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# The Influence of Phytohormones on Antioxidative and Antibacterial Activities in Callus Cultures of *Hypericum perforatum* L.

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**Abstract:** Natural extracts of *Hypericum perforatum* L. are widely used in the pharmaceutical industry due to their antiviral, antioxidant, antibacterial, antidiabetic, and antidepressant activities. Plant biotechnological methods are gaining more attention as efficient and biosustainable approaches for the production of high value compounds by using plant callus cultures in vitro. The aim of this research was to evaluate the influence of phytohormones on the formation of *Hypericum perforatum* L. callus culture, to explore antioxidant and antibacterial activities, and to determine the amounts of phytochemicals in the analyzed extracts. Callus cultures were induced on Murashige and Skoog basal medium supplemented with phytohormones (auxins, cytokinins, and salicylic acid), and containing newly synthesized compound *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide. The antioxidant activities of callus cultures were evaluated using DPPH and FRAP assays and the enzyme antioxidants (superoxide dismutase, catalase, and ascorbate peroxidase) were evaluated. The antibacterial activity of plant extracts was screened against *Escherichia coli* and *Bacillus subtilis* bacteria by the diffusion agar method. There was a significant positive correlation between total phenolic content and high antioxidant efficiency in *Hypericum perforatum* L. callus cultures, which were formed on Murashige and Skoog medium supplemented with auxin, cytokinin, and salicylic acid (100 μM). Our results have demonstrated that callus cultures generated on MS medium supplemented with *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide and cytokinin BAP (0.5 mg $L^{-1}$) exhibited improved antioxidant activities for the first time.

**Keywords:** plant cell cultures; plant hormones; oxidative stress; enzymatic antioxidants; phytometabolites



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## 1. Introduction

Natural bioactive products or extracts of *Hypericum perforatum* L. (*Hypericaceae*) have beneficial potential in many industrial applications (plant biotechnology, cosmetics, pharmaceutical and food industries, and sustainable agriculture). The market of *Hypericum perforatum* L. extracts is divided into segments according to the type (crude or standardized extracts). It is well known that these extracts possess antioxidant, antibacterial, antiviral, antidepressant, antidiabetic, and anticancer activities. They can help improve symptoms of anxiety and depression and the gut microbiota [1]. The main biologically active compounds present in extracts are as follows: naphthodiantrone derivatives (hypericin, pseudohypericin, and protohypericin), flavonoids, biflavones, phenolic acids (vanillic, caffeic, *p*-coumaric, ferulic, and chlorogenic acids), phloroglucinols (hyperforin and adhyperforin), proanthocyanidins, and essential oils [2]. Bioflavonoid and catechin-rich extracts have been shown to have a multitude of significant functions, such as the scavenging of harmful reactive oxygen species (ROS), protecting cells from oxidative stress or virus damage [3]. Quercetin, rutin, biapigenin,

hyperoside, quercitrin, and isoquercitrin have been identified to be the most useful flavonoids in the pharmaceutical and food industries [4].

Natural antioxidants have recently attracted increasing attention from researchers. These useful compounds are extracted mainly from various natural sources, particularly medicinal plants. Many bioactive compounds extracted from *Hypericum perforatum* L. are used as natural antioxidants and prebiotics [5]. Extracts of *Hypericum perforatum* L. demonstrate reactive oxygen-quenching activity, inhibit lipid peroxidation, and regulate the activity of various antioxidant enzymes [6,7]. The antioxidants in these extracts are phenolic compounds (flavonoids, phenolic acids), and carotenoids. Based on numerous research results, these valuable extracts possess cytotoxic activity against tumor formation [8–10].

Hyperforin, obtained from *Hypericum perforatum* L., is a cyclic terpene ketone phytometabolite that exhibits antibacterial activity against *Staphylococcus aureus* [11] and demonstrates a cellular protective effect [12,13]. The butanol extract of *Hypericum perforatum* L. showed anti-*Helicobacter pylori* properties [14], while the ethanol extract inhibited the growth of *Streptococcus mutans*, *Streptococcus sobrinus*, *Fusarium oxysporum*, and *Alternaria alternata* [15].

Hypericin (red dye), which is one of the most exciting photosensitizing compounds, possesses antidepressant, wound healing, and antiviral activities, is effective against enveloped viruses, inhibits the replication of encapsulated viruses, and the SARS-CoV-2-S protein:ACE2 complex [16,17].

In recent years, plant biotechnology has made a tremendous progress, and its techniques, if applied responsibly, have the potential to increase productivity of plants, control diseases and plant stresses, produce functional foods as diet supplements, and develop new effective pharmaceuticals. Plant cell cultures have been demonstrated to have potential for the synthesis of bioorganic compounds with unique structures [18]. In this respect, *Hypericum perforatum* L. has been explored for the production of bioactive phytometabolites using callus cultures. Various classes of phytohormones, such as salicylic acid, indole-3-acetic acid, cytokinins, and gibberellic acid, can improve the productivity of useful metabolites in plant cell cultures [19]. The main advantages of plant cell culture systems over conventional cultivation of whole plants are as follows: cultured cells are free from pathogens; plant pharmaceuticals can be obtained under controlled conditions; and growth of cells, from which these phytochemicals can be extracted, can be controlled in large-scale bioreactor systems [20]. The production of hypericin in plant cell suspension cultures is known to be biosustainable compared to that of field-grown plants [21].

Plant cell cultures could be useful to investigate the relationship between abiotic factors (phytohormones) and the antioxidant scavenging activity. Therefore, it is useful to integrate three plant biotechnology targets, i.e., application of phytohormones, initiation of growth and development of plant callus cultures, and regulation of metabolite processes to obtain useful organic substances in bioindustry. In the future, these strategies will enhance the value of plant cell cultures as phytomedicinal resources.

The aim of the present study was to explore whether the biological activity of callus culture extracts could be enhanced by supplementing *Hypericum perforatum* L. callus cultures with phytohormones compared to those of field-grown plants. Therefore, the influence of phytohormones (auxins, cytokinins, and salicylic acid) and the newly synthesized compound *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide on the formation of *Hypericum perforatum* L. callus cultures was assessed, the antioxidant and antibacterial activities of callus culture extracts were screened, and the quantities of phytochemicals in the analyzed extracts were determined. To the best of our knowledge, the influence of the newly synthesized compound on *Hypericum perforatum* L. callus cultures was explored for the first time. The present research has demonstrated the influence of phytohormones on three biological activities (antioxidative, antibacterial, and phytochemical) in *Hypericum perforatum* L. callus culture extracts. Here, we discuss the important challenges for understanding the role of plant hormones and the current strategies for improving plant callus characteristics in combination with antioxidant activity.

## 2. Materials and Methods

**Callus cultures of *Hypericum perforatum* L. formation**. Seeds of *Hypericum perforatum* L. were washed with 70% ethanol solution for 30 s, surface sterilized with 1.5% NaOCl for 15 min, rinsed 3 times in sterile deionized water, and cultured on Murashige and Skoog (MS) medium supplemented with 3% sucrose, phytohormones, newly synthesized compound, and solidified with 0.8% agar. The medium was adjusted to pH 5.7 before autoclaving (20 min at 120 °C). In vitro plants were grown in a growth chamber at 25 ± 1 °C under a photoperiod of 16 h light, with a relative humidity of 50–60%. Germinated 2-week-old seedlings of *Hypericum perforatum* L. plant in vitro were used as explants to obtain the callus cultures in vitro [22].

**Effects of phytohormones on *Hypericum perforatum* L. callus cultures**. The research was designed to explore the influence of these phytohormones: cytokinins—6-benzylaminopurine (BAP) and 6-furfurylaminopurine (kinetin); auxins—2,4-dichlorophenoxyacetic acid and 1-naphylacetic acid (NAA); salicylic acid, and the newly synthesized compound—*N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide [21] on *Hypericum perforatum* L. callus cultures). Callus photos of *Hypericum perforatum* L. are presented in Supplementary Table S1. The antioxidant activity of *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide screened by the DPPH radical scavenging assay was tested in previous experiments and was 1.4 times higher than that of a commercial antioxidant ascorbic acid [23].

**DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay**. The dried and finely ground *Hypericum perforatum* L. biomass (0.1 g) was extracted in methanol (99%, 1 mL) at 25 °C for 0.5 h. It was then centrifuged for 10 min at 9000× *g* (Hettich Universal 320 Randreas Hettich GmbH & Co., Tuttlingen, Germany). A solution of DPPH (1 µM) was prepared in methanol (99%, 50 mL). Test samples were prepared by mixing 77 µL of *Hypericum perforatum* extract and 3 mL of prepared DPPH solution. The analyzed samples were incubated at room temperature in the dark for 15 min. The absorbance of the samples was recorded at 517 nm using a Schimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) [24].

**Ferric ion reducing antioxidant power (FRAP) assay.** The dried and finely ground *Hypericum perforatum* L. biomass (0.5 g) was extracted in methanol (99%, 5 mL) at 45 °C for 0.5 h. Then, it was centrifuged for 10 min at 9000× *g* (Hettich Universal 320Randreas Hettich GmbH & Co., Tuttlingen, Germany). The FRAP reagent was prepared by mixing 300 mM acetate buffer, 10 mM TPTZ solution, and 20 mM $FeCl_2$ solution. Then, the FRAP reagent was mixed with 20 µL of methanol extract for 5 min before measuring the absorbance of the solution at 593 nm using a Schimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The antioxidant activity according to the FRAP method was determined based on the $FeSO_4 \cdot 7H_2O$ calibration curve [25].

**Measurement of the concentration of total phenolics using the Folin–Ciocâlteu method**. The total phenolics content was determined using the Folin–Ciocâlteu method [26]. Dried and finely ground *Hypericum perforatum* L. plant samples (0.05 g) were placed in 10 mL of aqueous acetone (70%). The samples were transferred to the centrifuge tubes and centrifuged for 10 min at approximately 9000× *g* at 4 °C. The supernatant was collected and kept on ice. The extract samples were mixed with 250 µL of 1 M Folin–Ciocâlteu reagent for 5 min and then 1.25 mL of 75 g $L^{-1}$ aqueous sodium carbonate solution was added. After incubation in darkness for 40 min, the absorbance of the reaction mixture was measured at 725 nm using a Schimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The concentration of total phenolics (mg/100 g) was estimated according to the calibration curve of tannic acid.

**Measurement of the activity of the antioxidant enzyme superoxide dismutase (SOD).** Superoxide dismutase activity was determined by measuring the inhibition in photoreduction of nitroblue tetrazolium (NBT) by the SOD enzyme [27]. The dried and finely ground *Hypericum perforatum* L. biomass (0.1 g) was extracted in 4 mL of 50 mM sodium phosphate buffer (pH 7.6), containing 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl

fluoride, and 1–3 mg polivinylpyrrolidone at 25 °C for 0.5 h. The samples were then centrifuged for 10 min at 9000× *g* (Hettich Universal 320 Randreas Hettich GmbH & Co., Tuttlingen, Germany). The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.6), 0.1 mM EDTA, 50 mM sodium carbonate, 12 mM L-methionine, 50 µM NBT, 10 µM riboflavin, and 100 µL of crudeenzyme extract in a final volume of 3.0 mL. A control reaction was performed without the crude extract. The SOD reaction was carried out by exposing the reaction mixture to white light at room temperature for 30 min. After 30 min of incubation, absorbance was recorded at 560 nm using a Schimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) [27].

**Catalase (CAT) activity assay.** CAT activity was measured spectrophotometrically at room temperature by monitoring the decrease in absorbance at 240 nm resulting from the decomposition of $H_2O_2$. The dried and finely ground *Hypericum perforatum* L. biomass (0.1 g) was extracted in 4 mL of 50 mM sodium phosphate buffer (pH 7.8), containing 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 1–3 mg of polivinylpyrrolidone at 25 °C for 0.5 h. Then, samples were centrifuged for 10 min at 9000× *g* (Hettich Universal 320 Randreas Hettich GmbH & Co., Tuttlingen, Germany). Subsequently, the CAT activity was measured according to the method of Aebi et al. [28].

**Ascorbate peroxidase (APX) activity assay**. The dried and finely ground *Hypericum perforatum* L. biomass (0.1 g) was extracted in 4 mL of cold 0.1 M sodium phosphate buffer (pH 7), containing 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride at 25 °C for 0.5 h. The samples were then centrifuged for 10 min at 9000× *g* (Hettich Universal Hettich GmbH & Co., Tuttlingen, Germany). Afterwards, the reaction mixture for the peroxidase contained 50 mM potassium phosphate, 0.5 mM ascorbate (pH 7.0), 0.1 mM $H_2O_2$, and 0.1 mM EDTA in a total volume of 3 mL. The reaction was started by adding the enzyme and $H_2O_2$, and the absorbance decrease was recorded at 290 nm after 3 min using a Schimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) [29].

**Measurement of carotenoids concentration.** The dried and finely ground *Hypericum perforatum* L. biomass (1 g) was extracted in 10 mL of 96.3 % ethanol for 0.5 h. The extract was centrifuged at 9000× *g* for 10 min (Hettich Universal 320 Randreas Hettich GmbH & Co., Tuttlingen, Germany. The absorbance at 441 nm was determined by a Schimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) [30].

**Measurement of the antibacterial activity of plant extracts.** First, 1 g of *Hypericum perforatum* L. plant material was mixed with 10 mL of DMSO and kept at 25 °C for 24 h. The suspension was centrifuged, and the antibacterial activity of supernatant was tested. Two bacterial species *Escherichia coli* and *Bacillus subtilis* were employed as test organisms [31]. The bacteria were kept in Luria–Bertani (LB) medium. Inoculum was prepared by adding an overnight culture of bacteria in LB medium to obtain an $OD_{600}$ = 0.1. The cells were allowed to grow to approximately $10^8$ × CFU/mL. The suspension was then diluted 1:100 in LB medium to obtain $10^6$ CFU/mL. Sterile discs were soaked separately with 25 µL of each of the plant extracts prepared in DMSO. These discs were placed on LB agar plates, previously swabbed with the bacterial culture at a concentration of $10^6$ CFU/mL. The tested samples were incubated at 37 °C for 24 h. Antibacterial activity was defined as the diameter (cm) of the clear inhibitory zone formed around the discs [31]. Ciprofloxacin was used as a control antibiotic.

**Statistical analysis.** The results from three experiments were expressed as means ± SD. Statistically significant comparisons between callus cultures of *Hypericum perforatum* L. and field growing plant extract experiments were tested with unpaired two-tailed *t*-test, with a significance level set at $p < 0.01$. GraphPad Prism version 8.0.2 for Windows (San Diego, California USA) was used for statistical analysis.

## 3. Results

**Antioxidative analysis of *Hypericum perforatum* L. plant extracts by using DPPH radical scavenging assay**. The current research was designed to assess the antioxidative activity according to DPPH bioassay in methanolic extracts of *Hypericum perforatum* L.

(Experiments No. 1–10). This method is based on the antioxidant measurement of the scavenging capacity towards the stable DPPH radical [24].

The results of the investigation revealed (Figure 1) that the highest antioxidative activity (89.37%) according to the DPPH method was obtained in *Hypericum perforatum* L. callus cultures induced on MS medium containing auxin 2,4-D (1.0 mg $L^{-1}$), cytokinin BAP (0.5 mg $L^{-1}$), and auxin NAA (0.1 mg $L^{-1}$) (Experiment No. 3) ($p < 0.01$), in comparison to the extract of the field growing plants. Additionally, the high antioxidant activity (87.1%) according to the DPPH method was determined for the callus cultures grown on MS medium supplemented with BAP (0.5 mg $L^{-1}$) and *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide (0.5 mg $L^{-1}$) (Experiment No. 7). In summary, it could be concluded that callus cultures of *Hypericum perforatum* L. were formed successfully and antioxidant activity according to DPPH method was increased by adding cytokinin and the newly synthesized compound to the growth medium. The probable mechanism for the DPPH radical scavenging activity of *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide is presented in Scheme 1.

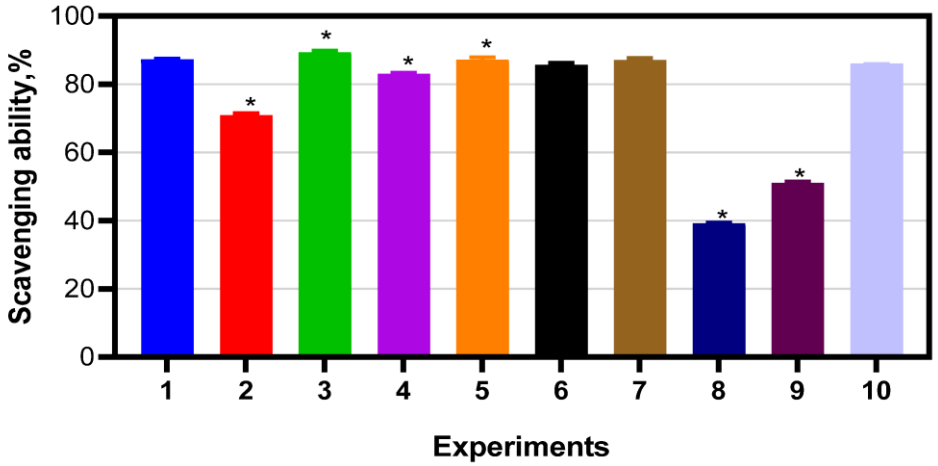
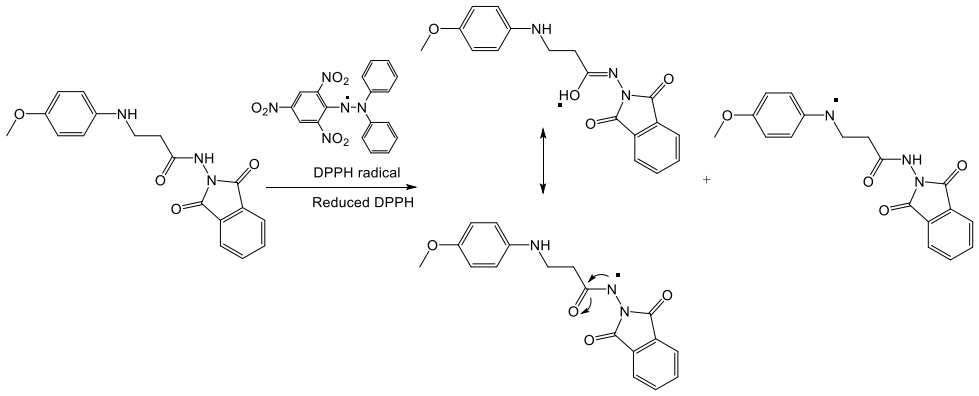



**Figure 1.** Antioxidative activity according to the DPPH method in *Hypericum perforatum* L. extracts. 1—Regenerated plants in MS medium in vitro; 2—MS medium with BAP (0.5 mg $L^{-1}$) and NAA (2.5 mg $L^{-1}$); 3—MS medium with 2,4-D (1.0 mg $L^{-1}$), BAP (0.5 mg $L^{-1}$), and NAA (0.1 mg $L^{-1}$); 4—MS medium with BAP (0.5 mg $L^{-1}$), NAA (2.5 mg $L^{-1}$), and salicylic (50 μM); 5—MS medium with BAP (0.5 mg $L^{-1}$), NAA (2.5 mg $L^{-1}$), and salicylic acid (100 μM); 6—MS medium and BAP (0.5 mg $L^{-1}$); 7—MS medium with BAP (0.5 mg $L^{-1}$) and *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide (0.5 mg $L^{-1}$); 8—MS medium with 2,4-D (0.9 μM) and kinetin (0.11 μM); 9—MS medium with 2,4-D (0.9 μM), kinetin (0.11 μM), and *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide (0.5 mg $L^{-1}$); 10—Field growing plants of *Hypericum perforatum* L. The error bars show the mean ± SD from three experiments. Asterisks show statistically significant comparisons between callus cultures of *Hypericum perforatum* L. and field growing plant extract experiments, which were tested with unpaired two-tailed *t*-test, with a significance level set at $p < 0.01$.

The lowest antioxidant activity (39.18%) of the extracts was in *Hypericum perforatum* callus cultures, which were formed on MS medium containing 2,4-D (0.9 μM) and (0.11 μM) kinetin ($p < 0.01$) (Experiment No. 8).

**Evaluation of ferric ion reducing antioxidant power (FRAP)**. The effect of phytohormones in MS medium on reducing antioxidant activity was examined using plant callus cultures. Plant hormones are the critical media components not only in determining the developmental pathway of plant cells, but also, they have a crucial impact on the biological activity.




**Scheme 1.** The probable mechanism for the DPPH radical scavenging activity of *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide.

As seen from the results presented in Figure 2, the highest reducing property (96.52 µmol/L) according to FRAP bioassay was found in *Hypericum perforatum* L. callus cultures induced on MS medium supplemented with BAP (0.5 mg $L^{-1}$), NAA (2.5 mg $L^{-1}$), and salicylic acid (100 µM) ($p < 0.01$) (Experiment No. 5). The antioxidant activity of the extracts was estimated to be three times higher compared to callus cultures in vitro grown on MS medium containing BAP (0.5 mg $L^{-1}$), NAA (2.5 mg $L^{-1}$), and salicylic acid (50 µM) ($p < 0.01$). Furthermore, the high antioxidant activity (56.79 µmol/l) was in plant callus cultures in vitro formed on MS medium supplemented with BAP (0.5 mg $L^{-1}$) and *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide (0.5 mg $L^{-1}$) ($p < 0.01$) (Experiment No. 7). Our studies have shown that the antioxidant activity of the extract increased by 3.6% when salicylic acid concentration in MS medium was two times higher (from 50 µM to 100 µM) ($p < 0.01$).

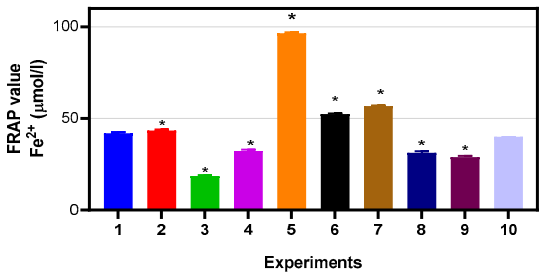



**Figure 2.** Antioxidative activity according to FRAP in *Hypericum perforatum* L. extracts. 1—Regenerated plants in MS medium in vitro; 2—MS medium with BAP (0.5 mg $L^{-1}$) and NAA (2.5 mg $L^{-1}$); 3—MS medium with 2,4-D (1.0 mg $L^{-1}$), BAP (0.5 mg $L^{-1}$), and NAA (0.1 mg $L^{-1}$); 4—MS medium with BAP (0.5 mg $L^{-1}$), NAA (2.5 mg $L^{-1}$), and salicylic (50 µM); 5—MS medium with BAP (0.5 mg $L^{-1}$), NAA (2.5 mg $L^{-1}$), and salicylic acid (100 µM); 6—MS medium and BAP (0.5 mg $L^{-1}$); 7—MS medium with BAP (0.5 mg $L^{-1}$) and *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide (0.5 mg $L^{-1}$); 8—MS medium with 2,4-D (0.9 µM) and kinetin (0.11 µM); 9—MS medium with 2,4-D (0.9 µM), kinetin (0.11 µM), and *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide (0.5 mg $L^{-1}$); 10—Field growing plants of *Hypericum perforatum* L. The error bars show the mean ± SD from three experiments. Asterisks show statistically significant comparisons between callus cultures of *Hypericum perforatum* L. and field growing plant extract experiments, which were tested with unpaired two-tailed *t*-test, with a significance level set at $p < 0.01$.

**Estimation of the total concentration of phenolics.** Phenolic compounds possess a wide variety of biological and pharmacological activities, and they have been attracting increasing attention because of their therapeutic activities. In this study, the quantity of total phenolics was evaluated.

The results demonstrated (Figure 3) that the highest concentration (3.63 mg/100 g) of total phenolics was found in the extracts of *Hypericum perforatum* L. callus cultures formed on MS medium containing BAP (0.5 mg $L^{-1}$), NAA (2.5 mg $L^{-1}$), and salicylic acid (100 µM) ($p < 0.01$) (Experiment No. 5).

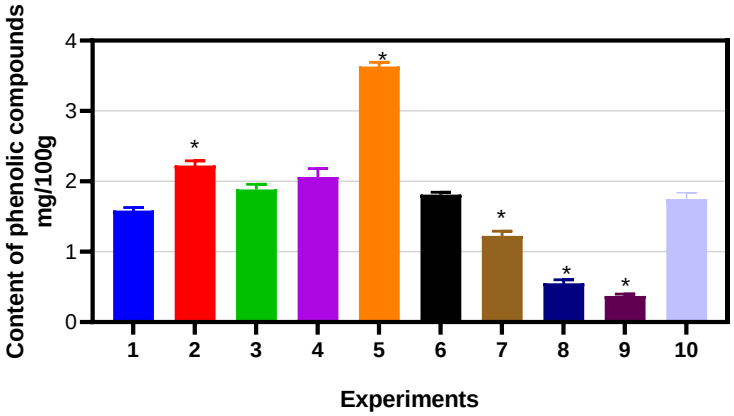



**Figure 3.** The concentration of total phenolics in the extracts of *Hypericum perforatum* L. 1—Regenerated plants in MS medium in vitro; 2—MS medium with BAP (0.5 mg $L^{-1}$) and NAA (2.5 mg $L^{-1}$); 3—MS medium with 2,4-D (1.0 mg $L^{-1}$), BAP (0.5 mg $L^{-1}$), and NAA (0.1 mg $L^{-1}$); 4—MS medium with BAP (0.5 mg $L^{-1}$), NAA (2.5 mg $L^{-1}$), and salicylic (50 µM); 5—MS medium with BAP (0.5 mg $L^{-1}$), NAA (2.5 mg $L^{-1}$), and salicylic acid (100 µM); 6—MS medium and BAP (0.5 mg $L^{-1}$); 7—MS medium with BAP (0.5 mg $L^{-1}$) and *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide (0.5 mg $L^{-1}$); 8—MS medium with 2,4-D (0.9 µM) and kinetin (0.11 µM); 9—MS medium with 2,4-D (0.9 µM), kinetin (0.11 µM), and *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide (0.5 mg $L^{-1}$); 10—Field growing plants of *Hypericum perforatum* L. The error bars show the mean ± SD from three experiments. Asterisks show statistically significant comparisons between callus cultures of *Hypericum perforatum* L. and field growing plant extract experiments, which were tested with unpaired two-tailed *t*-test, with a significance level set at $p < 0.01$.

This amount of total phenolics is 1.76 times higher than that determined in *Hypericum perforatum* callus cultures formed on MS medium supplemented with BAP (0.5 mg $L^{-1}$) and NAA (0.5 mg $L^{-1}$) ($p < 0.01$) (Experiment No. 2), thus indicating the crucial role of salicylic acid. Salicylic acid is known as a signaling molecule and has been used for increased accumulation of phytochemicals in plant cell cultures [32]. Therefore, it can be concluded that salicylic acid stimulates the formation of secondary metabolites only at higher concentrations.

**Evaluation of superoxide dismutase (SOD) activity.** The superoxide dismutase enzyme converts the superoxide anion to $H_2O_2$ and molecular oxygen. These enzymes are metalloenzymes found in plant cells as isoenzymes of Cu/Zn-SOD, Mn-SOD, and Fe-SOD [33].

Our studies (Figure 4) demonstrated that the highest activity (137.28 U/mg protein) of superoxide dismutase was in *Hypericum perforatum* L. callus cultures grown in vitro on MS medium (Experiment No. 1), while the lowest activity of superoxide dismutase (7.64 U/mg protein) was in callus culture extracts grown in MS medium supplemented with 2,4-D (0.9 µM) and kinetin (0.11 µM) (Experiment No. 8) ($p < 0.01$). The in vitro extracts of *Hypericum perforatum* L. callus cultures induced on MS medium with 2.4-D (1.0 mg $L^{-1}$), BAP (0.5 mg $L^{-1}$), and NAA (0.1 mg $L^{-1}$) had the highest activity (76.36 U/mg protein) compared to the extracts of the callus cultures in vitro grown on culture medium supplemented with salicylic acid (Experiment No. 3) ($p < 0.01$). The activity of SOD decreased when a higher concentration of salicylic acid was used.

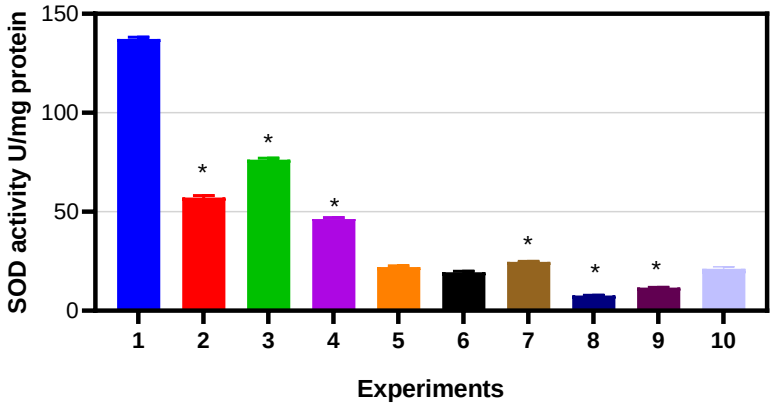



**Figure 4.** Superoxide dismutase activity in *Hypericum perforatum* L. extracts. 1—Regenerated plants in MS medium in vitro; 2—MS medium with BAP (0.5 mg $L^{-1}$) and NAA (2.5 mg $L^{-1}$); 3—MS medium with 2,4-D (1.0 mg $L^{-1}$), BAP (0.5 mg $L^{-1}$), and NAA (0.1 mg $L^{-1}$); 4—MS medium with BAP (0.5 mg $L^{-1}$), NAA (2.5 mg $L^{-1}$), and salicylic (50 µM); 5—MS medium with BAP (0.5 mg $L^{-1}$), NAA (2.5 mg $L^{-1}$), and salicylic acid (100 µM); 6—MS medium and BAP (0.5 mg $L^{-1}$); 7—MS medium with BAP (0.5 mg $L^{-1}$) and *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide (0.5 mg $L^{-1}$); 8—MS medium with 2,4-D (0.9 µM) and kinetin (0.11 µM); 9—MS medium with 2,4-D (0.9 µM), kinetin (0.11 µM), and *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide (0.5 mg $L^{-1}$); 10—Field growing plants of *Hypericum perforatum* L. The error bars show the mean ± SD from three experiments. Asterisks show statistically significant comparisons between callus cultures of *Hypericum perforatum* L. and field growing plant extract experiments, which were tested with unpaired two-tailed *t*-test, with a significance level set at $p < 0.01$.

**Ascorbate peroxidase (APX) activity in extracts of *Hypericum perforatum* L.** Enzymatic antioxidants, such as ascorbate peroxidase, catalase, glutathione peroxidase, peroxiredoxins, and thioredoxins, are also present in plants [34,35].

As seen from the results presented in Figure 5, the highest activity of ascorbate peroxidase (0.33 mmol/mg protein) was observed in extract of *Hypericum perforatum* L. callus cultures formed on MS medium supplemented with BAP (0.5 mg $L^{-1}$) and *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide (0.5 mg $L^{-1}$) (Experiment No. 7). The lowest activity (0.06 mmol/mg protein) of ascorbate peroxidase was observed in the extract of the regenerated plants in MS medium in vitro (Experiment No. 1).

**Evaluation of catalase activity in analyzed *Hypericum perforatum* L. extracts.** Catalase enzyme converts $H_2O_2$ into water and molecular oxygen in peroxisomes and cytoplasm. It is known that inhibition of catalase with salicylic acid plays an important role in estimating the stress response [33].

The results of the study (Figure 6) confirmed that salicylic acid inhibits the activity of the enzyme catalase. Salicylic acid increases endogenous $H_2O_2$ levels associated with hypersensitivity or photosynthesis and oxidative phosphorylation. The highest catalase activity (7.98 U/mg protein) was determined in *Hypericum perforatum* L. plants, which were grown in MS medium, because the enzyme was not affected by external factors. The high antioxidative activity (6.37 U/mg protein) of catalase was determined in *Hypericum perforatum* L. callus cultures formed on MS medium supplemented with BAP (0.5 mg $L^{-1}$) and *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide compound (0.5 mg $L^{-1}$) ($p < 0.01$) (Experiment No. 7). The extract of *Hypericum perforatum* L. obtained from field-grown plants, as in the case of the SOD study, had the lowest enzyme activity due to external abiotic factors.

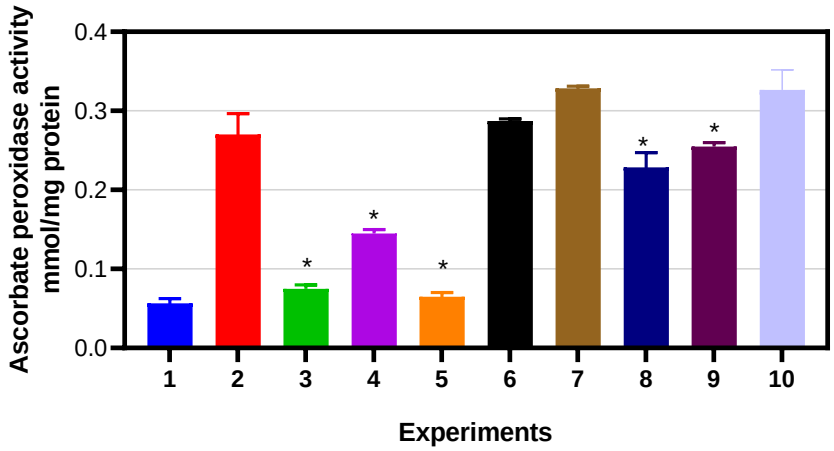



**Figure 5.** Ascorbate peroxidase activity in *Hypericum perforatum* L. extracts. 1—Regenerated plants in MS medium in vitro; 2—MS medium with BAP (0.5 mg $L^{-1}$) and NAA (2.5 mg $L^{-1}$); 3—MS medium with 2,4-D (1.0 mg $L^{-1}$), BAP (0.5 mg $L^{-1}$), and NAA (0.1 mg $L^{-1}$); 4—MS medium with BAP (0.5 mg $L^{-1}$), NAA (2.5 mg $L^{-1}$), and salicylic (50 µM); 5—MS medium with BAP (0.5 mg $L^{-1}$), NAA (2.5 mg $L^{-1}$), and salicylic acid (100 µM); 6—MS medium with BAP (0.5 mg $L^{-1}$); 7—MS medium with BAP (0.5 mg $L^{-1}$) and *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide (0.5 mg $L^{-1}$); 8—MS medium with 2,4-D (0.9 µM) and kinetin (0.11 µM); 9—MS medium with 2,4-D (0.9 µM), kinetin (0.11 µM), and *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide (0.5 mg $L^{-1}$); 10—Field growing plants of *Hypericum perforatum* L. The error bars show the mean ± SD from three experiments. Asterisks show statistically significant comparisons between callus cultures of *Hypericum perforatum* L. and field growing plant extract experiments, which were tested with unpaired two-tailed *t*-test, with a significance level set at $p < 0.01$.

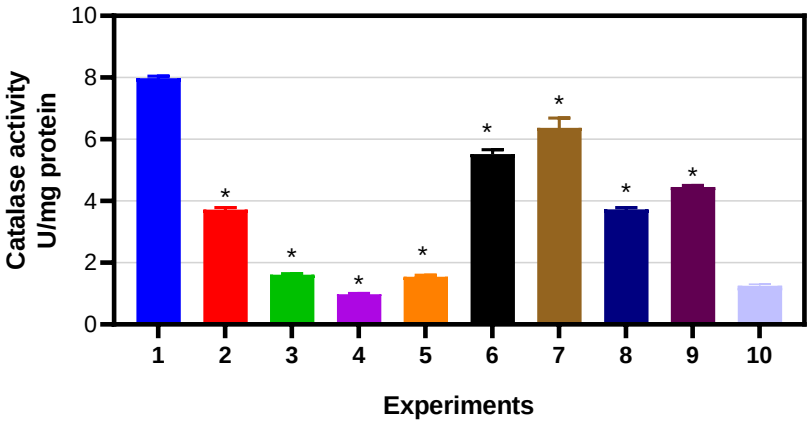



**Figure 6.** Catalase activity in *Hypericum perforatum* L. extracts. 1—Regenerated plants in MS medium in vitro; 2—MS medium with BAP (0.5 mg $L^{-1}$) and NAA (2.5 mg $L^{-1}$); 3—MS medium with 2,4-D (1.0 mg $L^{-1}$), BAP (0.5 mg $L^{-1}$), and NAA (0.1 mg$L^{-1}$); 4—MS medium with BAP (0.5 mg $L^{-1}$), NAA (2.5 mg $L^{-1}$), and salicylic (50 µM); 5—MS medium with BAP (0.5 mg $L^{-1}$), NAA (2.5 mg $L^{-1}$), and salicylic acid (100 µM); 6—MS medium with BAP (0.5 mg $L^{-1}$); 7—MS medium with BAP (0.5 mg $L^{-1}$) and *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide (0.5 mg $L^{-1}$); 8—MS medium with 2,4-D (0.9 µM) and kinetin (0.11 µM); 9—MS medium with 2,4-D (0.9 µM), kinetin (0.11 µM), and *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide (0.5 mg $L^{-1}$); 10—Field growing plants of *Hypericum perforatum* L. The error bars show the mean ± SD from three experiments. Asterisks show statistically significant comparisons between callus cultures of *Hypericum perforatum* L. and field growing plant extract experiments, which were tested with unpaired two-tailed *t*-test, with a significance level set at $p < 0.01$.

**Measurement of carotenoids concentration in *Hypericum perforatum* L. plant extracts.** Carotenoids represent the most common group of isoprenoid derivatives biosynthesized by plants. They are potential antioxidants as they play an essential role in protecting the photosystems by detoxifying ROS and are also effective during plant stress [32].

The study results (Figure 7) demonstrated that the highest concentrations (21.97 mg/100 g) of bioactive carotenoids were in the extracts of *Hypericum perforatum* L. plants grown in MS medium and in the callus cultures (21.03 mg/100 g) induced on MS medium supplemented with BAP (0.5 mg $L^{-1}$) and NAA (2.5 mg $L^{-1}$) ($p < 0.01$) (Experiment No. 2). The addition of salicylic acid (100 µM) to MS medium with BAP (0.5 mg $L^{-1}$) and NAA (2.5 mg $L^{-1}$) increased the concentration of carotenoids 1.12 times ($p < 0.01$) in comparison with experiment No. 4 when the salicylic acid concentration was 50 µM.

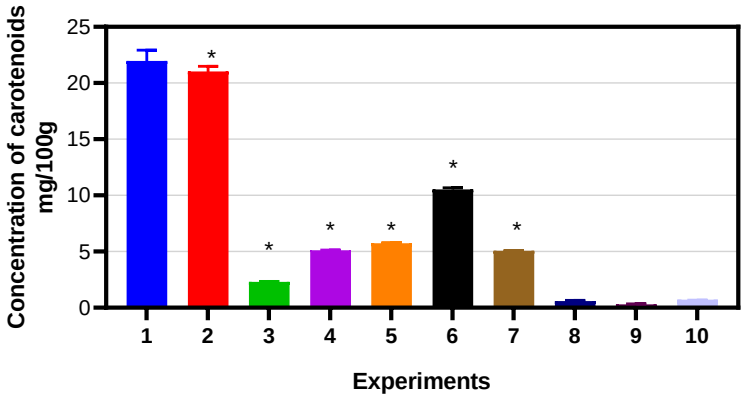



**Figure 7.** Measurement of carotenoids concentration in *Hypericum perforatum* L. extracts. 1—Regenerated plants in MS medium in vitro; 2—MS medium with BAP (0.5 mg $L^{-1}$) and NAA (2.5 mg $L^{-1}$); 3—MS medium with 2,4-D (1.0 mg $L^{-1}$), BAP (0.5 mg $L^{-1}$), and NAA (0.1 mg $L^{-1}$); 4—MS medium with BAP (0.5 mg $L^{-1}$), NAA (2.5 mg $L^{-1}$), and salicylic (50 µM); 5—MS medium with BAP (0.5 mg $L^{-1}$), NAA (2.5 mg $L^{-1}$), and salicylic acid (100 µM); 6—MS medium and BAP (0.5 mg $L^{-1}$); 7—MS medium with BAP (0.5 mg $L^{-1}$) and *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide (0.5 mg $L^{-1}$); 8—MS medium with 2,4-D (0.9 µM) and kinetin (0.11 µM); 9—MS medium with 2,4-D (0.9 µM), kinetin (0.11 µM), and *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide (0.5 mg $L^{-1}$); 10—Field growing plants of *Hypericum perforatum* L. The error bars show the mean ± SD from three experiments. Asterisks show statistically significant comparisons between callus cultures of *Hypericum perforatum* L. and field growing plant extract experiments, which were tested with unpaired two-tailed *t*-test, with a significance level set at $p < 0.01$.

**Determination of the antibacterial activity of the *Hypericum perforatum* L. plant extracts.** The extract of field growing plants *Hypericum perforatum* L. (Experiment No. 10) has the highest antibacterial activity against *E. coli* and *B. subtilis* bacteria (Figures 8 and 9).

*Hypericum perforatum* L. callus cultures formed on MS medium supplemented with BAP (0.5 mg $L^{-1}$), NAA (2.5 mg $L^{-1}$), and salicylic acid (50–100 µM) ($p < 0.01$), also demonstrated high antibacterial activity against *E. coli* and *B. subtilis* bacteria. Increasing the concentration of salicylic acid in MS medium led to more effective antibacterial properties against *E. coli*. Antibacterial activity can be related to the high content of total phenolics as defensive compounds. Callus cultures of *Hypericum perforatum* L. grown on MS medium containing 2,4-D (0.9 µM) and kinetin (0.11 µM) were the least active against *B. subtilis*. Callus cultures formed on MS medium containing 2,4-D (0.9 µM), kinetin (0.11 µM), and *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide (0.5 mg $L^{-1}$) (Experiment No. 9) demonstrated the lowest antibacterial properties against *E. coli*.

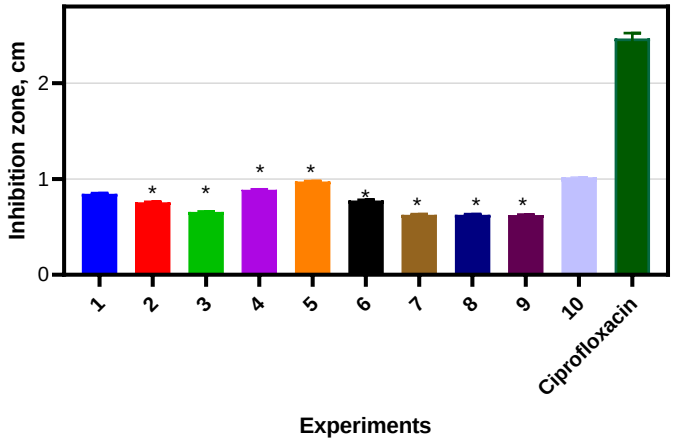



**Figure 8.** Determination of antibacterial activity in *Hypericum perforatum* L. extracts against *E. coli.* 1—Regenerated plants in MS medium in vitro; 2—MS medium with BAP (0.5 mg $L^{-1}$) and NAA (2.5 mg $L^{-1}$); 3—MS medium with 2,4-D (1.0 mg $L^{-1}$), BAP (0.5 mg $L^{-1}$), and NAA (0.1 mg $L^{-1}$); 4—MS medium with BAP (0.5 mg $L^{-1}$), NAA (2.5 mg $L^{-1}$), and salicylic (50 µM); 5—MS medium with BAP (0.5 mg $L^{-1}$), NAA (2.5 mg $L^{-1}$), and salicylic acid (100 µM); 6—MS medium and BAP (0.5 mg $L^{-1}$); 7—MS medium with BAP (0.5 mg $L^{-1}$) and *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide (0.5 mg $L^{-1}$); 8—MS medium with 2,4-D (0.9 µM) and kinetin (0.11 µM); 9—MS medium with 2,4-D (0.9 µM), kinetin (0.11 µM), and *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide (0.5 mg $L^{-1}$); 10—Field growing plants of *Hypericum perforatum* L. The error bars show the mean ± SD from three experiments. Asterisks show statistically significant comparisons between callus cultures of *Hypericum perforatum* L. and field growing plant extract experiments, which were tested with unpaired two-tailed *t*-test, with a significance level set at $p < 0.01$.

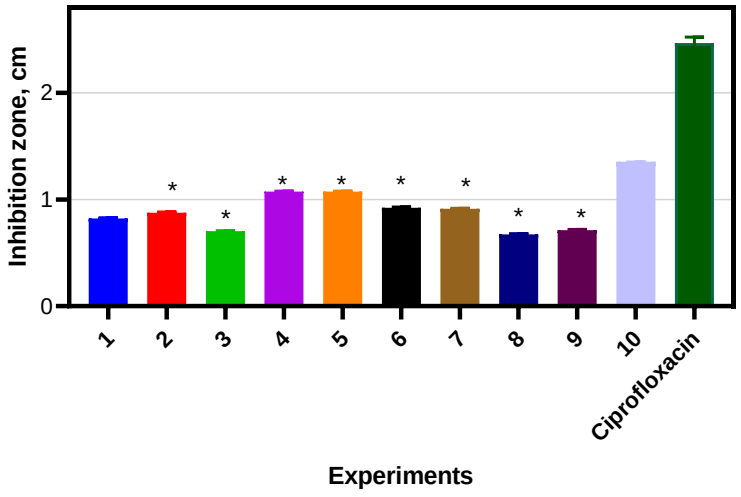



**Figure 9.** Determination of antibacterial activity in *Hypericum perforatum* L. extracts against *B. subtilis.* 1—Regenerated plants in MS medium in vitro; 2—MS medium with BAP (0.5 mg $L^{-1}$) and NAA (2.5 mg $L^{-1}$); 3—MS medium with 2,4-D (1.0 mg $L^{-1}$), BAP (0.5 mg $L^{-1}$), and NAA (0.1 mg $L^{-1}$); 4—MS medium with BAP (0.5 mg $L^{-1}$), NAA (2.5 mg $L^{-1}$), and salicylic (50 µM); 5—MS medium with BAP (0.5 mg $L^{-1}$), NAA (2.5 mg $L^{-1}$), and salicylic acid (100 µM); 6—MS medium and BAP (0.5 mg $L^{-1}$); 7—MS medium with BAP (0.5 mg $L^{-1}$) and *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide (0.5 mg $L^{-1}$); 8—MS medium with 2,4-D (0.9 µM) and kinetin (0.11 µM); 9—MS medium with 2,4-D (0.9 µM), kinetin (0.11 µM), and *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide (0.5 mg $L^{-1}$); 10—Field growing plants of *Hypericum perforatum* L. The error bars show the mean ± SD from three experiments. Asterisks show statistically significant comparisons between callus cultures of *Hypericum perforatum* L. and field growing plant extract experiments, which were tested with unpaired two-tailed *t*-test, with a significance level set at $p < 0.01$.

Finally, the results of the study demonstrated that the antibacterial activity of *Hypericum perforatum* L. plant extracts was higher against *B. subtilis* compared to *E. coli*. Therefore, it can be assumed that *Hypericum perforatum* L. extracts are more active against Gram-positive than Gram-negative bacteria.

## 4. Discussion

The findings of this study have clearly shown that phytohormones are significant signaling molecules and the critical media components in the development of plant callus cultures and the production of valuable bioactive antioxidants. In this study, we examined the relationship between endogenous phytohormones and the antioxidant, antibacterial activity of callus cultures. To achieve this, we induced callus cultures of *Hypericum perforatum* L. on Murashige and Skoog medium with various classes of phytohormones and their concentrations. According to the reviewed literature [19], plant hormones (auxins, cytokinins, and salicylic acid) can regulate and enhance the bioactivity of natural antioxidant defense systems. Our investigation has revealed that the highest antioxidative activity evaluated by the DPPH radical scavenging method was in *Hypericum perforatum* L. callus cultures, which were formed on MS medium containing auxin 2,4-D (1.0 mg $L^{-1}$), cytokinin BAP (0.5 mg $L^{-1}$), and auxin NAA (0.1 mg $L^{-1}$). High antioxidant activity according to the DPPH and FRAP assays, was also shown by plant callus cultures, which were grown on MS medium supplemented with BAP (0.5 mg $L^{-1}$) and *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide (0.5 mg $L^{-1}$). Therefore, it can also be concluded that *Hypericum perforatum* L. callus cultures were effectively formed and antioxidant activity was increased by supplementing MS medium with cytokinin and the newly synthesized compound.

It Is worth mentioning that the antioxidant activity of *Hypericum perforatum* L. extract was increased by 3.6% when the concentration of salicylic acid in MS medium was higher (from 50 μM to 100 μM) as determined by the FRAP assay. The investigation has also revealed that the highest amount of total phenolic compounds was found in *Hypericum perforatum* L. callus cultures grown on MS medium supplemented with BAP (0.5 mg $L^{-1}$), NAA (2.5 mg $L^{-1}$), and salicylic acid (100 μM). Additionally, the antibacterial activity in *Hypericum perforatum* L. callus cultures grown on MS medium containing BAP (0.5 mg $L^{-1}$), NAA (2.5 mg $L^{-1}$), and salicylic (50–100 μM) enhanced activity compared to with media without salicylic acid additive. These findings support the conclusion that salicylic acid can regulate antioxidant activity and biosynthesis of defensive compounds [19].

It can be concluded that the highest and enhanced ascorbate peroxidase activity was observed in *Hypericum perforatum* L. callus cultures initiated on MS medium with added cytokinin BAP (0.5 mg $L^{-1}$) and *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide (0.5 mg $L^{-1}$).

In the future, additional studies are needed to analyze the molecular–genetic mechanism between phytohormone modulation and medicinal plant cell cultures in vitro.

## 5. Conclusions

Knowledge about the influence of phytohormones on the antioxidant, antibacterial, and phytochemical activities in *Hypericum perforatum* L. callus cultures opens possibilities for the future research of new antioxidant metabolism pathways. For the first time, the bioactive compound *N*-(1,3-dioxoisoindolin-2-yl)-3-((4-methoxyphenyl)amino)propanamide along with cytokinin BAP (0.5 mg $L^{-1}$) added to MS medium has been shown to exhibit enhanced antioxidative (according to DPPH and FRAP assays) activity as well as stimulate the activity of enzyme antioxidants (catalase and ascorbate peroxidase).

In conclusion, the present study confirmed that salicylic acid present at higher concentration (100 μM) in the nutrient medium supplemented with different phytohormones increased the accumulation of bioactive secondary metabolites (phenolic compounds) and showed antibacterial activity in extracts of *Hypericum perforatum* L. callus cultures.

The present work provides new findings on the influence of phytohormones on the growth of callus cultures in vitro and the biological activity of the extracts analyzed. Our results show a significant interaction between the importance of plant hormones and phytochemical constituents in extracts of *Hypericum perforatum* L. The results obtained during this study show a positive influence of auxins, cytokinins, and salicylic acid on the antioxidant efficiency. Furthermore, it has been shown that the application of phytohormones in plant callus cultures can activate genes involved in different metabolic pathways.

The results obtained could be helpful to reveal new sources of antioxidant and antibacterial phytochemicals and proving that modulation with signaling molecules can be an attractive approach for the potential biotechnological application of suspension cultures of *Hypericum perforatum* L. callus or other medicinal plants. Further, plant cell and tissue culture techniques can be used to scale up the bioprocess on an industrial level of phytocompound production with potential for commercial applications.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13081543/s1, Table S1: Callus photos of *Hypericum perforatum* L.

**Author Contributions:** Conceptualization, I.J., I.T., K.K. and R.S.; methodology, I.J., I.T., K.K. and R.S.; formal analysis, I.J., I.T., R.S. and K.K.; investigation, I.J., R.S. and I.T.; resources, I.T. and I.J.; writing—original draft preparation, I.J., I.T., R.S. and K.K.; writing—review and editing, I.J., I.T. and K.K.; visualization, I.J., I.T. and K.K. All authors have read and agreed to the published version of the manuscript.

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