling cultures of *H. perforatum* were obtained after seed sterilization and germination on solid hormone-free medium supplemented with MS macro- and oligoelements, Gamborg B5 vitamin mixture (0.1 mg·L<sup>-1</sup> thiamine-HCl, 0.1 mg·L<sup>-1</sup> nicotinic acid and 1 mg·L<sup>-1</sup> pyridoxine-HCl), 3% sucrose, 0.7% agar, 0.2 g·L<sup>-1</sup> casein hydrolysate, and 0.1 g·L<sup>-1</sup> myo-inositol [25].

Three-week-old in vitro seedlings were used as stock cultures for the establishment of multiple shoots and calli in the following elicitation experiments with JA. These in vitro seedling, shoot, and callus cultures were kept in a growth chamber at 24 °C, with a photoperiod of 16 h, irradiance of 50  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , and relative humidity of 60%.

Apical segments with 2–3 leaf pairs were isolated from in vitro seedlings and inoculated in test tubes on MS/B5 medium with 0.5 mg·L<sup>-1</sup> N<sup>6</sup>-benzyladenine (BA) to obtain multiplied shoot cultures. Subcultures were carried out every 14 days. After two subcultures, multiple shoots (2–2.5 cm) were isolated and transferred to the medium supplemented with JA. For the induction of callus cultures, leaf explants were isolated from in vitro seedlings and inoculated in Petri dishes on MS/B5 medium supplemented with 1.0 mg·L<sup>-1</sup> 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg·L<sup>-1</sup> BA, and 0.1 mg·L<sup>-1</sup> 1-naphthaleneacetic acid (NAA). The callus tissues induced from the leaf explants were

isolated and subcultured every 14 days. After two subcultures, green calli were transferred to a MS/B5 medium supplemented with JA.

Elicitation experiments on calli and shoots were performed with exogenous application of different concentrations of JA at a final concentration of 50, 100, and 250  $\mu\text{M}$  in the culture medium. The JA (Sigma-Aldrich) was dissolved in ethanol and this stock solution was filter-sterilized through a 0.22  $\mu\text{m}$  syringe filter. Control shoots and calli were treated with an appropriate volume of ethanol.

Elicited and control calli were harvested at days 1, 4, 7, 14, and 21 of post-elicitation, while elicited and control shoots were harvested at days 7, 14, and 21 of post-elicitation. This selection of harvest days for both cultures was based on our previous elicitation experiments suggesting that differentiated shoots did not provide a clear answer to the applied elicitors in the early stage of post-elicitation, while undifferentiated cell cultures showed fast proliferation and strong response in the biosynthesis of secondary metabolites [26]. The harvested biomass of calli and shoots was weighed for fresh weight analysis, lyophilized and ground for phytochemical analyses and non-enzymatic antioxidant capacity, or frozen in liquid nitrogen and stored at  $-80\text{ }^{\circ}\text{C}$  for determination of antioxidant and phenylpropanoid/flavonoid enzymes activities.

### 2.3. Total Phenolic Compound Contents

**Extract preparation.** Phenolic compounds were extracted from homogenized plant material (0.2 g) with 5 mL 80% (*v/v*) methanol in ultrasonic bath at  $4\text{ }^{\circ}\text{C}$  for 30 min. After centrifugation (10 min at 12 000 rpm), the residue was re-extracted with 80% methanol under the above-mentioned conditions. The supernatants from both extractions were combined and used for quantification of total phenolics (TP), total flavonoids (TF), total flavan-3-ols (TFL), and total anthocyanins (TANT) contents.

**Total phenolics (TP) contents.** The TP content was determined according to the Folin–Ciocalteu assay, which is based on electron transfer from phenolic compounds to phosphomolybdic/phosphotungstic acid blue complex in alkaline medium. The assay was performed as follows: 20  $\mu\text{L}$  of methanolic extract were mixed with 100  $\mu\text{L}$  Folin–Ciocalteu reagent (Carlo Erba, Cornaredo, Italy) diluted 10 times with distilled water and 80  $\mu\text{L}$  0.7 M  $\text{Na}_2\text{CO}_3$  [27]. After the incubation of samples for 5 min at  $50\text{ }^{\circ}\text{C}$ , the absorbance was measured at 765 nm and the concentration of TP was calculated using gallic acid as a standard. The TP contents were expressed as milligrams of gallic acid (GA) equivalents per gram of dry weight ( $\text{mg GAE}\cdot\text{g}^{-1}\text{ DW}$ ).

**Total flavonoids (TF) contents.** The TF content was determined according to a method based on the nitration of flavonoids with  $\text{NaNO}_2$  followed by the development of a red-colored flavonoid– $\text{Al}^{3+}$  complex using  $\text{AlCl}_3$  in alkaline conditions. The assay was performed by mixing of 10  $\mu\text{L}$  methanolic extract with 5  $\mu\text{L}$  5%  $\text{NaNO}_2$ , 5  $\mu\text{L}$  10%  $\text{AlCl}_3$ , 80  $\mu\text{L}$  1 M  $\text{NaOH}$ , and 100  $\mu\text{L}$  distilled water [28]. Absorbance was measured at 510 nm and the concentration of TF was calculated using quercetin as a standard. The TF contents were expressed as milligrams of quercetin (Q) equivalents per gram of dry weight ( $\text{mg QE}\cdot\text{g}^{-1}\text{ DW}$ ).

**Total flavan-3-ols (TFL) contents.** The TFL contents were analyzed in plant extracts with 4-dimethylaminocinnamaldehyde (DMACA) reagent. This method is based on the formation of a green-colored complex between DMACA reagent and *meta*-oriented hydroxyl groups from flavane molecule with a single bond in the 2–3 position of heterocyclic ring [29]. An aliquot of 180  $\mu\text{L}$  DMACA reagent (100 mg DMACA dissolved in 100 mL of methanolic sulfuric acid) was added to 20  $\mu\text{L}$  of methanolic extracts. After incubation of the samples for 20 min at room temperature, the absorbance was measured at 637 nm and the concentration

of TFL was calculated using catechin as a standard. The TFA contents were expressed as milligrams of catechin (C) equivalents per gram of dry weight ( $\text{mg CE}\cdot\text{g}^{-1}\text{ DW}$ ).

Total anthocyanins (TANT) contents. The TANT content was analyzed by the method described by Giusti et al. [30]. The extraction of anthocyanins from plant samples was performed with 1% HCl/CH<sub>3</sub>OH (15/85, *v/v*). Absorbance was measured at 530 nm and the concentration of TANT was calculated using the molar extinction coefficient of cyanidin-3-glucoside ( $\epsilon_{530} = 34,300\text{ M}^{-1}\cdot\text{cm}^{-1}$ ) in acidic methanol. The TANT contents were expressed as milligrams of cyanidin-3-glucoside (CG) equivalents per gram of dry weight ( $\text{mg CGE}\cdot\text{g}^{-1}\text{ DW}$ ).

#### 2.4. Phenylpropanoid/Flavonoid Pathway Enzyme Assays

Extraction of enzymes. The extraction procedure was based on the homogenization of frozen tissues in 100 mM potassium phosphate buffer (pH 8.0) containing 2 mM EDTA and 1% (*w/v*) polyvinylpyrrolidone. This homogenate was centrifuged (12 000 rpm for 20 min) at 4 °C. The supernatant (enzyme extract) was collected for the determination of soluble protein content and enzyme activities.

Phenylalanine ammonia-lyase (PAL). The PAL activity was determined according to method described by Gadzovska et al. [26]. The reaction mixture consisted of 20  $\mu\text{L}$  enzyme extract and 180  $\mu\text{L}$  2% (*w/v*) L-phenylalanine in 50 mM Tris-HCl buffer (pH 8.8) and was incubated for 1 h at 40 °C. The PAL activity was determined as its absorbance increased at 290 nm by the measuring the rate of formation of *trans*-cinnamic acid. The molar extinction coefficient of cinnamate was  $\epsilon_{290} = 19,600\text{ M}^{-1}\cdot\text{cm}^{-1}$ . The specific activity of PAL was expressed in  $\text{pkat}\cdot\text{mg}^{-1}$  proteins.

Chalcone isomerase (CHI). The CHI activity was assayed according to the method presented by Gadzovska et al. [26]. The CHI assay was performed in 60 mM phosphate buffer (pH 8) with 50 mM KCN to inhibit peroxidase activity. The reaction was initiated by the addition of 25  $\mu\text{L}$  of enzyme extract in 225  $\mu\text{L}$  0.1% ethanolic solution of 2',4',6-tetrahydrochalcone and the mixture was incubated at 30 °C for 45 min. The enzyme reaction was monitored as its absorbance decreased at 400 nm. The molar extinction coefficient of 2',4',6-tetrahydrochalcone was  $\epsilon_{400} = 33,113\text{ M}^{-1}\cdot\text{cm}^{-1}$ . The specific activity of CHI was expressed in  $\text{pkat}\cdot\text{mg}^{-1}$  proteins.

#### 2.5. Chromatographic Identification and Quantification of Hypericin and Pseudohypericin

The protocols for the extraction of hypericin (HYP) and pseudohypericin (PHYP), as well as the separation, identification, and quantification of these naphthodianthrones in plant extracts were previously presented by Gadzovska et al. [25]. Briefly, chromatographic separation of HYP and PHYP were conducted on a Hypersil reversed-phase C18 column (150  $\times$  4.6 mm, 5  $\mu\text{m}$ ) at room temperature. The mobile phase consisted of two solvents: 10 mM triethylammonium acetate buffer (pH 7.0) and a mixture of methanol and acetonitrile (5:4, *v/v*). The gradient program, with a flow rate of 1.5  $\text{mL}\cdot\text{min}^{-1}$  and with a 20  $\mu\text{L}$  injected volume, was as follows: 60% B (0–3 min), 92% B (4–9 min), and 100% B (10 min). The HYP and PHYP standard solutions were prepared in a concentration of 1–100  $\mu\text{g}\cdot\text{mL}^{-1}$ , while chromatograms were read at 590 nm. The mass spectra of naphthodianthrones were acquired in negative ionization mode using a LCQ Deca mass spectrometer coupled with an atmospheric pressure chemical ionization (APCI). The APCI conditions were as follows: sheath gas (65 psi), auxiliary nitrogen gas (10 psi), while the capillary temperature, the needle voltage and capillary voltage were set at 250 °C, 4.5 kV, and –12 V, respectively. The HYP and PHYP contents in plant extracts were expressed as micrograms per gram of dry weight ( $\mu\text{g}\cdot\text{g}^{-1}\text{ DW}$ ).

The sensitivity of the method was assessed by determining the limits of detection (LOD) and quantification (LOQ), calculated as  $LOD = 3.3 \times \sigma/S$  and  $LOQ = 10 \times \sigma/S$ , where  $\sigma$  is the standard deviation of the  $y$ -intercept and  $S$  is the slope of the calibration curve at low concentration levels (0.100–1.00  $\mu\text{g}\cdot\text{mL}^{-1}$ ). The LOD values were 0.050  $\mu\text{g}\cdot\text{mL}^{-1}$  for HYP and 0.075  $\mu\text{g}\cdot\text{mL}^{-1}$  for PHYP, while the corresponding LOQ values were 0.167  $\mu\text{g}\cdot\text{mL}^{-1}$  and 0.250  $\mu\text{g}\cdot\text{mL}^{-1}$ . Method accuracy, evaluated through a recovery test, yielded sample concentrations of 0.283  $\mu\text{g}\cdot\text{mL}^{-1}$  for HYP and 0.538  $\mu\text{g}/\text{mL}$  for PHYP.

#### 2.6. Non-Enzymatic Antioxidant Properties (NEAOP) and Enzymatic Antioxidant Assays

NEAOP assay. The NEAOP of methanolic extracts were estimated by the  $\beta$ -carotene bleaching method modified by Marron et al. [31]. The analyses were performed by mixing 20  $\mu\text{L}$  methanolic extract, 30  $\mu\text{L}$  80% methanol, and 250  $\mu\text{L}$  of linoleic acid- $\beta$ -carotene emulsion (10 mg linoleic acid, 0.2  $\text{mg}\cdot\text{mL}^{-1}$  chloroformic  $\beta$ -carotene solution, and 0.1 g Tween 40), and the reaction mixture was incubated at 50 °C. The control reaction included 50  $\mu\text{L}$  80% methanol and 250  $\mu\text{L}$  emulsion. The absorbance of the samples was measured at 470 nm for 1 h and the antioxidant activity was expressed as inhibition percentage relative to the control according to the following equation:  $NEAOP = ((b - a)/b) \times 100$ , where  $a$  is variation in sample absorbance between 0 and 1 h;  $b$  is variation in control absorbance between 0 and 1 h.

Catalase (CAT) activity. The assessment of CAT activity in extracts was conducted according to Fu and Huang [32]. The reaction mixture contained 60 mM potassium phosphate buffer, 3% ( $v/v$ )  $\text{H}_2\text{O}_2$ , and deionized water. The enzyme reaction was initiated by the addition of 10  $\mu\text{L}$  enzyme extract to 190  $\mu\text{L}$  of reaction mixture. The reduction of  $\text{H}_2\text{O}_2$  was monitored for a period of 1 min as absorbance decreased at 240 nm. The rate of change in absorbance per minute was used to quantify CAT activity using the molar extinction coefficient of  $\text{H}_2\text{O}_2$   $\epsilon_{240} = 43.6 \text{ M}^{-1}\cdot\text{cm}^{-1}$ . The CAT-specific activity was expressed in  $\text{nkat}\cdot\text{mg}^{-1}$  proteins.

Peroxidase (POD) activity. The assessment of POD activity in plant extracts was performed according to method of Gonzales et al. [33] with minor modifications. The reaction mixture contained 100 mM sodium phosphate buffer (pH 6.0), 20 mM guaiacol, and 0.1% ( $v/v$ )  $\text{H}_2\text{O}_2$ . Reaction was initiated by addition of 10  $\mu\text{L}$  diluted enzyme extract to 190  $\mu\text{L}$  of reaction mixture and the absorbance was monitored at 420 nm for a period of 1 min. The rate of change in absorbance per minute was used to quantify POD activity using the molar extinction coefficient of the oxidized product tetraguaiacol ( $\epsilon_{420} = 6400 \text{ M}^{-1}\cdot\text{cm}^{-1}$ ). The POD-specific activity was expressed in  $\text{nkat}\cdot\text{mg}^{-1}$  proteins.

#### 2.7. Statistical Analysis

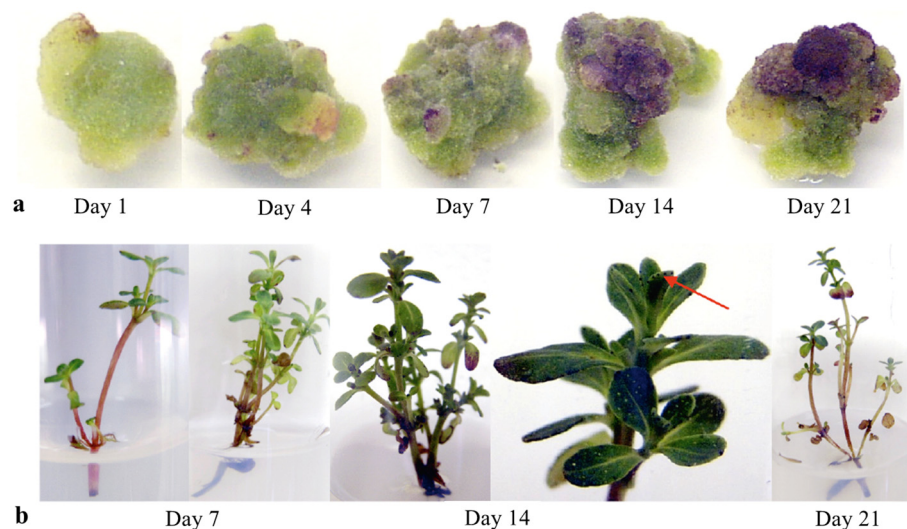
In this elicitation experiment, all analyses were conducted in biological triplicates. The statistical analyses were performed with the SPSS software program (v. 11.0.1, SPSS Inc., Chicago, IL, USA). The results for all parameters were presented as mean values with standard deviation and analyzed by one-way ANOVA to detect significant differences between samples. The significant differences between elicitor treatments and control samples at each post-elicitation period were post hoc evaluated using Student Newman Keuls' test. All statistical tests were considered to be statistically significant at  $p < 0.05$ .

### 3. Results

#### 3.1. Growth of *H. perforatum* Callus and Shoot Cultures

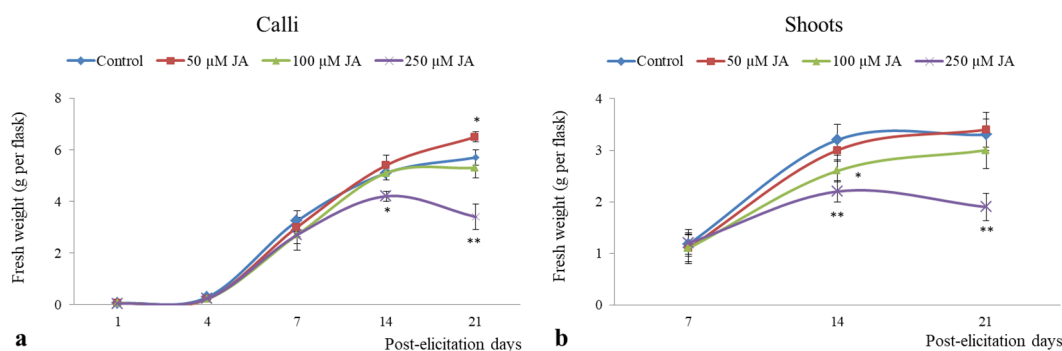
The influence of JA on the growth of *H. perforatum* callus and shoot cultures was monitored during 21 days of post-elicitation. Present results demonstrated that the green color of callus was changed into red and brown during the post-elicitation period (Figure 1a).

The control and elicited shoots had a reddish color on the stem and developed dark glands on the leaf margins. In comparison to healthy control shoots, treatment for a long time with JA (day 21) induced leaf browning and senescence (Figure 1b).



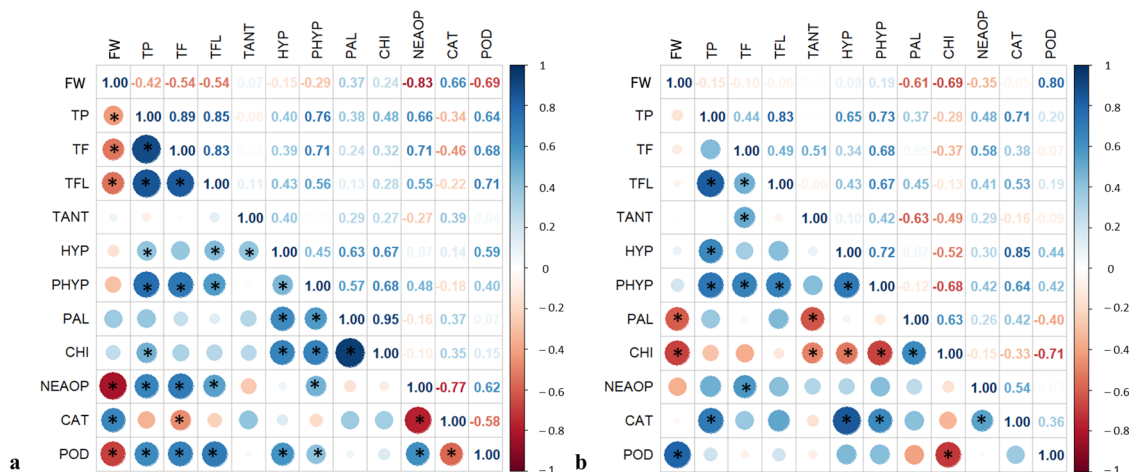
**Figure 1.** Morphology of *Hypericum perforatum* (a) calli and (b) shoots elicited with 100  $\mu\text{M}$  jasmonic acid at different post-elicitation periods.

In comparison to control callus cultures, the FW production of calli elicited with 50 and 100  $\mu\text{M}$  JA was not changed until the day 14 post-elicitation, where only 250  $\mu\text{M}$  JA caused significantly decreased biomass production. At the end of the post-elicitation period, the application of the lowest JA dose (50  $\mu\text{M}$ ) showed a 1.2-fold increase FW production, while the highest JA dose (250  $\mu\text{M}$ ) significantly decreased callus growth (1.7-fold) in comparison to control callus (Figure 2a). Regarding shoot growth under elicitor treatments (Figure 2b), high doses of JA (100 and 250  $\mu\text{M}$ ) significantly declined FW production in shoot cultures (up to 1.7-fold) compared to control shoots at day 14 and 21 of post-elicitation.



**Figure 2.** Biomass production of *Hypericum perforatum* (a) calli and (b) shoots elicited with jasmonic acid (JA). Asterisks indicate statistically significant differences compared to the control culture at each post-elicitation period (\*  $p < 0.05$ , \*\*  $p < 0.01$ ).

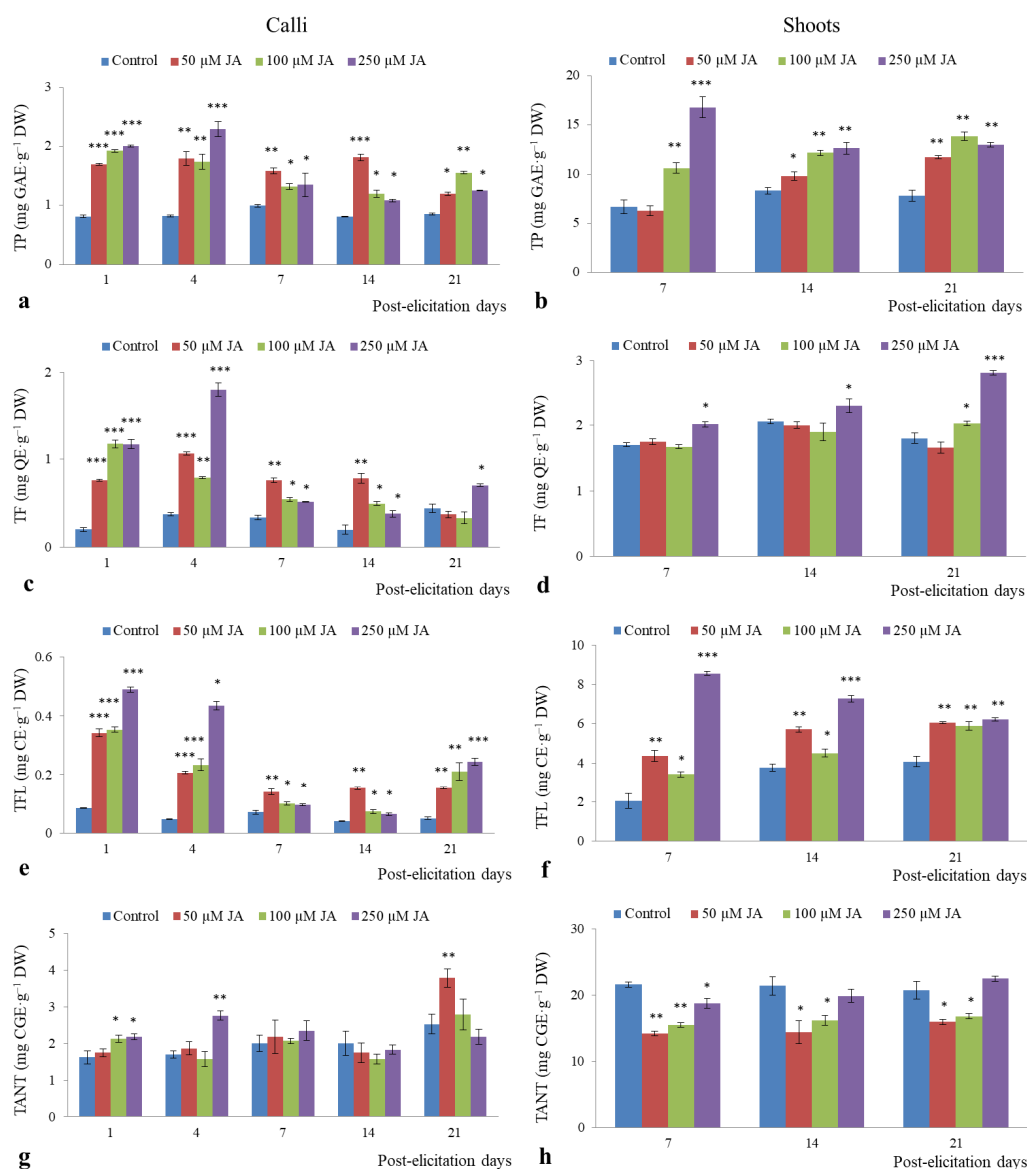
Correlation analysis showed that FW in JA-elicited calli had a significant negative relationship with phenolic compounds production (TP, TF, and TFL), as well as with antioxidant activities (NEAOP and POD), while significant positive correlation was found only for CAT activity (Figure 3a). In JA-elicited shoots, significant negative correlations were observed between FW and the activities of phenylpropanoid/flavonoid pathway enzymes (PAL and CHI), while positive correlation was shown between FW and POD activity (Figure 3b).



**Figure 3.** Correlation analyses between growth, phenolic compounds, and naphthodianthrone production and phenylpropanoid enzyme activities, non-enzymatic, and enzymatic antioxidant activities in *Hypericum perforatum* calli (a) and shoots (b) elicited with jasmonic acid. Blue-colored circles presented positive correlation, while red-colored circles showed negative correlation. Black asterisks represent the significance of correlation ( $p < 0.05$ ). FW: fresh biomass; TP: total phenolics; TF: total flavonoids; TFL: total flavan-3-ols; TANT: total anthocyanins; HYP: hypericin; PHYP: pseudohypericin; PAL: phenylalanine ammonia-lyase; CHI: chalcone isomerase; NEAOP: non-enzymatic antioxidant properties; CAT: catalase; POD: peroxidase.

### 3.2. Phenolic Compounds Production in *H. perforatum* Callus and Shoot Cultures

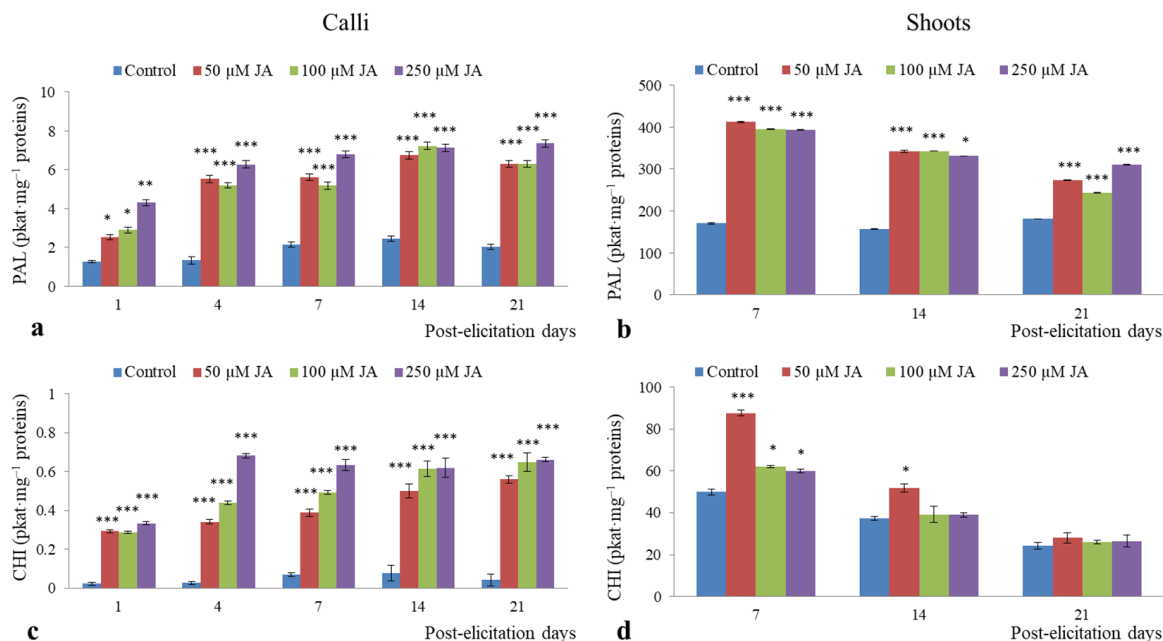
Phenolic compounds production (TP, TF, TFL, and TANT) in *H. perforatum* callus and shoot cultures was evaluated according to the dose of JA and duration of elicitation (Figure 4). The production of TP in callus cultures was constantly increased upon all doses of JA during the entire post-elicitation period (from 1.9- to 2.8-fold) in comparison to control calli (Figure 4a). Shoot cultures responded similarly to applied JA doses, inducing significantly enhanced TP production (from 1.2- to 2.5-fold) during the post-elicitation period compared to control shoots (Figure 4b). The TF contents in calli with all JA treatments were notably enhanced from day 1 to day 14 (up to 4.8-fold), while at day 21 only 250  $\mu$ M JA caused significantly increased TF production (1.6-fold) in comparison to control calli (Figure 4c). Concerning shoot cultures, only 250  $\mu$ M JA-elicited shoots exhibited increased TF contents (up to 1.5-fold) during entire post-elicitation period compared to control cultures (Figure 4d). In comparison to control cultures, all tested JA concentrations caused significantly enhanced TFL production in calli (from 1.4- to 9.2-fold) and shoots (from 1.3- to 4.3-fold) from the beginning to the end of post-elicitation (Figure 4e,f). Callus cultures displayed an early enhancement of TANT production upon treatments with 100 and 250  $\mu$ M JA (up to 1.6-fold) at day 1 and 4, while at the end of post-elicitation, only 50  $\mu$ M JA exhibited significantly increased TANT content (1.5-fold) compared to control calli (Figure 4g). On the other hand, shoot cultures elicited with 50 and 100  $\mu$ M JA exhibited significantly decreased TANT production (from 1.3- to 1.5-fold) in comparison to control shoots during the entire post-elicitation period (Figure 4h). Correlation data for JA-elicited calli and shoots (Figure 3) demonstrated that the levels of phenolic compounds (TP, TF, and TFL) were displayed a significant positive relationship with naphthodianthrone production (HYP and PHYP) and antioxidant activity (NEAOP, POD, and CAT). It is worth noting that TANT production in JA-elicited shoots was significantly negatively correlated with phenylpropanoid/flavonoid pathway enzyme (PAL and CHI) activities (Figure 3b).



**Figure 4.** Phenolic compounds contents in *Hypericum perforatum* callus and shoot cultures elicited with jasmonic acid (JA). (a,b) Total phenolic compounds (TP), (c,d) total flavonoids (TF), (e,f) total flavan-3-ols (TFL), (g,h) total anthocyanins (TANT). GAE: gallic acid equivalents; QE: quercetin equivalents; CE: catechin equivalents; CGE: cyanidin-3-glucoside equivalents; DW: dry weight. Asterisks indicate statistically significant differences compared to the control culture at each post-elicitation period (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

The PAL and CHI activities were also analyzed to investigate phenylpropanoid/flavonoid metabolic pathways in *H. perforatum* callus and shoot cultures upon elicitation with JA (Figure 5). Outgoing results demonstrated that both types of cultures responded to JA elicitation through up-regulation of PAL activity during the entire post-elicitation period. In this context, JA-elicited callus cultures showed a gradual enhancement in PAL activities from the beginning to the end of the post-elicitation period (from 2- to 3.6-fold) compared to the control callus (Figure 5a). Concerning JA-elicited shoots, the highest increment of PAL activity was observed at day 7 (about 2.4-fold), while at the end of post-elicitation, the enzyme activity was enhanced from 1.3- to 1.7-fold compared to control shoots (Figure 5b). Callus and shoot cultures showed distinct responses to CHI activity upon exogenously applied JA concentrations. The JA-elicited calli displayed an enormous increment of CHI activity (up to 25.3-fold) compared to control calli throughout the post-elicitation period

(Figure 5c). In comparison to control shoots, CHI activity in shoot cultures was significantly up-regulated (from 1.2- to 1.7-fold) at day 7, while only 50  $\mu\text{M}$  JA showed a 1.4-fold increased enzyme activity at day 14 of post-elicitation (Figure 5d).

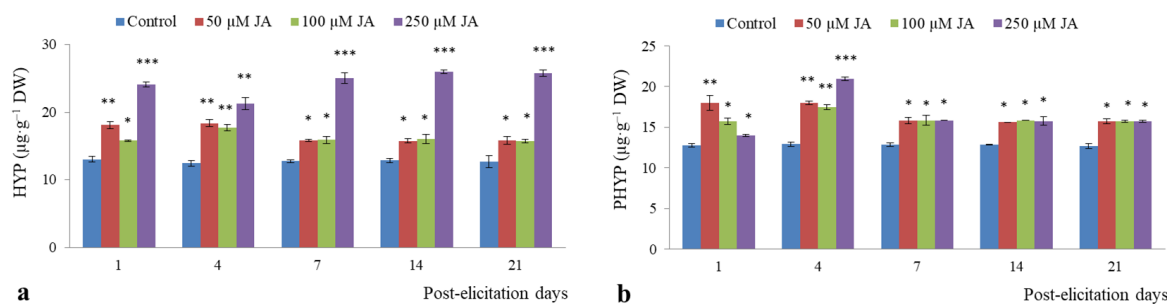


**Figure 5.** Phenylpropanoid enzyme activities in *Hypericum perforatum* callus and shoot cultures elicited with jasmonic acid (JA). (a,b) Phenylalanine ammonia-lyase (PAL). (c,d) Chalcone isomerase (CHI). Asterisks indicate statistically significant differences compared to the control culture at each post-elicitation period (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

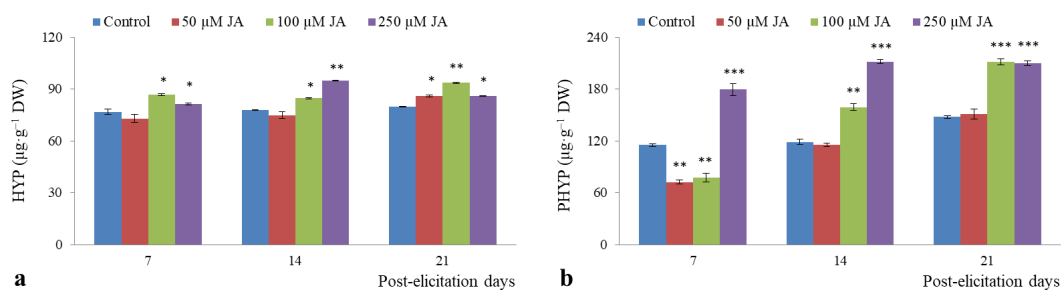
According to correlation analysis, the PAL and CHI enzyme activities in JA-elicited calli (Figure 3a) showed a significant positive relationship with naphthodianthrone production (HYP and PHYP). On the other hand, CHI activity in JA-elicited shoots was in significant negative correlation to HYP and PHYP levels, as well as to POD activity (Figure 3b).

### 3.3. Naphthodianthrone Production in *H. perforatum* Callus and Shoot Cultures

The chromatographic analysis revealed that JA elicitation differently affects naphthodianthrone (HYP and PHYP) production in callus (Figure 6) and shoot cultures (Figure 7).



**Figure 6.** Naphthodianthrone production in *Hypericum perforatum* callus cultures elicited with jasmonic acid (JA). (a) Hypericin (HYP). (b) Pseudohypericin (PHYP). DW: dry weight. Asterisks indicate statistically significant differences compared to the control culture at each post-elicitation period (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).



**Figure 7.** Naphthodianthrone production in *Hypericum perforatum* shoot cultures elicited with jasmonic acid (JA). (a) Hypericin (HYP). (b) Pseudohypericin (PHYP). DW: dry weight. Asterisks indicate statistically significant differences compared to the control culture at each post-elicitation period (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

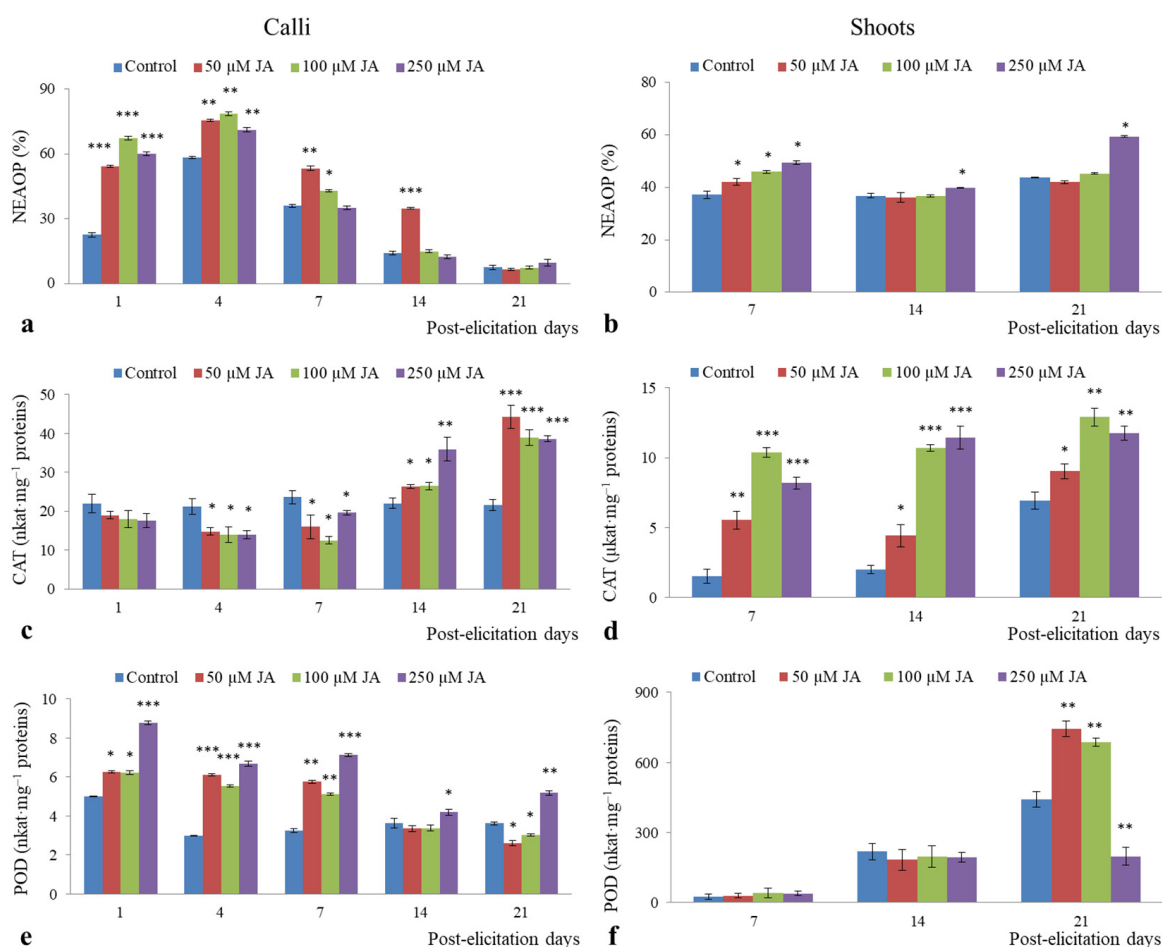
Over the course of the entire post-elicitation, HYP production was significantly enhanced in calli elicited with all tested JA doses, while a maximum HYP production (about twofold) was observed upon exogenous application of 250 µM JA (Figure 6a). Similarly, JA-elicited calli displayed increased levels of PHYP (up to 1.6-fold) during entire post-elicitation period compared to control calli (Figure 6b).

Contrary to calli, JA did not exhibit a strong effect on HYP amounts in shoot cultures, where addition of 100 and 250 µM JA caused slightly increased production of this naphthodianthrone (about 1.2-fold) in elicited shoots compared to control cultures during entire post-elicitation period (Figure 7a). Also, significantly higher HYP level was observed in 50 µM JA-elicited shoots compared to control cultures at the end of post-elicitation (day 21). The exogenous application of 50 µM JA in shoot cultures caused a significant decrease in PHYP amount at the beginning of the post-elicitation period (day 7), while this JA dose did not influence PHYP production until the end of the post-elicitation period. The PHYP production in shoots elicited with 100 µM JA was significantly declined at day 7, but it was notably elevated in the following period of post-elicitation (day 14 and 21) compared to control shoots. The PHYP contents in shoots treated with 250 µM JA was significantly increased (up to 1.8-fold) during the entire post-elicitation period compared to control cultures (Figure 7b).

Elicitation with JA demonstrated that HYP and PHYP levels in callus cultures (Figure 3a) were in significant positive correlation with antioxidant activities (NEAOP and POD), while those amounts in shoot cultures (Figure 3b) were positively related to CAT activity.

### 3.4. Antioxidant Activity in *H. perforatum* Callus and Shoot Cultures

Present results showed that all tested JA doses induced an early enhancement of NEAOP values in callus cultures (from 1.2- to 3-fold) up to day 4, while at the end of post-elicitation (day 21), there were no significant differences in antioxidant capacity between control and elicited calli (Figure 8a). With respect to shoot cultures, all JA doses caused significant enhancement of NEAOP at day 7 of post-elicitation (up to 1.3-fold), and thereafter, that elevation in antioxidant capacity compared to control cultures was maintained only upon treatment with 250 µM JA (Figure 8b). Concerning enzymatic antioxidant activities, the CAT activity in JA elicited calli was significantly declined during the intermediate post-elicitation period, and thereafter, applied elicitor doses induced a late and gradually elevation of enzyme activity (up to 2.1-fold) in comparison to control calli (Figure 8c). In contrast, all JA doses caused a significant increment of CAT activity in shoot cultures (from 1.3- to 6.9-fold) during entire post-elicitation period compared to control shoots (Figure 8d).



**Figure 8.** Antioxidant activities of *Hypericum perforatum* callus and shoot cultures elicited with jasmonic acid (JA). (a,b) Non-enzymatic antioxidant property (NEAOP), (c,d) catalase (CAT), (e,f) peroxidase (POD). Asterisks indicate statistically significant differences compared to the control culture at each post-elicitation period (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

Treatments of calli with all JA doses evoked an early enhancement of POD activity (up to 2.2-fold) compared to control calli and this increased enzyme activity was continued to the end of post-elicitation only with 250  $\mu\text{M}$  JA (Figure 8e). At day 21 of post-elicitation, a significant reduction in POD activity was observed in callus cultures upon treatment with 50 and 100  $\mu\text{M}$  JA. Significant differences in POD activity between control and elicited shoots were found only at the end of post-elicitation (Figure 8f), where 50 and 100  $\mu\text{M}$  JA induced about 1.6-fold enhancement in enzyme activity, while application of 250  $\mu\text{M}$  JA notably decreased POD activity (2.2-fold). Statistical analysis demonstrated that NEAOP in JA-elicited calli was in significant positive correlation to POD activity, but in negative relationship with CAT activity (Figure 3a). With regard to shoots elicited with JA, significant positive correlation was observed between NEAOP and CAT activity (Figure 3b).

## 4. Discussion

### 4.1. Effect of Elicitation on the Growth of *H. perforatum* Calli and Shoots

The productivity of plant in vitro culture systems upon elicitation depends not only on their capability for bioactive compounds production, but also on the competence to produce viable biomass [34]. The outgoing elicitation experiment with JA showed that a low elicitor dose of 50  $\mu\text{M}$  had a promoting effect on biomass production in callus cultures during the late post-elicitation period, while a high dose of 250  $\mu\text{M}$  was observed as a growth suppressor. In accordance with our results, Walker et al. [17] have noticed

that low-dose JA elicitation (up to 250  $\mu\text{M}$ ) stimulates the growth, while the addition of 500  $\mu\text{M}$  JA causes a decline in biomass production in undifferentiated cell suspensions of *H. perforatum*. Similarly, 10 and 50  $\mu\text{M}$  JA have been selected as optimal doses for fresh biomass increment in *H. perforatum* hairy roots during the late post-elicitation period [35]. Those authors have revealed that low JA doses caused a complete depletion of phosphates from the medium, resulting in cell proliferation and biomass accumulation. Even though we did not monitor nutrient composition in the medium during elicitation experiments, it seems that JA at low concentrations had a growth-promoting effect in callus cultures through increased utilization of nutrients that are crucial for boosting biomass production. Another explanation for the better growth of JA-elicited calli could be the enhancement of intrinsic concentrations of auxins and cytokinins, since it has been reported that jasmonates modulate signaling pathways of these phytohormones [36,37]. On the other hand, we have previously observed that the application of 100 and 250  $\mu\text{M}$  JA significantly inhibited the growth of *H. perforatum* undifferentiated cell suspensions [19], which is in agreement with present data. In this context, Gadzovska et al. [19] noticed that prolonged elicitation with a high JA dose induced a brown coloration, cell aggregation, and decreased the viability of *H. perforatum*-treated cells. These findings imply that the application of a non-physiologically high JA dose in *H. perforatum* undifferentiated cells or calli induced a stressful environment that suppressed cell growth but may activate secondary metabolism as a part of its defense mechanism. In this view, declined biomass production in elicited calli was strongly related to the increased levels of phenolic compounds (TPL, TF and TFL) and antioxidant activity (NEAOP). This relationship could be explained by the growth–defense hypothesis suggesting that high JA concentrations caused stressful conditions for callus cells resulting in energy channeling towards biosynthesis of antioxidant phenolic compounds instead of primary metabolites responsible for biomass increment.

Although elicitation experiments on *Hypericum* shoots and plantlets with MeJA are well-documented [21–23,38], the effects of JA on the growth performance of *H. perforatum* shoots have not yet been investigated. With regard to shoot cultures, it was presently shown that treatment with 50  $\mu\text{M}$  JA did not affect biomass production, while high elicitor dosage (100 and 250  $\mu\text{M}$  JA) notably depressed shoot growth. In accordance with our data, growth retardation upon elicitation with 100–500  $\mu\text{M}$  JA has already been confirmed in *H. hirsutum* and *H. maculatum* shoot cultures [39]. Since endogenous JA concentration in plant cells is very low, it seems that elicitation with high JA concentration had detrimental effects on shoot biomass through inducing of shoot senescence and necrosis [38], as well as decreasing of leaf area, chlorophyll pigments levels, photosynthesis rate, and carbohydrates contents in the leaves [40]. Additionally, it has been proposed that the exogenous application of JA might indirectly repress shoot growth by antagonizing the gibberellic acid pathway, resulting in the down-regulation of photosynthesis [41]. Taking into account that shoot cultures of different *Hypericum* species have shown a great variability in endogenous JA levels [42], it would be of great importance to adjust and select optimal elicitor dose for obtainment of fast growing and highly productive *H. perforatum* in vitro cultures. The growth–defense hypothesis could also be validated in elicited shoot cultures since the inhibited biomass production coincided with the up-regulated activities of PAL and CHI as key enzymes of phenylpropanoid/flavonoid biosynthetic pathways. Altogether, present data indicated that optimization of JA concentration, exposure period, and type of in vitro culture could be an important point in the elicitation studies to diminish the elicitor-mediated growth suppression and to maintain a feasible plant biomass, enriched with desired metabolites.

#### 4.2. Effect of Elicitation on Phenolic Compounds Production in *H. perforatum* Calli and Shoots

Present results showed that JA efficiently elicits biosynthesis of phenolic compounds (TP, TF, and TFL) in callus cultures. Similarly to present data, we have already reported that these JA concentrations (50–250  $\mu\text{M}$ ) have stimulatory effects on the overproduction of phenylpropanoids in *H. perforatum* cell suspensions during entire period of post-elicitation for 21 days [19]. Recently, it has been found that 10  $\mu\text{M}$  JA exhibited late response towards eliciting phenolic compounds and flavonoids, while JA at a dose of 100  $\mu\text{M}$  continuously stimulated phenylpropanoid metabolites throughout elicitation period in *H. perforatum* hairy roots [35]. Nevertheless, the influence of JA on phenylpropanoid metabolism between calli cultured under photoperiod and dark-grown hairy roots could not be properly compared since phenolic compounds production depends on dark/light conditions, cell differentiation status, and type of in vitro culture [26,43]. The results of this study showed that elicited calli with all tested JA concentrations exhibited a strong and progressive up-regulation of PAL and CHI activity during post-elicitation period that was positively related to the content of phenolic and flavonoid compounds. In accordance, the coordinated induction of PAL and CHI activity has also been noticed in *H. perforatum* cell suspensions elicited with JA [19]. Thus, significant correlations of phenolic compounds enhancement and increased activities of PAL and CHI confirmed the partitioning between the phenylpropanoid/flavonoid pathways in JA-elicited calli of *H. perforatum*. All these observations suggested that JA is an efficient elicitor for PAL- and CHI-mediated activation of phenylpropanoid metabolism and increased production of phenolic compounds in callus cultures of *H. perforatum*.

With respect to elicited shoots, PAL and CHI activities were boosted at the beginning of treatment with JA and thereafter, a gradual decline of their activities was observed to the end of post-elicitation. Accordingly, it has been noticed that flavonoid accumulation in in vitro plants is related to an early and transient increment of PAL activity after treatment with JA [44]. It is well-known that PAL is encoded by a multi-gene family with multiple isoforms that undergoes a complex regulation and differential expression depending on the type of cells/tissues, stages of development, as well as environmental stimuli [45]. It is interesting to point out that PAL and CHI activities did not give a clear correlation with TP, TF, and TFL production in JA-elicited shoots, which is in agreement with previous reports for *H. perforatum* shoot cultures elicited with SA [26] and polysaccharide elicitors [46], as well for transformed shoots regenerated from hairy roots [47]. Even though the PAL-mediated activation of phenylpropanoid metabolism is considered a common response to jasmonates, it has been shown that elicitation of *H. perforatum* could result in up-regulation of various classes of phenolic compounds. In this context, JA and MeJA have been proposed as effective elicitors for overproduction of xanthones [18,35], coumaroylquinic acids [40], as well as hypericins and hyperforin [39] in various *Hypericum* in vitro cultures. Numerous investigations have confirmed that xanthones represent inducible phytoalexins that are strongly accumulated in *H. perforatum* upon elicitation with bacterial and fungal elicitors, chitosan, and acetic acid [18,48–50]. Franklin et al. [48] have observed de novo production of xanthones in *H. perforatum* cell suspensions co-cultivated with *Agrobacterium tumefaciens* through up-regulation of PAL and 4-coumarate:CoA ligase (4CL) genes from the general phenylpropanoid metabolism and benzophenone synthase (BPS) gene from the xanthone biosynthetic pathway. These findings indicated that the induction of PAL activity in JA-elicited shoots is probably related to the accumulation of xanthones instead of flavonoids, although other alternative enzymes from the phenylpropanoid/flavonoid pathways cannot be excluded in the process of elicitation. Thus, further research should be focused on the evaluation of JA elicitation on different enzymes to determine the channeling of phenylpropanoid/flavonoid metabolism in *H. perforatum* shoot cultures.

Surprisingly, calli exhibited early induction of TANT production upon high JA doses, while the lowest elicitor concentration stimulated maximal productivity of these compounds at the end of post-elicitation. Similarly to our data, several studies have reported that low JA/MeJA concentrations have a great performance in anthocyanin accumulation in callus cultures of *Hibiscus sabdariffa* [51], *Allium jesdianum* [52] and *Azadirachta indica* [53]. Additionally, Chettri et al. [54] have observed that high JA concentration (100  $\mu\text{M}$ ) could not sustain induced production of anthocyanins over a prolonged period of post-elicitation in callus cultures of *Rosa* spp. Those results indicated that JA concentration and the time of elicitation are of particular importance for the production of anthocyanins in *H. perforatum* callus cultures. In contrast, TANT production in JA-elicited shoots continuously declined during the entire elicitation period. According to Aıkgöz et al. [55], the inhibitory effect of MeJA on anthocyanin production in *Achillea gypsicola* cell suspensions could be attributed to redirecting the phenylpropanoid metabolic flux from anthocyanins toward other phenolic compounds. This scenario could also be supported through our data because decreased anthocyanin production in JA-elicited shoots was related to the increased activities of PAL and CHI activities. Considering these data, we could speculate that JA activates PAL- and CHI-mediated downstream reactions towards flavan-3-ols, while the anthocyanin biosynthetic route is suppressed. Our previous studies demonstrated that SA, JA, polysaccharides and fungal mycelia could be efficiently used for enhanced production of anthocyanins in *H. perforatum* undifferentiated cells and calli as highly responsive culture systems for elicitation [19,26,56,57]. On the other hand, anthocyanin accumulation in *H. perforatum* shoot cultures has not been really affected upon elicitation with SA [26], indicating that cell differentiation level takes a main part in the elicitation process in terms of stimulation of phenolic compounds production.

If we compare both culture systems in this study, it was evident that shoots are less prone to alteration in their capability for phenolic compounds production upon JA elicitation compared to calli. Namely, increased production of TP, TF and TFL in callus cultures was noticed for all applied JA doses, while only high JA concentration showed superior metabolite productivity in shoot cultures during the post-elicitation period. This finding indicated that shoot cells possess a higher threshold for stimulation of phenylpropanoid metabolism upon treatment with JA in comparison to callus cultures. This is in accordance with our previous study reporting that exogenous application of high JA dose (100  $\mu\text{M}$ ) exhibited better performance in enhancing phenolic compounds production in *H. perforatum* transformed roots [35]. Since perception of optimal elicitor concentration by the cell receptors has a crucial role in activation of secondary metabolites [58], it seems that binding of JA molecules to the receptors on calli cells somehow forms an equilibrium state that continuously activate signal transduction reactions leading to enhanced production of phenolic compounds. In favor of this hypothesis, homogenous and parenchymatous callus cells with intense metabolism and fast proliferation responded more strongly to elicitor molecules compared to various types of differentiated cells in shoots cultures [26].

#### 4.3. Effect of Elicitation on Naphthodianthrone Production in *H. perforatum* Calli and Shoots

The presence of naphthodianthrone, as well-known bioactive compounds in genus *Hypericum*, has already been reported in *H. perforatum* calli cultured on media with various growth regulators [25,26,59]. Those observations suggested that calli grown on phytohormone-supplemented medium acquired certain degree of differentiation, which is an important factor for production of naphthodianthrone. In this study, high JA dose of 250  $\mu\text{M}$  was found more efficient in enhancement of HYP contents, while all tested JA doses showed similar response toward increased PHYP production in elicited calli compared to control cultures. Although the effect of JA on naphthodianthrone production

in *H. perforatum* callus cultures remains unexplored, only limited studies have evaluated the role of this elicitor in stimulating hypericin and pseudohypericin accumulation in cell suspensions [17,19]. Accordingly, Walker et al. [17] observed that high doses of JA (250 and 500  $\mu\text{M}$ ) had a more pronounced effect on increased amount of hypericin in *H. perforatum* cell suspensions. On the other hand, Gadzovska et al. [19] have noticed that all applied JA doses (50–250  $\mu\text{M}$ ) similarly affected overproduction of hypericin and pseudohypericin in *H. perforatum* cell suspensions compared to control cells. These authors suggested that elicitors act as signal molecules stimulating both morphogenetic potential and biosynthetic productivity of callus cultures with respect to HYP and PHYP. Taking this into account, the variable response of *H. perforatum* cell and callus cultures towards exogenous application of JA could be ascribed to the degree of cell differentiation. In this context, it seems that penetration of JA in undifferentiated cells is easier than callus cultures [26] and this close contact between cells and elicitor facilitates the activation of signal transduction leading to enhanced production of naphthodianthrone. Interestingly, present study revealed that HYP and PHYP levels are significantly related to PAL and CHI activity in JA-elicited calli. The stimulation of the naphthodianthrone pathway with concomitant induction of PAL activity has already been observed in our previous study for *H. perforatum* cell suspension cultures elicited with JA [19]. To the best of our knowledge, the polyketide naphthodianthrone pathway is independent from the phenylpropanoid/flavonoid pathway since it includes successive condensation of acetate and malonate units and formation of emodin anthrone as a basic structural unit of hypericins [8]. One possible explanation for PAL-mediated increment of HYP and PHYP biosynthesis in JA-elicited calli could be related to the enhanced accumulation of carbon skeletons that are prerequisite for naphthodianthrone biosynthesis [40]. However, it is still unclear whether JA acted on increased production of HYP and PHYP in calli through up-regulated expression of the PAL gene or other polyketide synthase genes from the naphthodianthrone biosynthetic pathway.

With regard to shoot cultures, it was evident that only high JA doses (100 and 250  $\mu\text{M}$ ) had a capacity for significant enhancement of HYP and PHYP levels. Numerous studies have been performed to evaluate the effect of jasmonates on the biosynthesis of hypericins in *Hypericum* shoot cultures. Sirvent and Gibson [21] have observed that application of 200  $\mu\text{M}$  MeJA markedly elevated production of hypericins (HYP, PHYP, and protopseudohypericin) in *H. perforatum* plantlets. The remarkable effect on enhanced production of HYP has already been observed in *H. perforatum* in vitro plants upon treatment with 1  $\text{mg}\cdot\text{L}^{-1}$  MeJA [23]. The stimulatory effects of JA and MeJA on hypericins biosynthesis have also been confirmed in shoot cultures of other *Hypericum* species, such as *H. sampsonii* [22], *H. maculatum* and *H. hirsutum* [39], as well as *H. aviculariifolium* and *H. pruinatum* [60]. All these findings suggested that increased production of naphthodianthrone in *Hypericum* spp. represents an essential part of the plant defense system to exogenously applied JA, which is probably perceived as an external stress molecule.

#### 4.4. Effect of Elicitation on Antioxidant Activity in *H. perforatum* Calli and Shoots

For a better understanding of the elicitation effect on plant defense response, the non-enzymatic antioxidant property (NEAOP) and the activities of two important antioxidant enzymes (CAT and POD) under JA treatment were determined in callus and shoot cultures. The *H. perforatum* calli elicited with JA displayed fast and transient induction of non-enzymatic antioxidant properties, which is in accordance with previous reports for *Azadirachta indica* callus cultures elicited with JA [53] and *H. perforatum* cell suspensions treated with various polysaccharide elicitors [57]. Correlation analyses for antioxidant capacity demonstrated positive relationships with the content of phenolics, flavonoids, and flavan-3-ols, implying that these compounds, particularly catechins, markedly contributed

to the non-enzymatic antioxidant state in JA-elicited calli. Therefore, the strong antioxidant activity of JA-elicited calli could be related to the presence of various groups of phenolic compounds that were contained in the extracts, presumably to the epicatechin as a dominant monomeric flavan-3-ol in *H. perforatum* callus cultures [59]. The elicitation experiment on shoot cultures also confirmed that JA could rapidly increase accumulation of compounds with antioxidant properties. In this context, the antioxidant activity of JA-elicited shoots was exclusively related to the flavonoid levels. Since *H. perforatum* shoot cultures have been proposed as an excellent source of hyperoside, rutin, quercitrin, and quercetin [61], it seems that JA increased antioxidant activity in shoots through biosynthetic modulation of these flavonoids. Altogether, these findings indicated that *H. perforatum* non-enzymatic antioxidant system is represented by phenylpropanoid metabolites expressing an efficient response toward JA-triggered oxidative stress.

Many researchers have reported that elicitation with jasmonates leads to oxidative stress due to the overproduction of ROS that have a detrimental effect in plant cells. However, the ROS production in JA-elicited in vitro culture systems is tightly regulated through the activation of antioxidant enzymes in order to avoid elicitor-induced oxidative injury processes [35,62]. The investigation of enzymatic antioxidant response in *H. perforatum* callus cultures elicited with JA showed an early declining in CAT activity and delayed enhancement of enzyme activity during the post-elicitation period. In contrast, POD activity in JA-elicited calli displayed an opposite trend with fast induction of enzyme activity and lately reduction in this antioxidant enzyme. Accordingly, the preeminent enzymatic antioxidant defense strategy by POD and the lower response of CAT activity have been previously reported in *H. perforatum* cell suspensions elicited with fungal elicitors *Phoma*, *Fusarium* and *Botrytis* [56]. These findings indicated that down-regulation of CAT activity in callus cultures in the early post-elicitation period is probably compensated through the elevated POD activity. Since these antioxidant enzymes operate concurrently to reduce the toxic concentrations of H<sub>2</sub>O<sub>2</sub> in elicited cells [62], it could be hypothesized that POD had a crucial importance in the early protection of callus cells against JA-mediated overproduction of H<sub>2</sub>O<sub>2</sub>, while CAT provides a defensive strategy during the long-term elicitor treatment. Outgoing results also demonstrated that POD activity in callus cultures treated with JA was positively correlated with phenolic, flavonoid, and flavan-3-ol contents, as well as with the non-enzymatic antioxidant properties. This synergistic collaboration between non-enzymatic and enzymatic antioxidants in the maintenance of cellular redox homeostasis under JA elicitation has already been confirmed in *Pelargonium graveolens* cell cultures [63] and *H. perforatum* hairy roots [35]. Shoot cultures elicited with JA displayed a distinct enzymatic antioxidant response compared to callus cultures, because CAT activity was consistently up-regulated during post-elicitation, while only 50 and 100 µM JA induced a late increment of POD activity. It has been shown that CAT activity is maintained more elevated than POD activity in *H. perforatum* cells elicited with pectin, indicating that CAT is an essential H<sub>2</sub>O<sub>2</sub>-scavenging enzyme during stressful conditions [57]. Noteworthy, elevated CAT activity in JA-elicited shoots coincided with high contents of phenolics and naphthodianthrones, as well with the non-enzymatic antioxidant properties, indicating that shoot cultures had a superior protection against ROS-mediated oxidative injuries upon JA elicitation.

## 5. Conclusions

In summary, the present work showed that jasmonic acid is an efficient elicitor for the enhancement of secondary metabolite production in *H. perforatum* callus and shoot cultures. The exogenous application of 50 µM jasmonic acid stimulated fresh biomass production in calli, while an elicitor dose of 250 µM had a growth-inhibitory effect on both callus and

shoot cultures. A strong accumulation of phenolic compounds, flavonoids, and flavan-3-ols was related to markedly higher activities of phenylalanine ammonia lyase and chalcone isomerase in elicited callus cultures. Concerning antioxidant state, jasmonic acid caused a significant increase in non-enzymatic antioxidant properties, peroxidase, and catalase activities in calli and shoots during different periods of the post-elicitation period. One of the best achievements in this study was the continuous overproduction of hypericin in 250  $\mu\text{M}$  JA-elicited calli, implying that this culture system can be efficiently used for the extraction of naphthodianthrone. Although the elicitation of callus cultures with a high dose of jasmonic acid significantly enhanced naphthodianthrone biosynthesis, the substantial reduction in fresh biomass productivity remains a major challenge for bioprocessing applications. Nevertheless, the protocol developed in this study provides an indication of how secondary metabolism in *H. perforatum* responds to jasmonic acid, and may help to develop more precise strategies to achieve further increases in phenylpropanoids and naphthodianthrone in callus and shoot cultures.

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