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ORIGINAL ARTICLE

Elicitation of *Hypericum perforatum* L. hairy root cultures with salicylic acid and jasmonic acid enhances the production of phenolic compounds and naphthodianthrones with biological activities

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Abstract

The aim of this study was to investigate the effect of salicylic acid (SA) and jasmonic acid (JA) on the growth, phenolic compounds and naphthodianthrones production, antioxidant status and in vitro neuroprotective and antihyperlipidemic activities in *Hypericum perforatum* hairy roots (HR). Application of all SA concentrations (50–250 µM) significantly declined HR biomass, while JA (10 and 50 µM) stimulated HR growth during the late post-elicitation period. Growth suppression in elicited HR was related to decreased utilization of nutrients (NH_4^+, NO_3^-, PO_4^{3-}, and residual sugars) from the culture medium. Low doses of SA (50 and 100 µM) exhibited fast accumulation of total phenolics (TP), flavonoids (TF) and flavan-3-ols (TFA), whereas high JA dose (100 µM) continuously improve metabolite productivity of HR. Positive relationships of TP, TF and TFA contents with antioxidant assays suggested that these phenolics represent main contributors to the antioxidant activities of elicited cultures. Concerning antioxidant enzymes, SA induced an early up-regulation of superoxide dismutase (SOD), but permanently reduced peroxidase (POD) that results in increment of H_2O_2 and MDA levels. In contrast, JA-treated HR displayed consistent enhancement of POD and late decreased in SOD activity causing a suppression of oxidative stress markers. Strong accumulation of catechins (catechin, epicatechin and procyanidins), naphthodianthrones (hypericin, pseudohypericin and protopseudohypericin) and xanthones in elicited HR at day 4 of post-elicitation coincided with increased acetylcholinesterase, lipase and cholesterol esterase inhibition. Present results indicated that elicitation of *H. perforatum* HR with SA and JA represents an efficient strategy for the production of bioactive compounds.

Key message

Elicitation of *Hypericum perforatum* hairy roots with salicylic acid and jasmonic acid represents an efficient strategy for increased production of phenolic compounds and naphthodianthrones with neuroprotective and antihyperlipidemic activity.

Keywords Elicitation · Hairy roots · *Hypericum perforatum* · Nutrient utilization · Oxidative stress · Phenolic compounds

Introduction

Hypericum perforatum L. (St. John's wort) is the most popular medicinal plant species in the world that attract a considerable interest in the scientific society due to its richness in biologically active compounds. The aerial flowering parts of this plant contain structurally diverse compounds, such as naphthodianthrones, acyl-phloroglucinols, phenolic acids, flavonoids, xanthones, and volatile compounds with well-established medicinal properties (Suryawanshi et al. 2024). These metabolites provide pharmacological benefits through

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their antioxidant, antimicrobial, antiviral, wound-healing, anticancer, anti-inflammatory, anti-obesity, antidiabetic, neuroprotective and antidepressant activities (Velingkar et al. 2017; Silva et al. 2021). Taking this into account, numerous dietary and medicinal preparations containing extracts or individual compounds from *H. perforatum* are among the top-selling products on the market worldwide for therapeutic properties (Barnes et al. 2019). However, final commercial products containing *H. perforatum* were prepared using crude plant material from wild or cultivated plants that are characterized with naturally occurring heterogeneity in the quality and quantity of bioactive compounds (Scotti et al. 2019). Thus, a plausible attention should be paid to the collection, cultivation and processing of *H. perforatum* raw material in order to obtain well-defined and standardized final products. One efficient approach to overcome chemical inconsistency of crude material represents the implementation of techniques for plant cultivation in controlled in vitro environment (Shasmita et al. 2023).

Over two decades, *H. perforatum* has been the point of comprehensive investigations for the establishment of in vitro cell, callus, shoot and adventitious root cultures as perspective eco-friendly systems for continuous and reliable production of pharmacologically valuable metabolites (Shasmita et al. 2023; Al-Khayri et al. 2024). More importantly, *H. perforatum* in vitro cultures have been subjected to various biotechnological strategies to improve the biosynthesis of existing and novel bioactive compounds using optimization of culture conditions and nutrient medium, bioreactor technology, genetic transformation, and elicitation (Murthy et al. 2014; Hou et al. 2016; Shakya et al. 2019; Al-Khayri et al. 2024). Among these approaches, elicitation has been recognized as an attractive tool to enhance high-value molecules and biological activities of *H. perforatum* (Shakya et al. 2019). Elicitors can be defined as environmental stimuli or signal molecules that triggers series of transduction cascade processes in plant cells resulting in expression of genes involved in secondary metabolism. According to their nature, they can be categorized as biotic elicitors deriving from cell walls of microorganisms and plants (chitosan, chitin, oligosaccharides) or abiotic elicitors that represent physical or chemical components. Physical elicitors are represented by UV radiation, temperature, osmotic and drought stress, while chemical elicitors comprise heavy metals, inorganic compounds, and signalling molecules including salicylic acid (SA) and jasmonic acid (JA), (Jeyasri et al. 2023). To the best of our knowledge, SA, JA, and methyl jasmonate (MeJA) have been widely used as efficient elicitors for enhanced secondary metabolite production in various *H. perforatum* in vitro cultures. In this context, cell suspensions of *H. perforatum* have been recognized as favored culture systems for SA- and JA-mediated elicitation of naphthodianthrones, phenylpropanoids, flavonoids and xanthones (Walker et al. 2002; Conceiçao et al. 2006; Gadzovska et al. 2007, 2013; Wang et al. 2015; Zubrická et al. 2015). Even though *H. perforatum* cell suspensions have shown promising outcomes using elicitation strategy, the commercial application of this technology is still constrained due to their slow growth rate and lack of glandular structures as the main accumulation sites of hypericins and hyperforins (Murthy et al. 2014). Concerning differentiated cultures, *H. perforatum* shoots and plantlets with well-developed translucent and dark glands on the leaves have been widely evaluated for overproduction of hypericin, pseudohypericin, and hyperforin upon elicitation with SA, JA and MeJA (Sirvent and Gibson 2002; Liu et al. 2007; Gadzovska et al. 2013). On the other hand, adventitious roots of *H. perforatum* have not been extensively used in elicitation studies with these signalling molecules and only one research reported notable increment of hypericin in bioreactor-cultured roots elicited with MeJA (Wu et al. 2014). According to available literature data, numerous elicitors such as red light, chitosan and acetic acid have been efficiently applied for enhanced biosynthesis of phenolic compounds in *H. perforatum* adventitious roots (Tocci et al. 2012; Valletta et al. 2016; Sobhani Najafabadi et al. 2019). However, several reports suggested that optimization of culture medium composition and implementation of multi-step culturing system for increased root biomass is usually necessary for stable metabolite production in *H. perforatum* adventitious roots upon elicitation (Cui et al. 2010a, b; Tocci et al. 2012). These time-consuming and optimization procedures during elicitation experiments for increased production of bioactive metabolites could be overcome using *H. perforatum* hairy root (HR) cultures.

Agrobacterium rhizogenes-induced HR cultures represent a superior platform for large-scale production of bioactive molecules in comparison to intact plants or undifferentiated cell or callus cultures. The main biotechnological advantages of HR cultures are related to their extensive growth on auxin-free medium, genetic stability, and concomitant increase of biomass accumulation and secondary metabolites biosynthesis (Gerszberg and Wiktorek-Smagur 2022). After the first report for successful *A. rhizogenes*-mediated transformation of *H. perforatum* (Di Guardo et al. 2003), numerous studies have lately been focused on the evaluation of biomass production, phenolic compounds contents, antioxidant status and biological activities in different *Hypericum* HR clones (Vinterhalter et al. 2015; Zubrická et al. 2015; Tusevski et al. 2019, 2023). Those studies indicated that agrobacterial T-DNA *rol* and *aux* gene expression or their integrated

copy numbers in plant genome significantly influenced the variability in the growth and phytochemical profile of *Hypericum* HR clones. Our previous report for screening of dark-grown *H. perforatum* HR clones resulted in the selection of HR F clone as the best-growing and superior line with respect to total phenylpropanoids and antioxidant activities (Tusevski et al. 2019). The detailed phytochemical analyses of this elite HR line confirmed its superior potential for the production of phenolic acids, flavan-3-ols, flavonols and xanthones, while enzyme inhibitory screening assays pointed out its in vitro antidepressant, neuroprotective and antihyperglycemic activity (Tusevski et al. 2023). Further experiments involving exposition of HR clones to light conditions revealed an activation of defense response in root cells upon photo-oxidative stress and enhanced contents of naphthodianthrones and xanthones with acetylcholinesterase, α-glucosidase, lipase, and cholesterol esterase inhibitory activities (Tusevski and Gadzovska 2023; Tusevski et al. 2024b). These observations suggested that *H. perforatum* HR clones as responsive culture systems to physical factors could be used for modulation of bioactive compounds production using elicitation strategy.

In the current study, previously selected elite HR F clone was used for the first time in elicitation experiments for enhanced production of phenolic compounds and naphthodianthrones, as well as for evaluation of antioxidant defense responses upon treatments with SA and JA. Elicited HR cultures were also examined for in vitro neuroprotective and antihyperlipidemic activity through enzyme inhibitory assays. Additionally, the effects of these hormonal elicitors on the utilization of nutrients (ammonium, nitrates, phosphates and residual sugars) from the culture medium and the growth of *H. perforatum* HR cultures were evaluated.

Materials and methods

Establishment of hairy root cultures

Establishment of HR cultures using *A. rhizogenes* A4-mediated transformation of *H. perforatum* was described in our previous study (Tusevski et al. 2013). Fifteen HR clones were monthly subcultured on a hormone-free solid MS/B5 medium with 3% sucrose and 0.7% agar. These stock cultures were maintained in a growth room under darkness at 24 ± 1 °C and relative humidity of 60%. The screening of HR clones revealed that HR line denoted as HRF is characterized with the best growth performance and superior production of phenolic compounds (Tusevski et al. 2019). Thus, HRF was selected as elite clone for elicitation experiments performed here.

Elicitor preparation and treatments

Root tips from one-month-old HR F clone (about 500 mg) were inoculated into 250 mL Erlenmeyer flasks containing 100 mL hormone-free liquid medium with MS salts (Murashige and Skoog 1962) and B5 vitamins (Gamborg et al. 1968), and further incubated under darkness at 25 ºC on a rotary shaker (100 rpm). After 7 days of cultivation, different concentrations of SA (50 µM, 100 µM and 250 µM) and JA (10 µM, 50 µM and 100 µM) were added in the culture medium as final concentrations per flask. The SA (Sigma-Aldrich) and JA (Cayman Chemical Company) stock solutions were prepared in 50% (v/v) ethanol and filter-sterilized using a 0.2 µm syringe filter (Ministart RC 25, Sartorius Stedim Biotech, Germany). Control or non-elicited HR cultures were treated with adequate volume of 50% ethanol. Control and elicited HR were cultured under the above-mentioned conditions and harvested at day 1, 4, 7, 14, 21, and 28 of post-elicitation. The HR biomass was harvested by filtration, rinsed with sterile water and gently blotted at filter paper to remove excess water before fresh biomass measurements. Thereafter, HR tissues were lyophilized by vacuum (0.22 mbar) and the values that correspond to initial dry inoculum and final dry biomass were used for calculation of dry weight. Collected dry HR biomass was ground into powder for phytochemical analyses and enzyme inhibitory activities or immediately frozen in liquid nitrogen and stored at -80 °C for enzymatic antioxidant activities and oxidative stress marker contents. Additionally, the culture media were collected by filtration and stored at -20 °C until analysis.

Culture medium analysis

The culture medium of control and elicited HR was used for analysis of nutrient contents (NH_4^+, NO_3^-, PO_4^{3-}, and residual sugars), as well as the levels of extracellular total phenolics (eTP) and hydrogen peroxide (eH_2O_2).

Ammonium (NH_4^+). The NH_4^+ concentration was analyzed according to method described by Condori et al. (2010) with minor modifications. Reaction mixture was consisted of diluted medium, phenol-nitroprusside solution (0.5% phenol and 5% sodium nitroprusside) and alkaline hypochlorite solution (0.5% NaOH and 5% NaOCl). The samples were incubated at 37 °C for 30 min and the absorbance was read at 625 nm. The NH_4^+ concentration was calculated with NH_4NO_3 as a standard and the results were expressed as milligrams of NH_4^+ per litre medium.

Nitrate (NO_3^-). The NO_3^- concentration was determined using the modified method of Condori et al. (2010). Reaction mixture consisted of diluted medium and 0.5% diphenylamine dissolved in concentrated sulfuric acid was

incubated 30 min at room temperature and the absorbance was read at 590 nm. The NO_3^- concentration was calculated with KNO_3 as a standard and the results were expressed as milligrams of NO_3^- per litre medium.

Phosphate (PO_4^{3-}). The PO_4^{3-} concentration in the medium was determined by the method of Towler et al. (2007) with the following modifications. An aliquot of diluted medium was mixed with phosphate reagent (8% H_2SO_4, 0.2% ammonium molybdate, 2% polyvinylpyrrolidone (PVP), and 0.4% ferrous ammonium sulfate), incubated for 10 min at room temperature and the absorbance was read at 650 nm. The PO_4^{3-} concentration was calculated with KH_2PO_4 as a standard and the results were expressed as milligrams of PO_4^{3-} per litre medium.

Residual sugars (RS). The RS concentration was analyzed by mixing of tested medium, 5% phenol, and concentrated sulfuric acid (Condori et al. 2010). The reaction mixture was incubated for 20 min at room temperature and the absorbance was read at 490 nm. The RS concentration was calculated with glucose as a standard and the results were expressed as grams of glucose equivalents per litre medium.

Extracellular total phenolics (eTP). The eTP concentration in the medium was determined according to Folin-Ciocalteu method (Singleton and Rossi 1965). Reaction mixture consisted of tested medium, Folin-Ciocalteu reagent and 0.7 M Na_2CO_3 was incubated at 50 ºC for 15 min and the absorbance was read at 765 nm. The eTP concentration was calculated with gallic acid as a standard and the results were expressed as milligrams of gallic acid equivalents per litre medium.

Extracellular hydrogen peroxide (eH_2O_2). The eH_2O_2 concentration in the medium was analyzed by Ferric-xylenol orange (FOX) method reported by Franklin et al. (2009). Reaction mixture was consisted of culture medium, xylenol orange reagent (0.5 mM NH_4FeSO_4, 50 mM H_2SO_4, 0.2 mM xylenol orange, and 0.2 M sorbitol). The samples were incubated under dark for 30 min at room temperature and the absorbance was measured at 560 nm. The eH_2O_2 concentration was calculated with H_2O_2 as a standard and the results were expressed as micromoles of H_2O_2 per litre medium.

Total phenolic compounds and antioxidant activities in hairy root cultures

Extract preparation. Lyophilized and powdered biomass of control and elicited HR cultures were used for preparation of methanolic extracts that were used for determination of total phenolics (TP), total flavonoids (TF) and total flavan-3-ols (TFA) contents, as well as for antioxidant activities including cupric reducing antioxidant activity (CUPRAC), DPPH radical scavenging, ferrous chelating activity (FCA), and phosphomolybdenum (PM) assay. Methanolic extracts were prepared by homogenization of 0.05 g dried biomass with 2 mL 80% CH_3OH in an ultrasonic bath for 20 min at room temperature. Thereafter, methanolic homogenates were centrifuged at 10,000 rpm for 15 min and collected supernatants were used for spectrophotometric determination of total phenolic compounds and antioxidant activities.

The contents of TP, TF and TFA in HR extracts were determined according to Folin-Ciocalteu, aluminium chloride and 4-dimethylaminocinnamaldehyde (DMACA) methods, respectively (Tusevski and Gadzovska Simic 2023; Tusevski et al. 2024a). The protocols for determination of antioxidant activities (CUPRAC, DPPH and FCA) in HR extracts were described in our previous studies (Tusevski et al. 2019; Tusevski and Gadzovska Simic 2023). The PM assay was performed using the procedure of Prieto et al. (1999) with minor modifications. An aliquot of HR extract was mixed with PM reagent [0.6 M H_2SO_4, 28 mM Na_3PO_4 and 4 mM $(NH_4)_2MoO_4$] and the samples were incubated at 95 °C for 90 min. After cooling, the absorbance of green colored complex was measured at 695 nm. The PM antioxidant activity was calculated using ascorbic acid (AA) as a standard and the results were expressed as milligrams of AA equivalents per gram of dry mass.

Enzymatic antioxidant activities and oxidative stress marker contents in hairy root cultures

Enzymatic antioxidant status of HR cultures was analyzed through peroxidase (POD) and superoxide dismutase (SOD) activities. Enzyme extracts were prepared by homogenization of frozen HR tissues with 50 mM potassium phosphate buffer (pH 7) containing 1% PVP. After centrifugation (12000 rpm) for 15 min at 4 °C, the supernatants were used for determination of protein contents and activities of antioxidant enzymes. The procedures for determination of POD activity by guaiacol oxidation method and SOD activity using photochemical reduction of nitroblue tetrazolium were described in our recent studies (Tusevski and Gadzovska Simic 2023; Tusevski et al. 2024a).

Regarding oxidative stress markers, the extracts for determination of endogenous H_2O_2 and malondialdehyde (MDA) contents were prepared from fresh HR biomass using 5% trichloroacetic acid, while $O_2^{\bullet-}$ production was determined in the extracts previously prepared for antioxidant enzymes activities. The protocols for determination of oxidative stress marker contents were presented in our previous studies (Tusevski et al. 2019; Tusevski and Gadzovska Simic 2023).

Chromatographic analysis of phenolic compounds and naphthodianthrones in hairy root cultures

Lyophilized and powdered root biomass was homogenized with 80% methanol in an ultrasonic bath for 30 min at 4 °C under darkness. After centrifugation at 12 000 rpm for 15 min, the methanolic supernatants were filtered using 0.2 µm filters prior to the chromatographic analyses. Identification and quantification of phenolic compounds was performed by HPLC/DAD/ESI-MS^n analysis, while naphthodianthrones were analyzed by UPLC-TUV system.

The HPLC analysis was carried out on Agilent 1100 system with diode array detector coupled with an ion trap mass detector. Chromatographic column Eclipse XDB-C18 (150 × 4.6 mm, 5 µm) was used for separation of phenolic compounds. Formic acid (1% (*v*/*v*) as solvent A and methanol as solvent B were used as mobile phase for separation in the following gradient program: 20% B (5 min, linear), up to 45% B (5–25 min), up to 80% B (25–35 min), up to 100% (35–50 min) and continue with 100% B linear for 15 min. The temperature of the column was set on 38 ºC. The flow rate was 0.4 mL·min^{-1} and injection volume of 10 µL. For MS detector, nitrogen was used as nebulizing gas at a pressure level of 50 psi and flow 12 L·min^{-1}. Capillary temperature and voltage were set at 350 ºC and 4 kV, respectively. The MS spectra were collected in the mass range from *m*/*z* 100 to 1200 in the negative ionization mode. Identification of the compounds was performed with comparison of their UV and MS spectra with standard compounds and literature data (Tusevski et al. 2013, 2017, 2023, 2024b, c). Quantification was made according to area under the peaks in UV chromatogram. The xanthones were quantified as mangiferin equivalent at 260 nm, flavan-3-ols as catechin equivalent at 280 nm, phenolic acid as caffeic acid equivalent at 330 nm and flavonol glycosides at 350 nm as quercetin equivalent.

Naphthodianthrones in HR extracts were analyzed on ACQUITY UPLC system (Waters, USA) with dual-wavelength TUV detector (Tusevski et al. 2024a). Chromatographic separation of hypericin (HYP), pseudohypericin (PHYP), and protopseudohypericin (PPHYP) was performed using ACQUITY UPLC HSS T3 column (100 × 2.1 mm, 1.8 µm; Waters) at 38 °C. The mobile phase included solvent A: 0.1% formic acid (*v*/*v*) and solvent B: acetonitrile in the following gradient program: 10–50% B (0.0–1.0 min), 50–100% B (1.0–14 min), 100% B (14–19 min), and 100%-10% B (19–25 min). The flow rate was 0.57 mL·min^{-1} and the injection volume was 10 µL. The assignment of naphthodianthrone peaks (HYP, PHYP, and PPHYP) was based on the comparison of their retention time to those of reference standards. The external standard method was used for quantification of naphthodianthrones in HR extracts at 590 nm according to their peak area.

Enzyme inhibitory activities of hairy root cultures

In vitro neuroprotective activity through acetylcholinesterase (AChE) inhibition and antihyperlipidemic activity through lipase (LIP) and cholesterol esterase (CE) inhibition were analyzed according to the previously described protocols (Tusevski et al. 2024b, c). In vitro biological activities were performed using dried methanolic extracts from HR dissolved in dimethyl sulfoxide. The stock solutions of HR were serially diluted and different extract concentrations (62.5–2000 µg·mL^{-1}) were used for performing enzyme inhibitory assays. Eserine (0.96–125 µg·mL^{-1}), orlistat (7.80–250 µg·mL^{-1}) and simvastatin (15.62–500 µg·mL^{-1}) were used as specific inhibitors of AChE, LIP and CE, respectively. The HR extract concentrations providing 50% enzyme inhibition (IC_{50}) was calculated using GraphPad Prism software.

Statistical analyses

In the elicitation experiments, three replicate flasks were assessed per treatment. The results for the analyzed parameters in the culture medium and elicited HR extracts were expressed as average values with standard deviation. Statistical analyses were done using STATISTICA for Windows (v. 8.0; StatSoft Inc., Tulsa, OK, USA). The average values between control and elicited samples were compared by one-way ANOVA and significant differences ($p < 0.05$) were post-hoc examined by Duncan's test.

Results

Growth of *H. perforatum* hairy root cultures

The effect of SA and JA on the fresh weight (FW) and dry weight (DW) of *H. perforatum* HR cultures was investigated during 28 days of post-elicitation. In addition, growth of control and elicited HR expressed as grams FW per litre medium is presented in Supplementary Fig. 1. Present results showed that SA-elicited HR began to brown gradually after 7 days of elicitation, while JA-treated HR did not exhibited significant change in the color of the biomass compared to control cultures (Fig. 1). The HR cultures displayed distinct response to exogenously applied SA and JA, indicating that biomass accumulation was related to the type of elicitor used. The FW production in HR elicited with all tested SA concentrations was significantly suppressed from day 7 to the end of post-elicitation compared

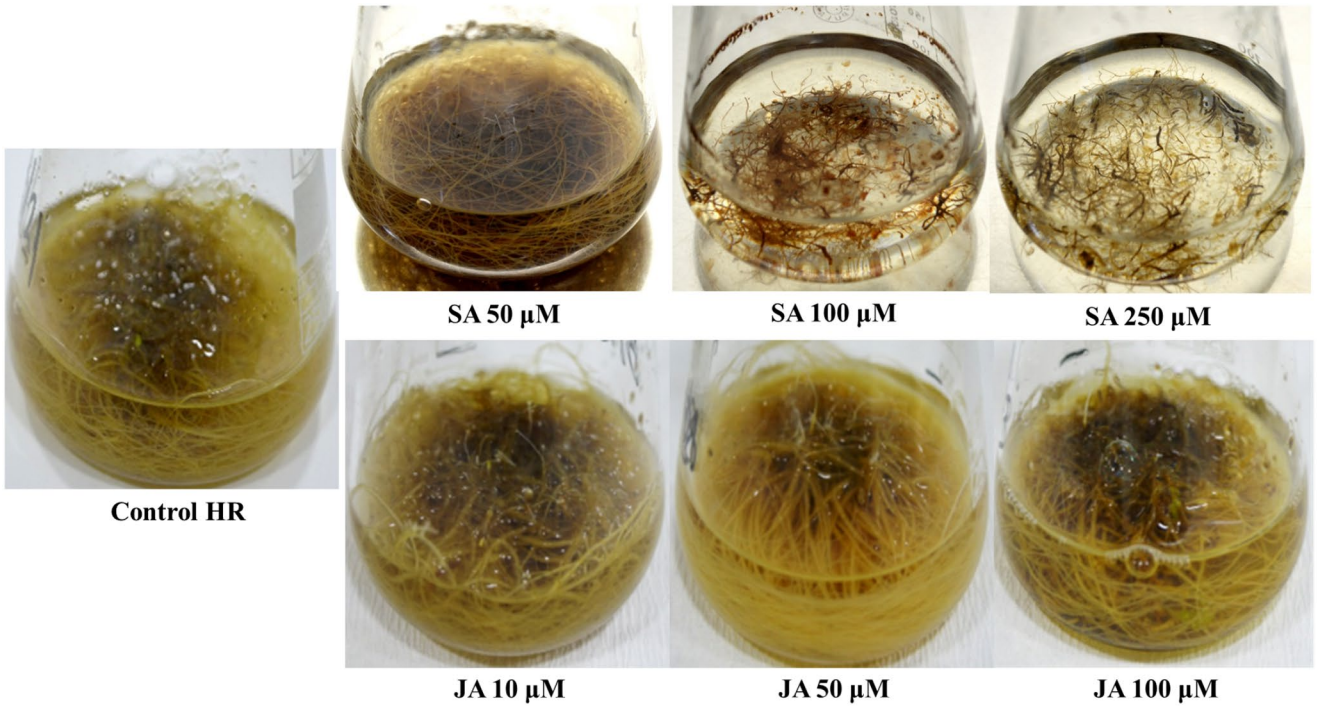

Fig. 1 Morphology of *Hypericum perforatum* hairy roots (HR) elicited with salicylic acid (SA) and jasmonic acid (JA) at day 28 of post-elicitation

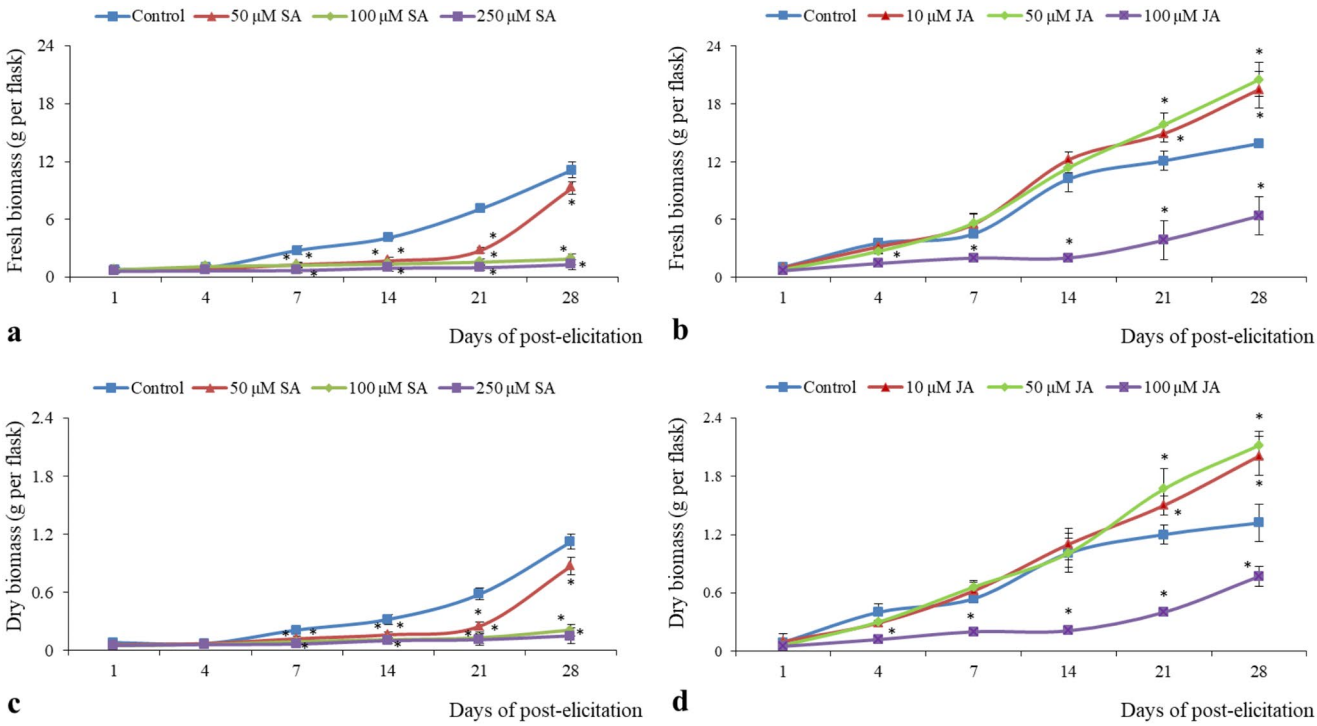

Fig. 2 Biomass production of *Hypericum perforatum* hairy roots elicited with salicylic acid (SA) and jasmonic acid (JA). a, b: fresh biomass, c,d: dry biomass. The values denoted with asterisk showed significant differences between control and elicited samples ($p < 0.05$)

to control (Fig. 2a). During this period, HR elicited with 100 and 250 µM SA exhibited almost equal growth inhibition with markedly decreased FW (about 8.5-fold) at the end of post-elicitation compared to control HR. On the other hand, HR elicited with 50 µM SA displayed a slight suppression in FW (1.2-fold) compared to control at the end of post-elicitation period. With respect to elicitation with JA, it was clear that elicitor concentrations differently affect FW production

in HR cultures (Fig. 2b). Application of 10 and 50 µM JA did not induce marked alteration in HR growth compared to control cultures till day 14 of post-elicitation. Noteworthy, from day 21 to day 28, these JA concentrations significantly increased FW production (from 1.2- to 1.4-fold) in elicited HR compared to control. In contrast, exogenous addition of 100 µM JA in culture medium continuously retarded FW production of HR during the entire post-elicitation period. The data for DW production in HR cultures elicited with SA and JA were similar as results obtained for FW. In this context, all tested SA concentrations significantly declined DW in elicited HR compared to control cultures from day 7 to the end of post-elicitation (Fig. 2c). Concerning JA elicitation, addition of 100 µM dose markedly decreased DW production in elicited HR compared to control during the entire post-elicitation period, while 10 and 50 µM JA significantly improved DW values in elicited HR at day 21 and 28 of post-elicitation (Fig. 2d). Correlation analysis showed that FW in HR elicited with both elicitors was in significant negative correlation with nutrient contents in the culture medium (NH_4^+, NO_3^-, PO_4^{3-}, and RS), as well with the production of H_2O_2 and MDA as oxidative stress markers (Fig. 3).

Nutrient contents in culture medium of *H. perforatum* hairy root cultures

The utilization of nutrients by *H. perforatum* HR cultures was evaluated by determination of NH_4^+, NO_3^-, PO_4^{3-}, and RS contents in culture medium (Fig. 4a-h). The NH_4^+ content in the medium of control HR and 50 µM SA-elicited HR showed similar descending trend during post-elicitation period. Contrary to this, culture medium of HR elicited with 100 and 250 µM SA displayed markedly higher NH_4^+ levels compared to control medium from day 14 to the end of post-elicitation (Fig. 4a). Application of all tested JA concentrations exhibited inhibition in NH_4^+ utilization from day 7 to day 21 that was evident through significantly higher NH_4^+content in the medium of JA-elicited HR compared to control (Fig. 4b). At the end of post-elicitation, only medium with 100 µM JA showed markedly higher NH_4^+ content (2.4-fold) in comparison to control medium. Present results demonstrated that NO_3^- contents in the medium of SA-elicited HR were significantly higher (from 1.2- to 1.6-fold) compared to control medium from day 7 to the end of post-elicitation (Fig. 4c). During this elicitation period, only medium of 100 µM JA-elicited HR demonstrated notably higher NO_3^- contents in comparison to that of control cultures (Fig. 4d). The PO_4^{3-} contents in the media of HR elicited with all SA concentrations were significantly higher (from 1.2- to 2.5-fold) compared to those of control HR from day 7 to day 28 (Fig. 4e). Exogenous application of all JA doses resulted in significantly greater PO_4^{3-} contents at day 4, while addition of 100 µM JA lead to continuously higher PO_4^{3-} levels in the medium of elicited HR compared to control to the end of post-elicitation (Fig. 4f). The RS contents in the medium of elicited HR were significantly higher with the application of all concentrations of SA (from 1.6- to 5.1-fold) and JA (from 1.2- to 19.8-fold) compared to control medium from day 4 to the end of post-elicitation (Fig. 4g, h). It is interesting to note that SA induced considerable increment of RS, while JA caused constant depletion of available RS in the medium of elicited HR cultures. According to correlation analysis for SA experiments, the nutrient contents in culture medium (NH_4^+, NO_3^-, PO_4^{3-}, and RS) positively correlated to phenolic compounds production (TP, TF and TFA), antioxidant activities (CUPRAC, DPPH, and PM) and oxidative stress markers (H_2O_2 and MDA), but negatively to POD activity (Fig. 3a). With respect to JA-elicited HR, nutrient contents in the medium were in significant positive correlations only with FCA and H_2O_2 levels (Fig. 3b).

Phenolic compounds production in *H. perforatum* HR cultures

The TP production enhanced more rapidly in HR cultures upon SA treatment up to day 21 (from 1.2- to 2.2-fold) and thereafter, TP content was significantly decreased in comparison to control HR (Fig. 5a). Elicitation with all JA doses caused significantly increased TP production (from 1.2- to 3.7-fold) in HR from day 4 to day 21 compared to control (Fig. 5b). At the end of post-elicitation, only 10 µM JA induced significantly higher TP production (1.5-fold) in HR compared to control cultures. Treatments of HR with SA significantly improved TF production from day 1 to day 21 (up to 3.1-fold), while 100 and 250 µM SA caused decreased TF contents compared to control at the end of post-elicitation (Fig. 5c). Noteworthy, all tested JA concentrations promoted TF production in HR (up to 1.8-fold) at day 28, while high JA dose (100 µM) increased TF contents more efficiently (up to fourfold) from day 4 to the end of post-elicitation compared to control HR (Fig. 5d). The stimulation of TFA production upon SA was observed from the beginning till day 21 (Fig. 5e), whereas treatments with JA promoted TFA accumulation from day 4 to the end of post-elicitation (Fig. 5f). Notably, all tested SA doses induced an early enhancement of TFA production at day 1 (from 2- to 2.5-fold), while JA doses evoked a late improvement of TFA accumulation at day 28 (from 2.3- to 3.4-fold) in HR cultures compared to control. Statistical analysis demonstrated that TP, TF, and TFA contents in SA and JA-elicited HR were in significant positive correlations with CUPRAC, DPPH, and PM antioxidant assays (Fig. 3). More specifically, phenolic

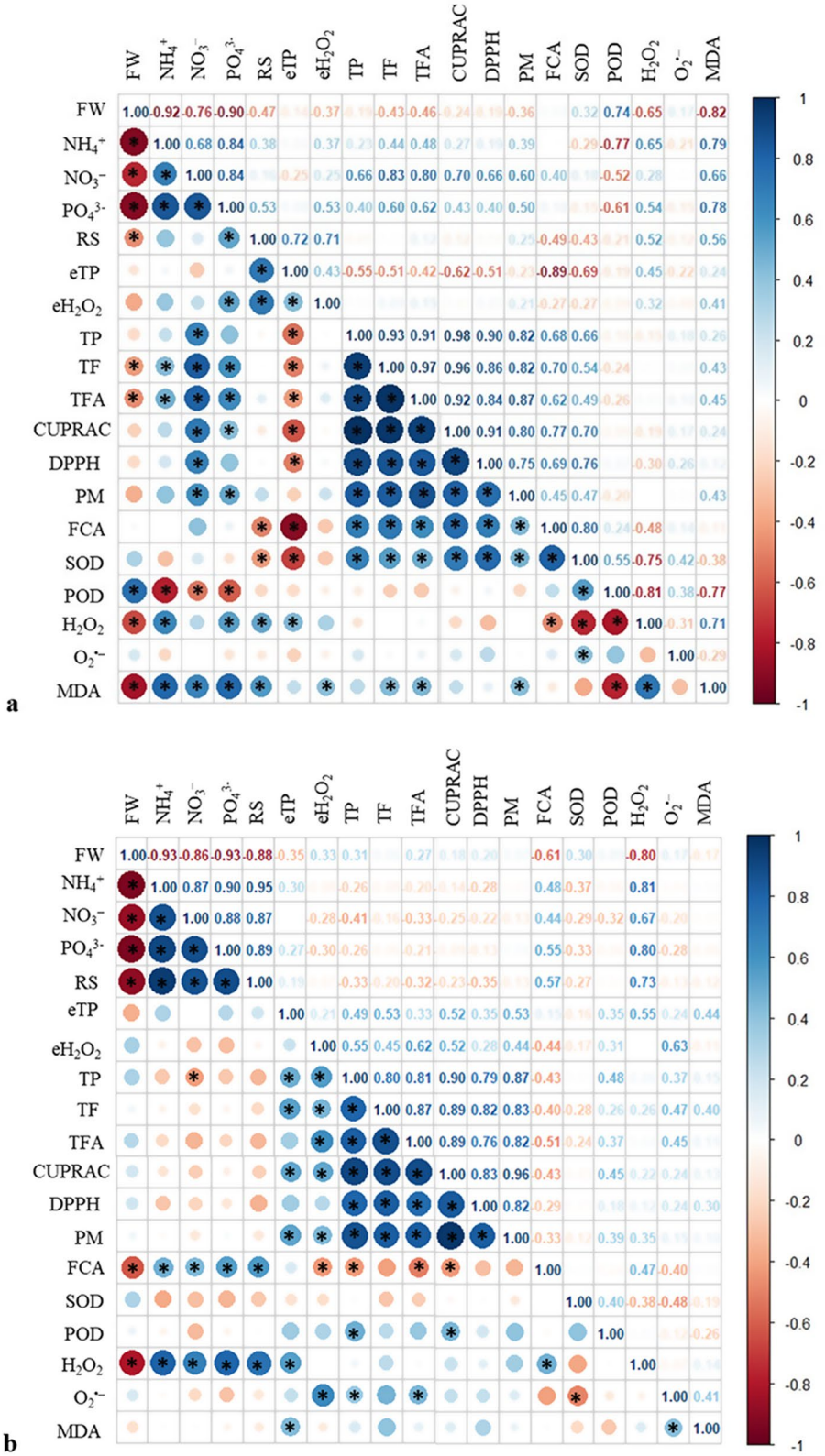

Fig. 3 Correlation analyses between growth, nutrient contents in culture medium, phenolic compounds production, non-enzymatic and enzymatic antioxidant activities and oxidative stress marker levels in *Hypericum perforatum* hairy roots elicited with **a**: salicylic acid (SA) and **b**: jasmonic acid (JA). Blue colored circles show positive correlation, while red colored circles indicate negative correlation. Black asterisks represent the significance of correlation ($p < 0.05$). *FW* fresh weight, NH_4^+ ammonium, NO_3^- nitrate, PO_4^{3-} phosphate, *RS* residual sugars, *eTP* extracellular total phenolics, eH_2O_2 extracellular hydrogen peroxide, *TP* total phenolics, *TF* total flavonoids, *TFA* total flavan-3-ols, *CUPRAC* cupric reducing antioxidant capacity, *DPPH* DPPH radical scavenging, *PM* phosphomolybdenum assay, *FCA* ferrous chelating activity, *SOD* superoxide dismutase, *POD* peroxidase, H_2O_2 hydrogen peroxide, $O_2^{\bullet-}$ superoxide production rate, *MDA* malondialdehyde,

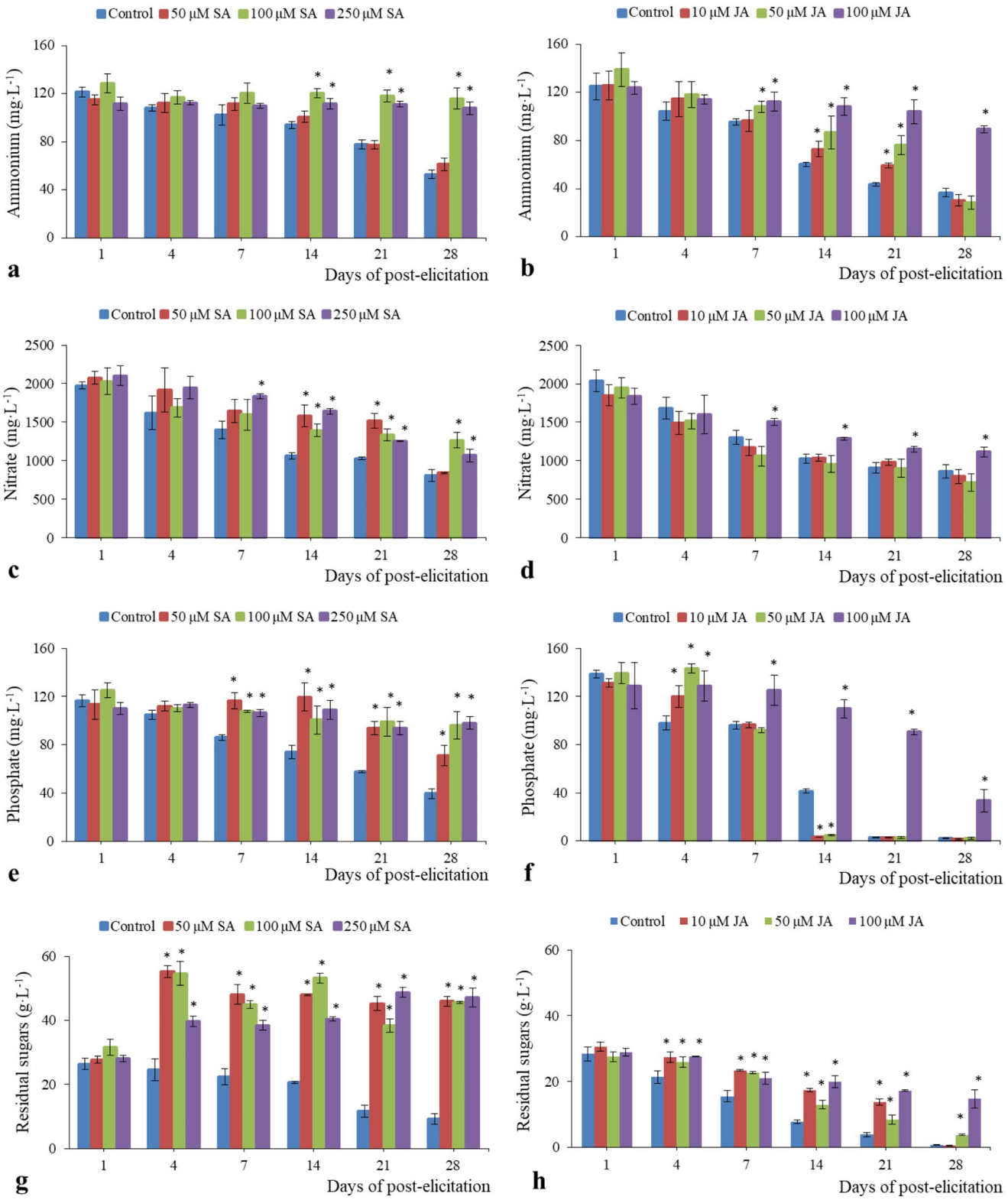

Fig. 4 Nutrient contents in culture medium of *Hypericum perforatum* hairy roots elicited with salicylic acid (SA) and jasmonic acid (JA). **a**, **b**: ammonium (NH_4^+), **c**, **d**: nitrate (NO_3^-), **e**, **f**: phosphate (PO_4^{3-}), **g**, **h**: residual sugars (RS). The values denoted with asterisk showed significant differences between control and elicited samples ($p < 0.05$)

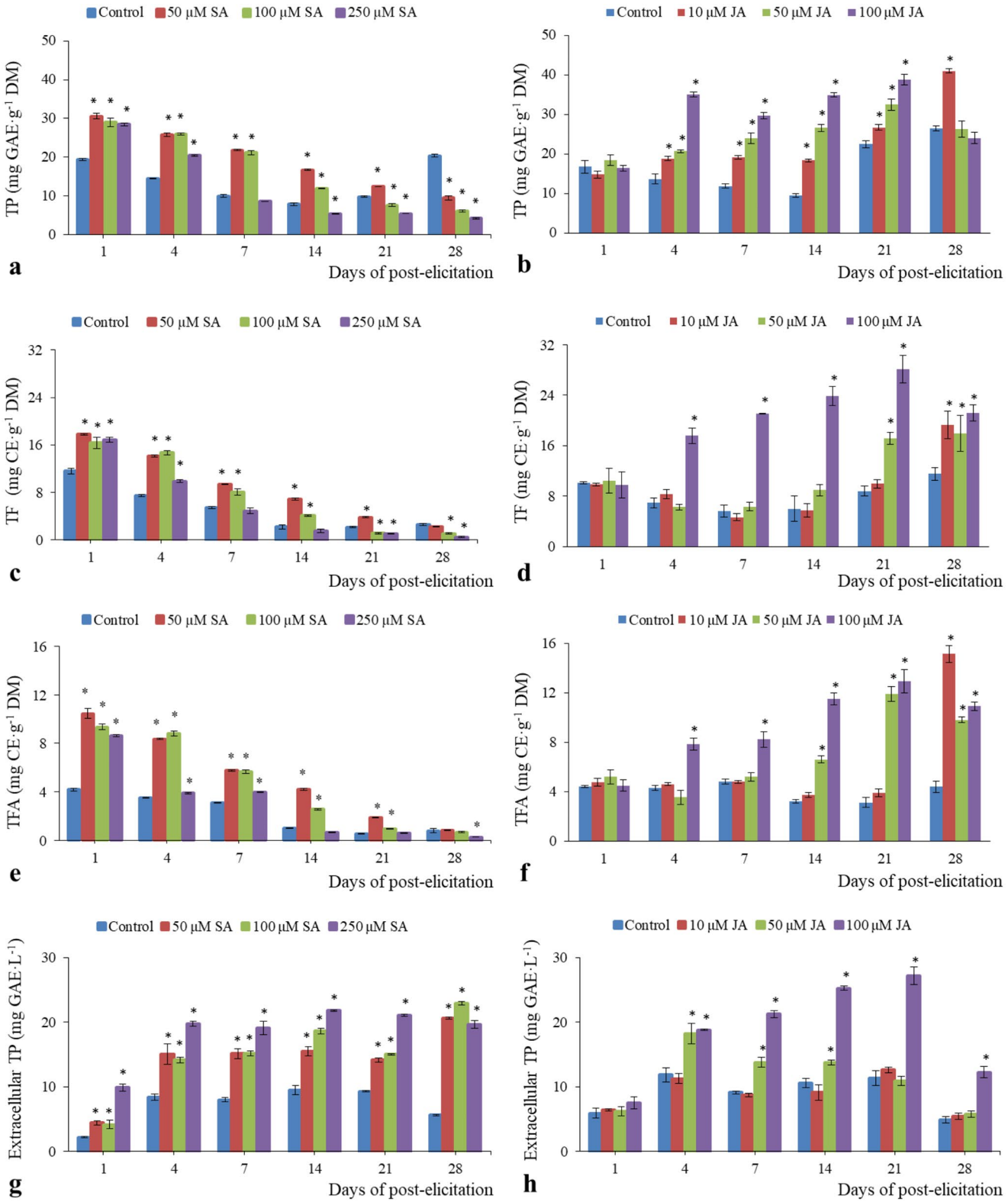

Fig. 5 Phenolic compounds contents in *Hypericum perforatum* hairy roots elicited with salicylic acid (SA) and jasmonic acid (JA). a, b: total phenolics (TP), c,d: total flavonoids (TF), e,f: total flavan-3-ols (TFA), g,h: extracellular total phenolics (eTP). *GAE* gallic acid equivalents, *CE* catechin equivalents, *DM* dry mass. The values denoted with asterisk showed significant differences between control and elicited samples ($p < 0.05$)

compounds content in SA-elicited HR was positively related to SOD activity and MDA level (Fig. 3a), while production of phenolics in JA-elicited HR was in positive correlation to $O_2^{\bullet-}$ production rate, but in negative correlation to FCA (Fig. 3b).

Extracellular TP contents were measured in the medium of control and elicited HR in order to evaluate releasing of intracellular metabolites due to possible stress-mediated alteration in membrane fluidity upon elicitation. Present results demonstrated that all SA treatments during entire post-elicitation period significantly increased TP secretion (up to 4.5-fold) in the medium of elicited HR compared to control medium (Fig. 5g). In contrast, only 50 and 100 µM JA induced a notable TP secretion in the medium (up to 2.4-fold) of elicited HR than control medium from day 4 to the end of post-elicitation (Fig. 5h). Correlation analysis showed that eTP content in SA-elicited HR was in significant negative relationship with phenolic compounds production (TP, TF, and TFA), non-enzymatic antioxidant assays (CUPRAC, DPPH, and FCA), as well to SOD activity (Fig. 3a). Concerning HR elicitation with JA, eTP contents were positively correlated to TP and TF contents, CUPRAC and PM antioxidant assays, as well to H_2O_2 and MDA levels (Fig. 3b).

Non-enzymatic and enzymatic antioxidant activity in *H. perforatum* HR cultures

The effect of SA and JA on non-enzymatic antioxidant activity in *H. perforatum* HR cultures was evaluated using CUPRAC, DPPH, FCA and PM assays (Fig. 6a-h). Outgoing results demonstrated that SA induced an early increment of CUPRAC values in HR (up to 1.5-fold), while prolonged exposure to elicitor markedly reduced antioxidant capacity (up to 7.5-fold) compared to control cultures (Fig. 6a). The addition of 50 and 100 µM JA resulted in enhancement of CUPRAC values (up to 2.6-fold) from day 4 to day 21, while 10 and 50 µM JA caused increased antioxidant activity (up to 2.1-fold) in HR at day 28 in comparison to control (Fig. 6b). The results for DPPH scavenging of elicited HR cultures were similar as data obtained for CUPRAC indicating that SA promoted fast and transient induction of antioxidant activity (Fig. 6c), while JA was involved in late increment of antioxidant activity (Fig. 6d). With respect to PM assay, SA was shown as efficient elicitor in the antioxidant activity increment (up to 2.2-fold) in HR from the beginning until day 21 (Fig. 6e), while JA exhibited stimulatory effects on antioxidant properties (up to 1.9-fold) from day 4 to the end of post-elicitation (Fig. 6f). Present data showed that applied elicitors differentially influenced FCA activity in HR cultures. After elicitation with SA, significant inhibition in FCA values (up to 3.3-fold) was noted from day 4 to the end of post-elicitation compared to corresponding control (Fig. 6g). In comparison to control, FCA values in HR treated with low dose of JA were slightly increased from day 1 to day 7, while longer exposure to high elicitor concentrations caused a declining in FCA values (Fig. 6h). Elicitation with SA showed that antioxidant assays were in significant positive correlation with SOD activity (Fig. 3a), while JA-elicitation revealed that CUPRAC, DPPH and PM assays negatively correlated to FCA (Fig. 3b).

Enzymatic antioxidant response in HR cultures upon elicitation with SA and JA was examined through determination of POD and SOD activity (Fig. 7a-d). The POD activity in SA-elicited HR was consistently decreased during the entire elicitation period, with exception of low elicitor dose where moderate enhancement of enzyme activity (about 1.5-fold) was observed at day 28 in comparison to control (Fig. 7a). On the other hand, elicited HR with all tested concentrations of JA displayed a strong up-regulation of POD activity (up to 3.3-fold) from the beginning to the end of post-elicitation compared to control HR (Fig. 7b). Concerning SOD, the application of SA induced a 1.3-fold increment of enzyme activity in HR cultures only at day 1, and thereafter, the SOD activity was gradually decreased (up to 3.2-fold) to the end of post-elicitation in comparison to control HR (Fig. 7c). The effect of JA on SOD activity in elicited HR was not significantly changed compared to control till day 7 of the treatment, while the remaining post-elicitation period was characterized with decrease in enzyme activity (from 1.2- to 1.9-fold) compared to control (Fig. 7d). Present results showed that SOD and POD activity in SA-elicited HR were in significant negative correlation with H_2O_2 and MDA levels, but in positive correlation to $O_2^{\bullet-}$ production rate (Fig. 3a). The elicitation with JA showed significant negative correlation between SOD activity and $O_2^{\bullet-}$ production rate (Fig. 3b).

Oxidative stress marker contents in *H. perforatum* HR cultures

Oxidative stress marker contents in control and elicited cultures were represented by H_2O_2, $O_2^{\bullet-}$, and MDA (Fig. 8a-f). Present data showed significant enhancement of H_2O_2 levels in elicited HR with SA (up to fivefold) and JA (up to 2.9-fold) during entire post-elicitation period compared to control cultures (Fig. 8a, b). Concerning the $O_2^{\bullet-}$ production rate, elicited HR displayed distinct response to exogenously applied elicitors. In this view, $O_2^{\bullet-}$ production rate in SA-elicited HR was significantly declined (from 1.4- to 2.4-fold) from day 14 to day 28, while this post-elicitation period was described with an enhancement in $O_2^{\bullet-}$ production (up to threefold) in JA-elicited HR compared to control (Fig. 8c, d). The HR cultures elicited with SA and JA also showed opposite trend with regards to MDA production. Namely, MDA contents in all SA

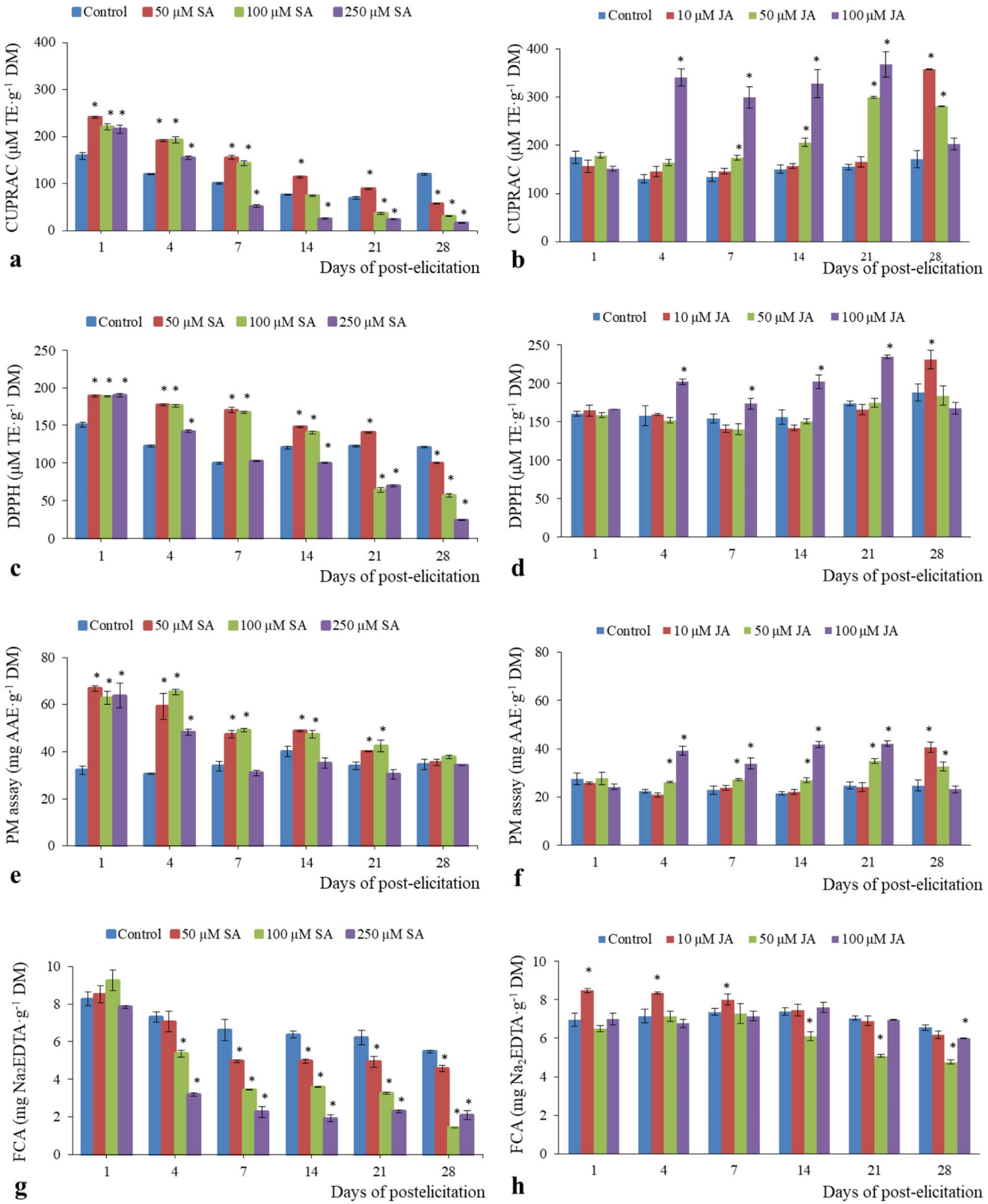

Fig. 6 Antioxidant activities of *Hypericum perforatum* hairy roots elicited with salicylic acid (SA) and jasmonic acid (JA). **a**, **b**: cupric reducing antioxidant capacity (CUPRAC), **c**, **d**: DPPH radical scavenging (DPPH), **e**, **f**: phosphomolybdenum assay (PM), **g**, **h**: ferrous chelating activity (FCA). *TE* trolox equivalents, *AAE* ascorbic acid equivalents, *DM* dry mass. The values denoted with asterisk showed significant differences between control and elicited samples ($p < 0.05$)

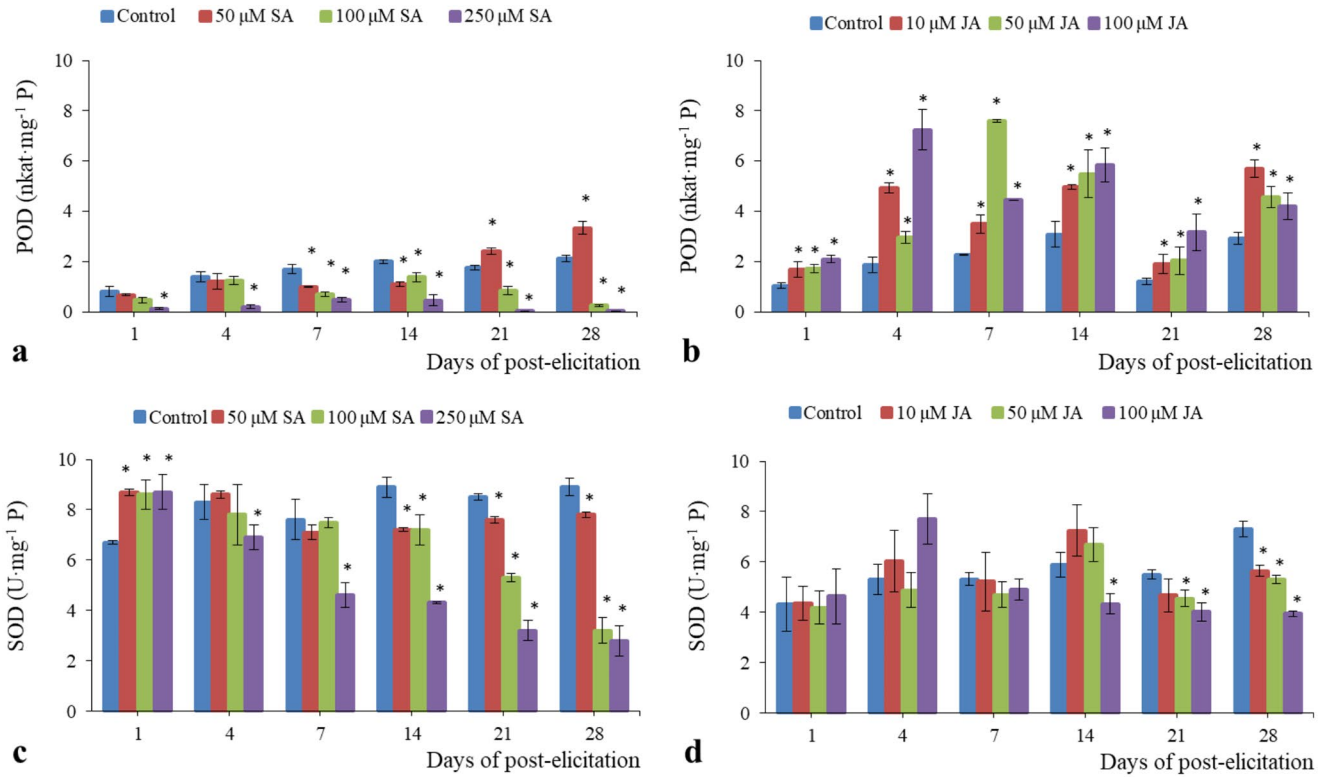

Fig. 7 Antioxidant enzyme activities of *Hypericum perforatum* hairy roots elicited with salicylic acid (SA) and jasmonic acid (JA). **a**, **b**: peroxidase (POD), **c**, **d**: superoxide dismutase (SOD). *P* proteins. The values denoted with asterisk showed significant differences between control and elicited samples ($p < 0.05$)

treatments were significantly enhanced (up to twofold) from day 4 to the end of post-elicitation compared to control cultures (Fig. 8e). In contrast, 10 and 50 µM JA caused declined MDA production (up to 2.2-fold), while 100 µM JA marginally enhance MDA content (1.2-fold) from day 14 to the end of post-elicitation (Fig. 8f). The analysis of H_2O_2 levels in the medium was performed to monitor possible transmission of intracellular stress in HR cells to the extracellular environment. In comparison to control medium, application of SA considerably enhanced H_2O_2 contents (up to 4.2-fold) in the medium of elicited HR from day 4 to the end of treatment (Fig. 8g), while JA administration caused significant increment of H_2O_2 levels (up to 2.8-fold) in the medium from day 7 to the end of elicitation (Fig. 8h). It is interesting to note that H_2O_2 contents in the medium of JA-elicited HR were in significant positive correlation with total phenolic compounds (TP, TF, and TFA), antioxidant activities (CUPRAC and PM), as well as $O_2^{\bullet-}$ production rate (Fig. 3b).

Chromatographic analysis of phenolic compounds and naphthodianthrones in *H. perforatum* HR cultures

According to present results for total phenolic compounds contents and our previous published data for treatment of different *H. perfortum in vitro* cultures with SA and JA (Gadzovska et al. 2007, 2013), elicited HR with 100 µM SA and JA along with control HR at day 4 of cultivation were chosen for detailed phytochemical analysis using HPLC/DAD/ESI-MS^n method (Supplementary Fig. 2). The chromatographic analysis showed the presence of hydroxycinnamic acids, catechins, flavonol glycosides (Table 1) and xanthones (Table 2).

Hydroxycinnamic acids. Control and elicited HR cultures were found to accumulate quinic acid (**F1**) and three hydroxycinnamic acids denoted as 3-cafeoylquinic acid (**F2**), 5-cafeoylquinic acid (**F3**) and *p*-coumaric acid (**F4**). It is interesting to point out that SA- and JA-elicited HR had comparable or even lower amounts of detected hydroxycinnamic acids compared to control cultures (Table 1).

Catechins. Qualitative analysis of catechins in HR extracts (Table 1) revealed the presence of monomeric catechin (**F7**) and epicatechin (**F10**), as well as several olygomeric procyanidins, such as procyanidin dimer A type (**F13**), several procyanidin dimers (**F5**, **F6**, **F9**, and **F11**) and procyanidin trimer (**F8**). The compounds F6, F8 and F10 were *de novo* synthesized in SA-elicited HR, while F11 was exclusively found in JA-elicited cultures. In comparison to control HR, SA-elicited HR showed 1.3-fold increased production of F9, whereas JA-elicited HR exhibited 2.5-fold enhanced

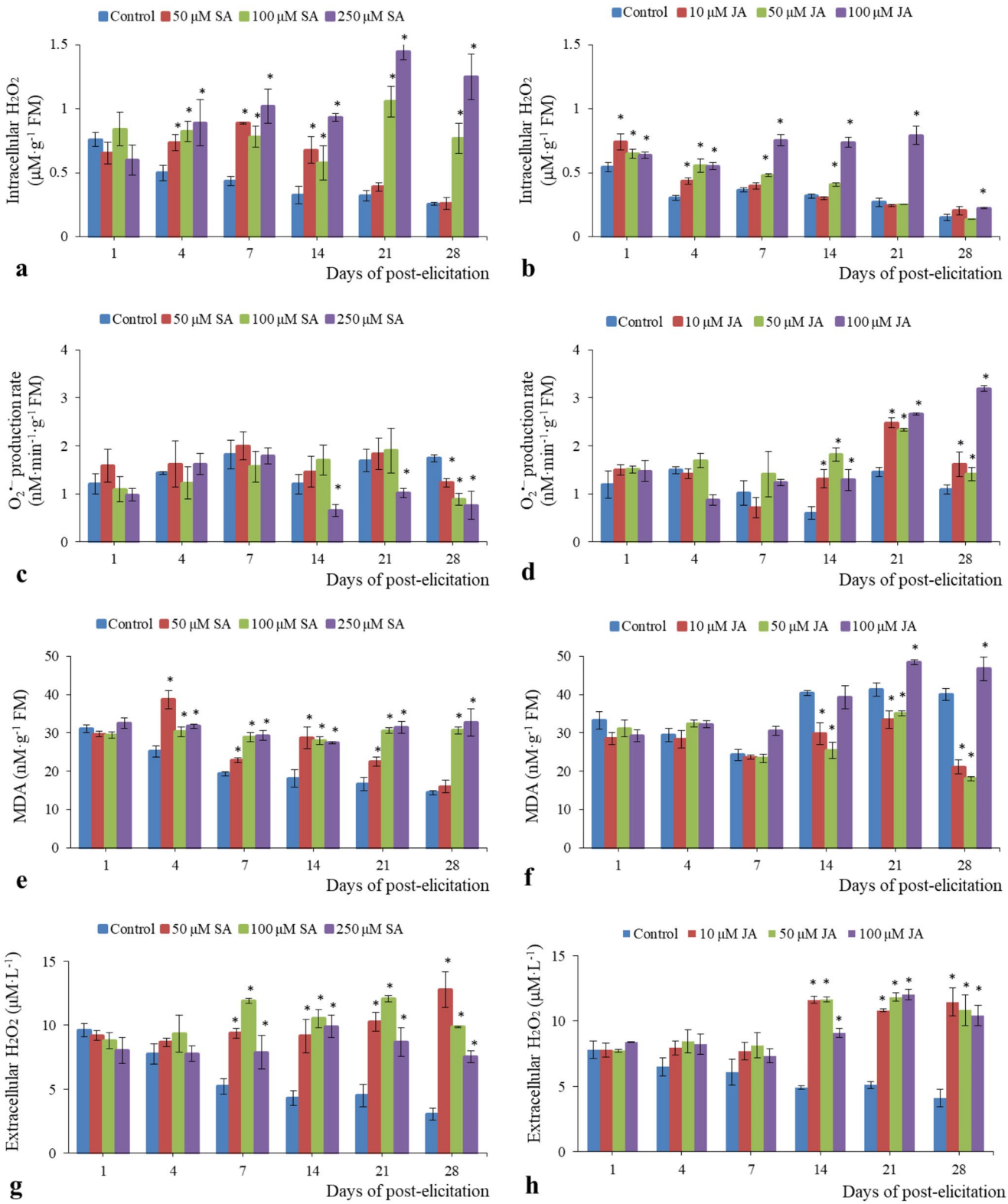

Fig. 8 Oxidative stress marker contents in *Hypericum perforatum* hairy roots elicited with salicylic acid (SA) and jasmonic acid (JA). **a**, **b**: hydrogen peroxide (H_2O_2), **c**, **d**: superoxide production rate ($O_2^{\bullet-}$), **e**, **f**: malondialdehyde (MDA). *FM* fresh mass. The values denoted with asterisk showed significant differences between control and elicited samples ($p < 0.05$)

Table 1 HPLC/DAD/ESI-MS^n analysis of phenolic compounds in *Hypericum perforatum* control and elicited hairy root cultures at day 4 of post-elicitation.[A]

Phenolic compounds:		t_R (min)	UV max (nm)	$[M–H]^–$	MS^2	Control HR	100 µM SA-elicited HR	100 µM JA-elicited HR
Phenolic acids and other acids								
F1	Quinic acid	4	262, 310	191	173, **127**	47.94 ± 3.15	16.26 ± 0.98*	13.02 ± 1.87*
F2	3-Cafeoylquinic acid	6.2	240, 294sh, 326	353	**191**, 179, 135	6.96 ± 0.55	5.72 ± 0.53	4.03 ± 0.69*
F3	5-Cafeoylquinic acid	6.8	240, 294sh, 326	353	191, **179**, 135	9.95 ± 1.08	2.27 ± 0.15*	3.48 ± 0.22*
F4	*p*-Coumaric acid	23.5	314	337	191, **163**	6.24 ± 0.77	6.90 ± 0.21	7.15 ± 0.54
Catechins								
F5	Proacyanidin dimer	24.1	280	577	559, 451, **425**, 407, 289	5.85 ± 0.33	6.10 ± 0.72	5.88 ± 0.24
F6	Proacyanidin dimer	24.5	280	577	559, 451, **425**, 407, 289	n.d.	9.33 ± 1.02	n.d.
F7	Catechin	26.2	280	289	245, 205	4.21 ± 0.66	n.d.	10.56 ± 0.82*
F8	Procyanidin trimer	26.6	280	865	847, 739, 695, **577**, 543, 407, 289	n.d.	38.25 ± 1.15	n.d.
F9	Procyanidin dimer	28.1	280	577	**559**, 451, 425, 407, 289	20.10 ± 0.68	26.15 ± 1.28*	n.d.
F10	Epicatechin	28.4	280	289	**245**, 205	n.d.	32.94 ± 0.71	n.d.
F11	Procyanidin dimer	32.3	244, 280	575	539, 449, **423**, 289	n.d.	n.d.	15.70 ± 1.19
F13	Procyanidin dimer A type	34.2	286	557	513, **463**, 405, 351	31.84 ± 1.56	61.28 ± 2.44*	53.78 ± 2.92*
Flavonol glycosides								
F12	Quercetin 6-*C*-glucoside	33.2	256, 356	421	331, **301**	n.d.	10.20 ± 0.61	n.d.
F14	Kaempferol hexoside	36.3	256, 266, 350	447	285	14.65 ± 1.09	35.83 ± 1.63*	n.d.

[a] Contents of detected compounds are expressed as milligrams per 100 g dry weight (mg·100 g^{-1} DW ± SD). The values in the row noted with an asterisk denoted significant differences between control and elicited samples at $p < 0.05$. *SA* salicylic acid, *JA* jasmonic acid, *n.d.* not detected, *S.D.* standard deviation, *[M–H]*$^–$ deprotonated molecular ion, $MS^2[M–H]^–$ collision fragment ions, sh: shoulder, t_R retention time. MS^2 ions in bold showed the base peak

accumulation of F7. Also, SA- and JA-elicited HR demonstrated significantly improved production of F13 (1.9- and 1.6-fold, respectively) compared to control HR.

Flavonol glycosides. Two flavonol glycosides identified as quercetin 6-*C*-glucoside (**F12**) and kaempferol hexoside (**F14**) were confirmed in control and SA-elicited HR cultures (Table 1). The SA-elicited HR displayed *de novo* production of F12, while F14 was found in 2.4-fold increased amount compared to control cultures.

Xanthones. The HPLC analysis showed that xanthones were the most divergent and abundant group of phenolic compounds in control and elicited HR as evidenced by twenty-three identified components (Table 2). The presence of the following xanthones: mangiferin (**X1**), mangiferin isomer (**X4**), trihydroxyxanthone-sulfonate (**X5**), 1,3,5,6-tetrahydroxyxanthone (**X8**), 1,3,6,7-tetrahydroxyxanthone (**X9**), 1,3,5,6-tetrahydroxyxanthone 8-prenyl isomer (**X11**), paxanthone (**X15**), gartanin isomer (**X16**), γ-mangostin isomer (**X17**), trihydroxy-1-metoxy-*C*-prenyl xanthone (**X18**), and γ-mangostin (**X19**) were confirmed in control cultures, as well as in SA- and JA-elicited HR. Three xanthones identified as 1,3,6,7-tetrahydroxyxanthone 8-prenyl xanthone (**X10**), α-mangostin (**X21**) and garcinone C (**X23**) were exclusively found only in control HR. Concerning SA-elicited HR, only two xanthones identified as tetrahydroxy-metoxy-C-prenyl xanthone (**X2**) and tetrahydroxyxanthone-*O*-hexoside (**X3**) were *de novo* synthesized, while tetrahydroxyxanthone-*C*-pentoside (**X7**), X8 and X19 were found in significant higher contents (from 2.7- to 10-fold) compared to control HR. In contrast, xanthones denoted as X1, X4, X5, tetrahydroxyxanthone-*C*-pentoside (**X6**), X9, X15, X17, and X18 were down-regulated in SA-elicited HR cultures. With respect to JA-elicited HR, the most of detected xanthones such as X1, X4, X8, X9, 1,3,7-trihydroxy-2-(2-hydroxy-3-methyl-3-butenyl)-xanthone (**X12**), 1,3,7-trihydroxy-6-methoxy-8-prenyl xanthone (**X13**), X16, garcinone E (**X20**) were up-regulated in comparison to control. The remaining detected xanthones in JA-elicited HR including X5, X11, 1,3,6,7-tetrahydroxyxanthone 2-prenyl xanthone (**X14**), X15, X17, and smeathxanthone B (**X22**) were found in comparable or decreased contents than control cultures.

Taking into account the importance of naphthodianthrones as main bioactive compounds in *H. perforatum*,

Table 2 HPLC/DAD/ESI-MS^n analysis of xanthones in *Hypericum perforatum* control and elicited hairy root cultures at day 4 of post-elicitation.[A]

Xanthones		t_R (min)	UV max (nm)	$[M–H]^-$	MS^2	Control HR	100 µM SA-elicited HR	100 µM JA-elicited HR
X1	Mangiferin	30.8	238, 256, 312, 362	421	331, 301, **258**	15.01 ± 0.77	10.55 ± 0.24*	31.39 ± 2.28*
X2	Tetrahydroxy-metoxy-C-prenyl xanthone	31.9	286, 314	341	326, 137	n.d.	5.69 ± 0.14	n.d.
X3	Tetrahydroxyxanthone-*O*-hexoside	33.8	254, 328	421	301, **257**	n.d.	2.98 ± 0.09	n.d.
X4	Mangiferin isomer	34.9	260, 318, 382sh	421	331, **301**	31.75 ± 2.71	22.11 ± 0.11*	300.63 ± 12.56*
X5	Trihydroxyxanthone-sulfonate	35.6	222, 314sh	323	243	18.26 ± 0.87	5.41 ± 0.45*	9.95 ± 1.15*
X6	Tetrahydroxyxanthone-*C*-pentoside	37.5	216, 252, 284sh, 326	391	331, 323, **301**	4.65 ± 0.55	0.96 ± 0.08*	n.d.
X7	Tetrahydroxyxanthone-*C*-pentoside	37.9	216, 252, 284sh, 326	391	331, **301**	0.83 ± 0.03	8.33 ± 0.12*	n.d.
X8	1,3,5,6-Tetrahydroxyxanthone	40.5	250, 282, 328	259	**229**, 213, 187	16.37 ± 0.78	44.70 ± 2.31*	25.21 ± 1.43*
X9	1,3,6,7-Tetrahydroxyxanthone	42.0	236, 254, 314, 364	259	231, **215**, 187, 147	6.38 ± 0.39	3.97 ± 0.22*	11.54 ± 0.63*
X10	1,3,6,7-Tetrahydroxyxanthone 8-prenyl xanthone	45.8	248, 312, 366	327	325, **297**, 258,201	1.36 ± 0.07	n.d.	n.d.
X11	1,3,5,6-Tetrahydroxyxanthone 8-prenyl isomer	47.1	242, 260, 320, 368	327	325, **297**, 258, 201	11.28 ± 1.32	12.07 ± 0.67	10.86 ± 0.87
X12	1,3,7-Trihydroxy-2-(2-hydroxy-3-methyl-3-butenyl)-xanthone	48.9	238, 260, 314, 388	327	**309**, 257	6.65 ± 0.45	n.d.	26.61 ± 1.68*
X13	1,3,7-Trihydroxy-6-methoxy-8-prenyl xanthone	49.5	240, 260, 318, 370	341	326, 311, **297**, 285	5.48 ± 0.37	n.d.	8.32 ± 0.44*
X14	1,3,6,7-Tetrahydroxyxanthone 2-prenyl xanthone	50.03	248, 312, 368	327	325, 283, **271**	3.53 ± 0.34	n.d.	3.00 ± 0.21
X15	Paxanthone	50.5	244, 264, 324, 386	339	**324**, 307	15.02 ± 1.14	9.19 ± 0.48*	0.54 ± 0.06*
X16	Gartanin isomer	51.4	254, 286, 324	395	326, 283, **271**	6.61 ± 0.46	n.d.	21.34 ± 2.09*
X17	γ-Mangostin isomer	52.0	260, 316, 370	395	351, **339**, 326, 283	44.43 ± 3.40	12.90 ± 0.54*	24.32 ± 1.79*

Table 2 (continued)

Xanthones	t_R (min)	UV max (nm)	$[M–H]^-$	MS^2	Control HR	100 µM SA-elicited HR	100 µM JA-elicited HR
X18 Trihydroxy-1-metoxy-*C*-prenyl xanthone	52.3	260, 286, 314	341	**325**	12.84 ± 0.69	2.34 ± 0.15*	17.89 ± 1.07*
X19 γ-Mangostin	52.6	246, 262, 320	395	351, 339, 326, **283**	6.40 ± 0.31	37.07 ± 2.77*	7.38 ± 0.56*
X20 Garcinone E	53.1	256, 286, 332	463	394, 351, **339**, 297, 285	94.68 ± 6.73	n.d.	109.28 ± 6.72*
X21 α-Mangostin	53.4	254, 330	409	394, **351**, 325, 272	6.73 ± 0.54	n.d.	n.d.
X22 Smeathxanthone B	54.5	262, 288, 322	393	**326**, 283	6.58 ± 0.52	n.d.	6.35 ± 0.43
X23 Garcinone C	55.7	286, 340	413	369, 344, **301**, 233	8.27 ± 0.48	n.d.	n.d.

[a] Contents of detected compounds are expressed as milligrams per 100 g dry weight (mg·100 g^{-1} DW ± SD). The values in the row noted with an asterisk denoted significant differences between control and elicited samples at $p < 0.05$. *SA* salicylic acid, *JA* jasmonic acid, *n.d.* not detected, *S.D.* standard deviation, *$[M–H]^-$* deprotonated molecular ion, *$MS^2[M–H]^-$* collision fragment ions, *sh* shoulder, t_R retention time. MS^2 ions in bold showed the base peak

Table 3 Naphthodianthrone contents in *Hypericum perforatum* control and elicited hairy root cultures at day 4 of post-elicitation.[a]

	HYP	PHYP	PPHYP
Control HR	0.68 ± 0.07	3.75 ± 0.76	4.23 ± 0.14
50 µM SA	0.45 ± 0.01*	3.84 ± 0.63	7.00 ± 0.56*
100 µM SA	0.55 ± 0.05	6.11 ± 0.72*	6.65 ± 0.50*
250 µM SA	1.16 ± 0.09*	19.70 ± 2.17*	18.14 ± 1.42*
10 µM JA	0.69 ± 0.05	3.64 ± 0.26	4.08 ± 0.28
50 µM JA	0.58 ± 0.06	6.50 ± 0.55*	11.21 ± 0.77*
100 µM JA	0.96 ± 0.08*	7.67 ± 0.65*	3.04 ± 0.22*

[a] Contents of detected compounds are expressed as micrograms per gram dry weight (µg·g^{-1} DW ± SD). The values in the column noted with an asterisk denoted significant differences between control and elicited samples at $p < 0.05$. *HR* hairy roots, *SA* salicylic acid, *JA* jasmonic acid, *S.D.* standard deviation, *HYP* hypericin, *PHYP* pseudohypericin, *PPHYP* protopseudohypericin

control cultures and elicited HR with all tested SA and JA concentrations were analyzed at day 4 of cultivation. The UPLC-TUV analysis revealed quantitative differences in the contents of HYP, PHYP and PPHYP between control and elicited HR (Table 3). Application of SA in low dose of 50 µM induced significant decrease in HYP content, while high elicitor dose of 250 µM caused 1.7-fold enhanced production of this naphthodianthrone compared to control HR. Similarly, elicitation with JA showed that only high elicitor dose of 100 µM significantly enhanced HYP production (1.4-fold) in comparison to control. Treatment of HR with 100 and 250 µM SA significantly improved biosynthesis of PHYP (up to 5.3-fold) and PPHYP (up to 4.3-fold) compared to control HR. The JA-elicited HR with 50 and 100 µM displayed notable increment of PHYP contents (from 1.7- to 2-fold) compared to control cultures. In comparison to control HR, addition of 50 µM JA caused 2.6-fold enhancement in PPHYP content, while the treatment with 100 µM JA lead to 1.4-fold decreased amount of this protoform.

Enzyme inhibitory activities of *H. perforatum* HR cultures

Present results for in vitro neuroprotective activity demonstrated that HR elicited with all tested SA concentrations had better anti-AChE activity (up to 1.6-fold) compared to control HR (Fig. 9a). Elicitation experiments with JA showed that treatments of HR with 10 and 50 µM induced significantly higher AChE inhibitory activity (up to 1.7-fold), while 100 µM JA caused markedly enhancement of enzyme inhibition (5.6-fold) in comparison to control cultures (Fig. 9b). Concerning in vitro anti-LIP activity, only HR elicited with 50 µM SA displayed significantly higher LIP inhibitory activity (1.9-fold), while all tested JA doses resulted in notable enhanced enzyme inhibition (from 1.5- to

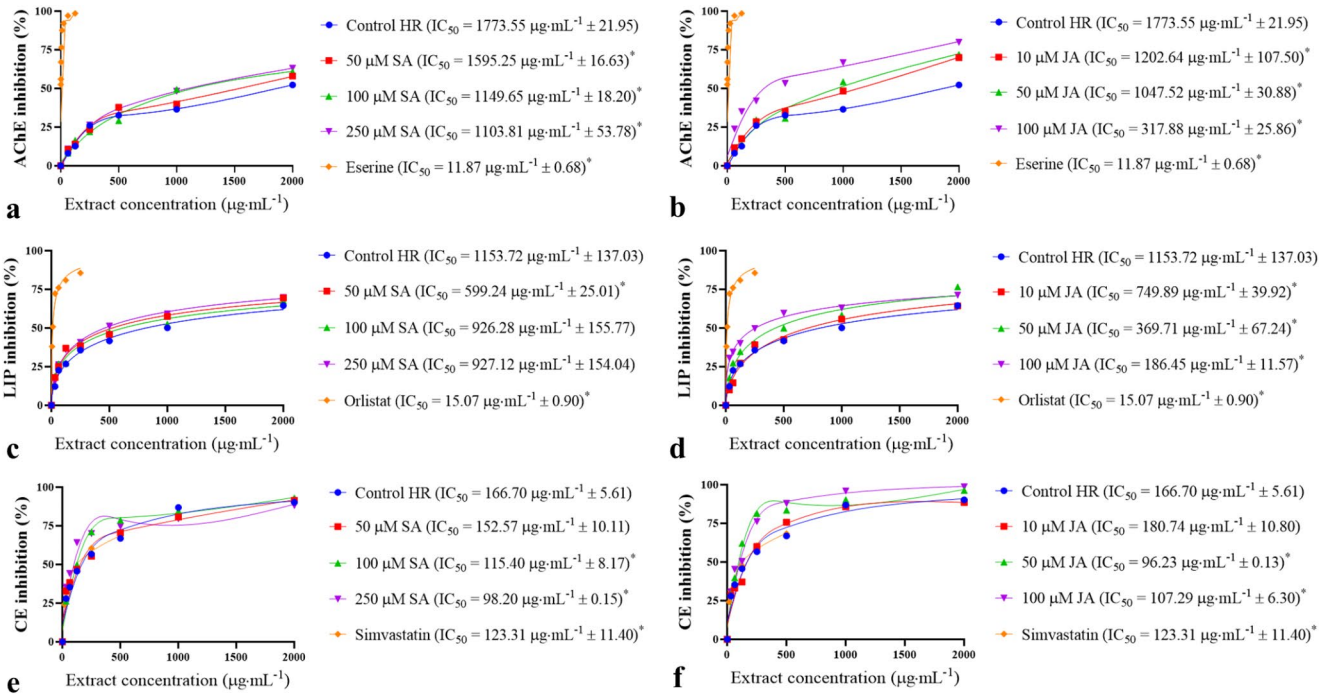

Fig. 9 Enzyme-inhibitory activity of *Hypericum perforatum* hairy roots (HR) elicited with salicylic acid (SA) and jasmonic acid (JA). **a**, **b**: acetylcholinesterase (AChE), **c**, **d**: lipase (LIP), **e**, **f**: cholesterol esterase (CE). IC_{50} extract concentration that inhibits 50% of enzyme activity. The values denoted with asterisk showed significant differences between control and elicited samples ($p < 0.05$)

6.2-fold) compared to control HR (Fig. 9c, d). With respect to anti-CE activity, significant enhancement of enzyme inhibition was observed for HR cultures elicited with 100 and 250 µM SA, as well with 50 and 100 µM JA (from 1.5- to 1.7-fold) compared to control (Fig. 9e, f). It is worth to point out that elicited HR cultures with SA and JA showed comparable or even lower IC_{50} values for CE inhibition than those observed for simvastatin as a specific enzyme inhibitor.

Discussion

Effect of elicitation on the growth and nutrient utilization by *H. perforatum* HR cultures

Results in this study showed that fresh biomass production in *H. perforatum* HR cultures depends on the type of elicitor used (SA and JA), their concentrations and post-elicitation period. The exogenous application of SA suppressed biomass production of HR, which is in agreement with previous data confirming growth inhibitory effect of SA in *H. perforatum* cell suspensions, calli (Walker et al. 2002; Gadzovska et al. 2013) and plantlets (Sirvent and Gibson 2002). The growth inhibition of HR upon elicitation might be associated with SA-mediated oxidative stress since excessive accumulation of reactive oxygen species (ROS) cause lipid peroxidation that lead to membrane deteriorations and tissue necrosis (Kováčik et al. 2009). This hypothesis could be confirmed by present data showing that declined biomass production in SA-elicited HR was significantly correlated with increased intracellular levels of oxidative stress markers (H_2O_2 and MDA). Concerning elicitation with JA, it was evident that low elicitor doses (10 and 50 µM) had a stimulating effect on biomass production in HR cultures during the late post-elicitation period. Similarly, Walker et al. (2002) have observed that elicitation with low JA concentration enhances biomass accumulation in *H. perforatum* cell suspensions. Even though growth stimulatory effect of jasmonates has not been considered as usual outcome during elicitation, this phenomenon has already been reported for HR cultures of *Psoralea corylifolia* (Zaheer et al. 2016) and *Gentiana dinarica* (Krstić-Milošević et al. 2017). It has also been shown that jasmonates modulates auxin signaling pathways in *Arabidopsis* through activation of auxin biosynthesis genes resulting in increment of endogenous levels of indol-3-acetic acid (Yan et al. 2016). This jasmonate-induced production of intrinsic auxins could explained better growth of elicited HR since transformed root cells expressing *rol* genes displayed increased sensitivity to auxins (Veena and Taylor 2007). On the other hand, prolonged treatment with high JA dose might cause stressful conditions that limit root growth and biomass production in HR cultures. As presently reported, high concentrations of jasmonates notably suppressed HR growth that could be

ascribed to direct toxic effects of elicitor or activation of defense mechanisms (Amani et al. 2024).

Four main components in the culture medium (NH_4^+, NO_3^-, PO_4^{3-} and RS) were monitored throughout the growth of *H. perforatum* HR in order to evaluate the effect of elicitation on nutrient utilization. Ammonium and nitrate as inorganic nitrogen source in the medium have an important role in the regulation of biomass production and secondary metabolism in plant in vitro cultures (Cui et al. 2010b). Outgoing results indicated that SA and JA elicitation significantly reduced the capacity of HR for utilization of available NH_4^+ and NO_3^- in the medium. However, NO_3^- was found as preferred nitrogen source for elicited HR in comparison to NH_4^+ during the late post-elicitation period. This is in accordance with previous study reporting that efficient utilization of NO_3^- from the medium has an important role for the growth of *H. perforatum* adventitious roots (Cui et al. 2010b). Phosphate as an essential inorganic source of phosphorus for plant tissues represents a constitutive element of nucleotides, ATP and membrane phospholipids, as well as key regulator of metabolic pathways in plants (Ticconi and Abel 2004). Present data demonstrated that exogenous application of SA had an inhibitory effect on PO_4^{3-} uptake by HR cultures that could be related to the application of non-physiological concentrations of elicitor and stressful conditions. On the other hand, elicitation with low JA doses caused almost complete depletion of PO_4^{3-} from the medium suggesting that utilization of this macronutrient has a major role in controlling the HR biomass production. Similarly to our results, Condori et al. (2010) have reported a complete consumption of PO_4^{3-} from MS medium by *Arachis hypogaea* HR cultures upon elicitation with sodium acetate. These results suggested that extracellular PO_4^{3-} concentration could be a relevant nutrient marker for cell survival and growth state in *H. perforatum* HR during elicitation with jasmonates.

Residual sugars were monitored in the medium in order to evaluate consumption of sucrose as a potential carbon and energy source for the growth and secondary metabolite production in *H. perforatum* HR. Our results showed that application of SA markedly increased residual sugars in the elicited medium implying that available sucrose was probably hydrolyzed to glucose and fructose. According to Cui et al. (2010a), this extracellular hydrolysis of sucrose is probably mediated by cell wall-bounded invertase that was secreted from *H. perforatum* adventitious roots. Even this additional increment of residual sugar levels was not confirmed in the medium upon treatment with JA, elicited HR displayed reduced uptake of sucrose during the entire post-elicitation period. Present results showed that the growth of SA- and JA-elicited HR was in significant negative correlation with NH_4^+, NO_3^-, PO_4^{3-} and RS contents in the medium indicating that those nutrients are essential substrates that control cell growth during elicitation period. Taking into account that *H. perforatum* elicited HR exhibited reduced uptake of nutrients from the medium, it could be assumed that root cells tend to adapt to stressful conditions through switching their primary metabolism toward secondary metabolite biosynthesis.

Effect of elicitation on total phenolic compounds and antioxidant activities of *H. perforatum* HR cultures

Present results demonstrated that total phenolic compounds (TP, TF and TFA) production in *H. perforatum* HR was significantly increased upon elicitation with SA and JA. In accordance with our data, SA and JA/MeJA have been efficiently used for overproduction of phenolics and flavonoids in HR cultures of *Lactuca indica* (Yi et al. 2019), *Galega officinalis* (Khezri et al. 2022), and *Ficus carica* (Amani et al. 2024). We have previously observed that SA and JA triggered the production of total phenolics, flavonols and flavanols in *H. perforatum* cell suspensions and calli through increase activity of phenylalanine ammonia lyase (PAL) and chalcone isomerase (CHI) as key enzymes of the phenylpropanoid/flavonoid pathway (Gadzovska et al. 2007, 2013). Several studies have reported that elicitation of HR cultures with SA and JA/MeJA up-regulated PAL expression and other phenlypropanoid-related genes that result in an enhancement of phenolic compounds biosynthesis (Li et al. 2020; Amani et al. 2024). Even though we have not evaluated the activity of enzymes involved in phenylpropanoid metabolism, it appears that SA and JA act as signaling molecules that cause specific changes in the expression of defense-related genes and consequently activate PAL-mediated biosynthesis of phenolic compounds (Li et al. 2020).

Outgoing results indicated that SA and JA doses and the time of exposure to elicitation have a considerable importance for phenolic compounds biosynthesis. In particular, low SA doses (50 and 100 µM) during early- to mid-elicitation were found more efficient in the stimulation of total phenolic compound contents, while high elicitor dose (250 µM) at late post-elicitation had an inhibitory effect on the productivity of HR cultures. This fast eliciting activity of SA toward biosynthesis of phenolic compounds in *H. perforatum* HR could possibly be explained by the hermetic response of plant cells exposed to mild stress (Vargas-Hernandez et al. 2017). As presently reported, declining of phenolic contents in HR cultures at prolonged exposure with high SA dose may be ascribed to the toxicity stress that potentially lead to damage of root cell membranes and releasing of synthesized metabolites into the extracellular medium (Rodríguez-Sánchez et al. 2020). This scenario

could be confirmed by the present data displaying that extracellular phenolics in the medium was negatively related to the accumulation of phenolic compounds in elicited HR cultures. In contrast to data obtained for SA elicitation, low JA dose (10 µM) exhibited late response towards eliciting of phenolic compounds, while high JA dose (100 µM) continuously improved the HR productivity throughout elicitation period. These findings suggested that *H. perforatum* HR cultures are characterized with high threshold value for elicitation of phenolic compounds upon elicitation with JA, which is in accordance with previously reported data by Rajan et al. (2020). However, current investigations showed that elicitation of HR with high JA dose markedly stimulated the secretion of extracellular total phenolics in the medium. Similarly to our results, Ruiz-May et al. (2009) have demonstrated that increase of MeJA doses (100 and 250 µM) significantly enhanced releasing of secondary metabolites in *Catharanthus roseus* HR cultures. Noteworthy, our results revealed that accumulation of TP, TF and TFA in JA-elicited HR coincided with secretion of phenolic metabolites in the medium. Taking into account that intracellular accumulation of metabolites might inhibit their own biosynthesis (Kang et al. 2006), the activation of releasing mechanism upon elicitation with high JA dose could explain superior production of phenolic compounds by HR cultures. Even though exact mechanisms for secretion of phenolic compounds by *H. perforatum* elicited HR cultures remain largely unknown, the effects of JA on the cell membrane fluidity through modification in the expression of trans-membrane proteins cannot be ruled out (Ruiz-May et al. 2009).

The *H. perforatum* HR elicited with SA and JA showed better capability to reduce Cu^{2+} and Mo^{6+} ions (CUPRAC and PM assay, respectively) and greater DPPH scavenging capacity compared to non-elicited cultures. The enhancement of antioxidant capacity upon elicitation with SA and jasmonates has already reported in HR cultures of various medicinal plants (Chung et al. 2016; Amani et al. 2024). Outgoing data revealed that antioxidant capacities of control and elicited HR followed the similar pattern as results for TP, TF and TFA contents. These findings were confirmed through significant positive correlations of those phenolics with CUPRAC, PM and DPPH suggesting that phenolic and flavonoid compounds represent the main contributors to the antioxidant activities of elicited HR. In favor of this hypothesis, we have previously reported that *H. perforatum* cell suspensions responded to fungal and polysaccharide elicitors through accumulation of phenolic compounds with ROS-scavenging activity in order to minimize the consequences of stressful conditions (Gadzovska et al. 2015a, b). Present study revealed that *H. perforatum* defense system was up-regulated by phenylpropanoid-mediated increment of non-enzymatic antioxidants in elicited HR cultures.

Effect of elicitation on antioxidant enzymes and oxidative stress markers in *H. perforatum* HR cultures

The evaluation of antioxidant enzymes in *H. perforatum* HR upon SA treatments revealed continuous reduction in POD activity during post-elicitation period, while only low elicitor dose caused late enhancement of this antioxidant enzyme. On the other hand, all applied SA concentrations induced an early SOD induction and subsequently declining of enzyme activity to the end of post-elicitation period. The SOD and POD as antioxidant enzymes synergistically acted into the maintenance of redox homeostasis and protection of plant cells from ROS-mediated cell damages (Gill and Tuteja 2010). In the present study, SOD was found to respond rapidly in order to protect HR cells from SA-induced oxidative stress since the early activation of SOD was followed by decreasing in superoxide anion production rate. Additionally, SOD activity in SA-elicited cells was positively related to the production of phenolics (TP, TF, and TFA) and total antioxidant activities (CUPRAC, DPPH, PM and FCA) indicating a potential synergistic interactions between non-enzymatic and enzymatic antioxidants towards ROS scavenging. The co-operative functioning of SOD and non-enzymatic antioxidant compounds as defensive mechanism for ROS neutralization upon elicitation with SA has already been confirmed in *Rauvolfia serpentina* root cultures (Dey et al. 2020). However, a decline in POD activity under elicitation with high SA concentration suggested a possible delay in neutralization of toxic peroxides, which was evident through notable accumulation of intracellular H_2O_2 and MDA in HR. Increased production of oxidative stress and lipid peroxidation biomarkers has also been observed in *Galega officinalis* HR elicited with SA (Khezri et al. 2022). It has been reported that application of excessive concentrations of SA inactivated H_2O_2-degrading enzymes in plant cells and induced ROS-mediated oxidative damage that ultimately lead to hypersensitive cell death (Rao et al. 1997). The significant positive correlations between intracellular H_2O_2 and MDA levels with extracellular phenolics suggested that SA caused evident oxidative burst in HR cells and further loss of membrane integrity as evidenced by the increase secretion of phenolic compounds in the medium. These findings implied that optimization of SA concentrations during elicitation experiments could be an important factor to minimize the oxidative stress-induced damage in HR cultures and to maintain production of viable biomass with stable production of bioactive metabolites.

An opposite trend in antioxidant enzymes was observed for JA-treated HR cultures, where POD displayed consistent up-regulated activity, while SOD enzyme was suppressed in the late post-elicitation period. Declining in SOD activity

after JA treatment indicated that the scavenging activity of this antioxidant enzyme was impaired due to the excessive production of superoxide anion radicals, which was presented here through strong negative relationship between SOD activity and $O_2^{\bullet-}$ production rate in HR cultures. In accordance with these findings, exogenous application of JA and MeJA has been related to the descending of SOD activity and overproduction of ROS in *Panax ginseng* and *Cnidium offinale* adventitious roots (Ali et al. 2006; Ho et al. 2020). Although mechanism responsible for SOD inhibition is unclear, recent elicitation study of *Arachis hypogaea* HR with a combination of MeJA, chitosan, and cyclodextrin revealed a strong up-regulation of *CuZn-SOD* gene expression immediately after the treatment, while prolonged elicitation resulted in suppressed gene expression (Chayjarung et al. 2022). Taking into account all these observations, it could be suggested that SOD enzymatic antioxidant system operates as an early elicitor response to ROS scavenging in HR cells. The comparison of antioxidant enzymes in JA-treated HR demonstrated that the lower response of SOD activity to elicitation may be compensated by the enhanced POD activity. These data are in agreement with previous studies reporting an increment in POD activities in adventitious root cultures upon elicitation with JA/MeJA (Ho et al. 2020; An et al. 2022). Even though we did not observe significant correlations between antioxidant enzyme activity and oxidative stress marker contents, it was shown that JA-mediated increment of POD slightly increased H_2O_2 production, while MDA levels was unaffected or decreased in HR cultures. More importantly, POD activity showed strong positive correlation with phenolic compounds production and non-enzymatic antioxidant activities indicating that HR tissues are better protected from oxidative damage and the activation of antioxidant system is likely to suppress lipid peroxidation upon JA treatment. Further investigations should be focused on the evaluation of relationship between activation of antioxidant enzyme activities and phenolic compounds biosynthesis in the maintenance of cell redox state in *H. perforatum* HR upon SA and JA elicitation.

Effect of elicitation on individual phenolic compounds and naphthodianthrones in *H. perforatum* HR cultures

Present results demonstrated that SA- and JA-elicited HR had comparable or even decreased production of quinic acid and phenolic acids (3-cafeoylquinic acid, 5-cafeoylquinic acid, and *p*-coumaric acid) compared to control cultures. Similarly to our data, Benavides et al. (2023) have reported that SA and MeJA did not have a general tendency to elicit the production of phenolic acids in *Phyllanthus acuminatus* HR cultures. In contrast, several studies have demonstrated that MeJA elicitation enhanced the production of caffeic acid, chlorogenic acid, and coumaric acid through up-regulation of phenylpropanoid pathway genes expression in *Ficus carica* and *Mentha spicata* HR (Yousefian et al. 2020; Amani et al. 2024). However, those investigations highlighted that possible delay between phenylpropanoid gene expression and phenolic acids biosynthesis could be the reason for non-significant correlations between these two processes. The reduced accumulation of quinic acid in SA- and JA-elicited cultures is not surprising outcome, since we have recently reported that photoperiod exposition strongly enhanced the accumulation of this intermediate from the shikimate pathway in *H. perforatum* HR (Tusevski et al. 2024b). The low productivity of quinic acid in elicited HR cultured under darkness might be a reason for suppressed biosynthesis of chlorogenic acids (3-cafeoylquinic acid, 5-cafeoylquinic acid) that represent the esters of quinic acid and caffeic acid.

The chromatographic results for catechins clearly showed that SA- and JA-elicited HR had markedly enhanced or *de novo* production of monomeric catechin and epicatechin, as well as procyanidin oligomers. In agreement with this finding, it has already been shown that SA and jasmonates are powerful elicitors for stimulation of catechins and procyanidins biosynthesis in various in vitro cultures (Bulgakov et al. 2011; López-Orenes et al. 2013). To the best of our knowledge, *H. perforatum* HR obtained by genetic transformation with *A. rhizogenes* strain A4M70GUS and A4 represent an excellent source of catechins (Tusevski et al. 2017, 2023). However, those studies suggested that those HR are highly responsive to photoxidative stress accumulating low amounts of epicatechin and procyanidin B2 due to possible photoperiod-mediated suppression of key enzymes from the flavonoid biosynthetic pathways (Tusevski et al. 2017, 2023). In the study of Paponov et al. (2021), elicitation of *H. perforatum* with MeJA increased production of catechin, epicatechin and procyanidin dimer, while far-red light had no effect or decreased the production of these catechins. It has also been shown that dihydroflavonol 4-reductase for conversion of dihydroflavonols to leucoanthocyanidins, and further transformation of these products to catechins represent a key regulatory enzyme for the biosynthesis of catechins (Kausar et al. 2020). Concerning this, we can hypothesized that exogenous application of SA and JA somehow affect the activity of dihydroflavonol 4-reductase and modulated phenylpropanoid flux towards enhancement of flavan-3-ols accumulation in *H. perforatum* HR.

As far as we know, *H. perforatum* HR have a limited capacity for the biosynthesis of flavonols and only quercetin and kaempferol glycosides have been identified in these culture types (Tusevski et al. 2023, 2024b). With the exception of our recent study for down-regulation of quercetin and

kaempferol glucosides in photoperiod-exposed *H. perforatum* HR, the literature data for overproduction of flavonols in transformed roots upon SA and JA elicitation is rather scarce. Noteworthy, outgoing elicitation experiments demonstrated that SA induced *de novo* production of quercetin 6-*C*-glucoside in elicited HR, while kaempferol hexoside was found in significantly enhanced amounts compared to control cultures. Several studies have also reported that SA at a concentration of 100–150 µM represent an efficient elicitor for increased accumulation of quercetin, quercetin-3-*O*-glucoside and kaempferol-3-rutinoside in various in vitro cultures (Wen et al. 2019; Abbasi et al. 2020). Surprisingly, JA-elicited HR did not display any capability for the accumulation of flavonols that were identified in control or SA-treated HR. These findings are in agreement with previous reports displaying reduced accumulation of quercetin and kaempferol derivatives in hydroponically grown *H. perforatum* plants upon MeJA treatment (Paponov et al. 2021). One possible explanation for this outcome could be related to the stimulatory effect of JA on the enzymatic transformation of flavonols towards flavan-3-ols, which may explain the superior productivity of elicited HR for catechins and procyanidins. Another one might be associated with the JA-mediated exudation of synthesized flavonols into the medium since the highest elicitor concentration was accompanied with maximum contents of extracellular phenolics. Thus, evaluation of SA and JA effects on the key enzymes from flavonoid biosynthetic pathway in *H. perforatum* HR or detailed phenolic profile of elicited culture medium is necessary in the following studies to confirm these hypotheses.

Outgoing data indicated that xanthones represented the most abundant and affected phenolic compounds in *H. perforatum* HR upon elicitation with SA and JA. It was observed that JA-elicited HR accumulated more than twofold higher contents of total identified xanthones compared to control cultures, while SA-treated HR displayed inferior productivity of these compounds. This heterogeneity in the accumulation of xanthones could be ascribed to the specific types of stress and signaling pathways induced by SA versus JA. The relevance of SA and MeJA as efficient elicitors for enhanced production of xanthones has already been reported in cell suspensions of *H. perforatum* (Conceição et al. 2006; Zubrická et al. 2015). Induction of xanthone accumulation in various *H. perforatum in vitro* cultures has also been confirmed upon elicitation with *Colletotrichum gloeosporioides* (Conceição et al. 2006), *A. tumefaciens* (Franklin et al. 2009), chitosan (Tocci et al. 2012) and acetic acid (Valletta et al. 2016). Present results showed that mangiferin, 1,3,5,6- and 1,3,6,7-tetraxydroxyxanthones, as well as γ-mangostin were dominant xanthones in control and elicited HR implying their dual function as constitutive phytoanticipins and inducible phytoalexins in stressful conditions. One of the most important achievements in the current study was the enormous enhancement of mangiferin and its isomer in JA-elicited HR exhibiting twofold and tenfold enhancement in comparison to control cultures, respectively. Since mangiferin play an important function as antioxidant and antimicrobial compound (Franklin et al. 2009), the elicitation of *H. perforatum* HR with JA might be recognized as perspective approach for obtainment of mangiferin-enriched extracts for medicinal purposes. However, elicitors used in this study exhibited specific effects on the accumulation of particular xanthones in HR indicating that SA and JA differentially affect some biosynthetic steps in xanthone metabolism or enzymatic reactions responsible for modification of xanthone structures. In this context, JA was found to stimulate the production of numerous prenylated xanthones (1,3,7-trixydroxy derivatives, gartanin isomer, garcinone E and α-mangostin), while the contents of those xanthones were significantly decreased or completely absent in response to SA elicitation. The high productivity of prenylated xanthones in JA-elicited HR could be explained by elicitor-induced activity of prenyltransferases, which catalyze addition of prenyl moieties to the xanthone skeleton. In agreement with this hypothesis, Fiesel et al. (2015) have observed that elicited *H. calycinum* cell cultures showed increased transcript level of membrane-bound prenyltransferase that subsequently resulted in accumulation of prenylated xanthones. In contrast, down-regulation of numerous prenylated xanthones or their disappearance in SA-elicited HR tissues is probably attributed to their easy secretion in the culture medium, which is in agreement with previous data confirming the presence of xanthones in the medium of elicited *H. perforatum* cells (Tocci et al. 2010). Since xanthones have been predominantly accumulated in the exodermis of *H. perforatum* roots (Tocci et al. 2018), the increased permeability of these surface cells for xanthones cannot be excluded upon SA elicitation. The putative accumulation of xanthones into elicited medium could be considered as a promising strategy for scaling-up of the production process and easy recovery of these bioactive compounds.

Despite the extensive coverage of elicitation in various *Hypericum in vitro* cultures (Coste et al. 2011; Gadzovska et al. 2007, 2013; Wu et al. 2014), the effect of SA and JA on hypericins biosynthesis in *H. perforatum* transformed roots has never been explored. Outgoing analysis of naphthodianthrones demonstrated that SA and JA had a stimulatory effect on hypericin, pseudohypericin and protopseudohypericin production in *H. perforatum* HR. Our recent studies revealed that photoperiod exposition markedly enhanced total hypericins, as well as pseudohypericin and protopseudohypericin contents in *H. perforatum* HR (Tusevski and Gadzovska Simic 2023; Tusevski et al. 2023). Contrary to these observations, Walker et al. (2002) have reported

that dark-grown *H. perforatum* cells elicited with SA and JA accumulated significantly higher amounts of hypericin compared to light-exposed cultures. However, the effects of these elicitors on naphthodianthrone production between cells and transformed roots cultured under dark or light conditions could not be relevantly compared because hypericins accumulation largely depends on type of in vitro cultures and degree of cell differentiation (Gadzovska et al. 2013). Present results demonstrated that SA has stronger capability for increment of naphthodianthrone production in HR compared to JA, which is in agreement with previous study for elicitation of *H. hirsutum* and *H. maculatum* shoot cultures (Coste et al. 2011). The different effects of SA and JA could be explained by the activation of distinct signaling pathways that result in up-regulation of genes involved in naphthodianthrone biosynthesis. It is interesting to note that naphthodianthrone contents in *H. perforatum* HR presented here are inferior compared to those for adventitious roots elicited with MeJA (Wu et al. 2014), cultured in bioreactor (Sobhani et al. 2021) or selected as high-producing root lines (Shafaei et al. 2024). It should be pointed out that all these strategies for enhanced production of naphthodianthrones have been performed on adventitious roots cultured in the presence of indol-3-butyric acid (IBA). Taking into account that auxins in the medium could modulate naphthodianthrone production in adventitious roots (Wu et al. 2014; Shafaei et al. 2024), it seems that hormone-free medium for cultivation of *H. perforatum* HR limits the effects of applied elicitors on hypericins biosynthetic reactions. These findings could be a starting point for further evaluation of potential synergistic interactions between elicitors and phytohormones in the culture medium for the establishment of *H. perforatum* transformed roots with promising production of hypericins.

Effect of elicitation on in vitro neuroprotective and antihyperlipidemic activity in *H. perforatum* HR cultures

The neuroprotective activity of *H. perforatum* extracts through inhibition of cholinesterases has been the subject of several studies (Altun et al. 2013; Tusevski et al. 2018). Present data for in vitro neuroprotective activity demonstrated that HR elicited with both SA and JA had significantly higher AChE inhibitory activities compared to control cultures. The strong anti-AChE activity of elicited HR could potentially be explained by their enrichment in xanthones. Accordingly, our recent in vitro and in silico investigations on *H. perforatum* transformed roots indicated that garcinone B and γ-mangostin as prenylated xanthones are the main contributors to the AChE inhibitory activity (Tusevski et al. 2023, 2024b). As previously reported, the prenyl groups of xanthones have been proposed as essential structural components for establishment of hydrophobic interactions with amino acids of the AChE active site (Khaw et al. 2014). In favor of these statements, the stronger AChE inhibitory activity of JA-elicited HR compared to those elicited with SA could be explained by their higher capability for accumulation of prenylated xanthones. Outgoing data indicated that JA represents an efficient elicitor that stimulates the production of prenylated xanthones as neuroprotective compounds in *H. perforatum* transformed roots that might be potentially used for prevention or treatment of Alzheimer's disease.

The lipase and cholesterol esterase as pivotal enzymes involved in the pathways of lipid hydrolysis and absorption are usually targeted for the management of obesity and hyperlipidemia-related diseases (He et al. 2023). It has already been shown that *H. perforatum* plants could be efficiently used for treatment of metabolic syndromes related to dyslipidemia due to their anti-lipase and cholesterol lowering activity (Tian et al. 2015; Hou et al. 2020). Outgoing data revealed superior lipase inhibition in HR elicited with the lowest SA dose and the highest JA concentration. Taking into account that those elicitor concentrations induced maximum production of total phenolic compounds (flavonoids and flavan-3-ols) at day 4 of post-elicitation, it can be suggested that catechins participate in lipase inhibition. In this context, Cao et al. (2024) have performed in vitro and in silico analyses and reported that catechin and epicatechin from coffee leaf extracts are the key components for inhibitory activity against pancreatic lipase. Previous molecular docking analyses of the most abundant phenolic compounds in *H. perforatum* HR-regenerated shoots revealed that epicatechin represent a promising inhibitor of pancreatic lipase (Tusevski et al. 2024c). All these observations indicated that SA and JA elicitors stimulated the production of catechin, epicatechin, and oligomeric procyanidin derivatives that might be responsible for better lipase inhibitory properties of *H. perforatum* HR cultures.

Concerning cholesterol esterase inhibition, outgoing data indicated that SA- and JA-elicited HR significantly increased enzyme inhibitory activity compared to control cultures. The exception was found only for HR cultures elicited with low doses of SA and JA displaying comparable anti-cholesterol esterase activity as non-treated HR. These results suggested that intermediate and high doses of SA and JA significantly increased the production of hypericin, pseudohypericin and protopseudohypericin with potential cholesterol esterase inhibitory activity in elicited HR cultures. In accordance, we have recently reported significant correlations between pseudohypericin production and inhibitory activity against cholesterol esterase in *H. perforatum* HR-regenerated shoots (Tusevski et al. 2024c). Those data indicated that pseudohypericin had superior cholesterol

esterase inhibitory activity due to its capability to form hydrogen bonds to amino acids of the enzyme active center. The study of Tian et al. (2015) has also demonstrated that *H. perforatum* fraction enriched in hypericin and pseudohypericin alleviated lipid metabolism disturbance exhibiting hypocholesterolemic effect in high-fat-diet induced obese mice. Even though hypericins contents in elicited HR were comparatively lower to those of other groups of phenolics, their contribution to cholesterol esterase inhibition could not be ruled out since in silico data displayed inhibition constant for pseudohypericin in nanomolar value (Tusevski et al. 2024c). Altogether, SA and JA-mediated biosynthesis of catechins and naphthodianthrones in *H. perforatum* transformed roots could be considered as a promising alternative in ameliorating postprandial hyperlipidemia and hypercholesterolemia.

Conclusion

This study describes for the first time the stimulatory effects of various concentrations of salicylic acid and jasmonic acid on the production of phenolic compounds and naphthodianthrones in *H. perforatum* transformed root cultures. During elicitation experiments, jasmonic acid was found to increase fresh biomass of transformed roots at low concentrations, while salicylic acid showed inhibitory effect on the growth irrespective of the concentrations used. The growth suppression in elicited transformed roots was related to their reduced utilization of nutrients from the culture medium under stressful conditions. Antioxidant activity in elicited transformed roots was significantly increased during different period of post-elicitation period suggesting a modification in the production of phenolic compounds with antioxidant properties. Transformed roots elicited with salicylic acid exhibited decreased activity of antioxidant enzymes that resulted in accumulation of oxidative stress markers (hydrogen peroxide and malondialdehyde) and secretion of phenolic compounds into culture medium. In contrast, the co-operating functioning of antioxidant enzymes and phenolic compounds upon elicitation with jasmonic acid can prevent oxidative damage and lipid peroxidation in transformed roots. Both elicitors exhibited enhanced accumulation of catechins (catechin, epicatechin and procyanidins) and naphthodianthrones (hypericin, pseudohypericin and protopseudohypericin), while jasmonic acid showed superior capacity for xanthones production. Noteworthy, elicited transformed roots displayed significantly higher acetylcholinesterase, lipase and cholesterol esterase inhibitory activities indicating their in vitro neuroprotective and antihyperlipidemic activity. Further studies should be focused on the optimization of culture conditions for simultaneous improvement of growth and bioactive metabolites production in *H. perforatum* transformed roots during elicitation experiments by using bioreactor technology.

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Declarations

Research involving human participants and/or animals Not applicable to the current study as this research did not involve any human or animal subject.

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