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The Content of Biologically Active Substances in *Crocosmia* × *crocosmiiflora* 'Lucifer' Tubers after Treatment with GA_3

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Abstract: This study was conducted to assess the influence of gibberellic acid (GA_3) on the content of biologically active substances in *Crocosmia* × *crocosmiiflora* 'Lucifer' tubers. These tubers are a promising source of potential antioxidants, and their extracts can be used in pharmaceutical industry, as well as in cosmetics products and antifungal and antibacterial substances. Four groups of biologically active substances were determined from tubers: saponins, phenolic acid, flavonoids and carotenoids. The antioxidant activity of the extracts from tubers increased proportionally to the GA_3 concentrations. GA_3 at concentrations of 200, 400 and 600 mg dm^{-3} increased the content of medicagenic acid by 42.9–57.1% and the content of polygalic acid by 50% without affecting the content of medicagenic acid 3-*O*-triglucoside. The GA_3 concentrations used in the experiment positively influenced the accumulation of caffeic acid, *p*-coumaric acid and gallic acid. The highest content of caffeic acid was noted in the tubers soaked in GA_3 concentrated at 400 and 600 mg dm^{-3}. GA_3 at a concentration used in the study stimulated the accumulation of kaempferol by 15%, quercetin by 7–8.2%, quercetin 3-*O*-glucoside by 1.8% (when GA_3 was applied at a concentration of 200 mg·dm^{-3}) and by 4.1% and 3.6% (when GA_3 was applied at concentrations of 400 and 600 mg·dm^{-3}) and kaempferol 3-*O*-rhamnosylglucoside by 1.5–3.4%. The soaking of the tubers in GA_3 increased the content of β-carotene by 7.9%, 5.2% and 7.9%, respectively, without affecting the content of crocin. For soaking of *Crocosmia* tubers, it is recommended to use GA_3 at a concentration of 400–600 mg·dm^{-3}.

Keywords: geophytes; GA_3; saponins; phenolic acid; flavonoids; carotenoids

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1. Introduction

Crocosmia × *crocosmiiflora* (Lemoine) N.E.Br. is a hybrid resulting from the cross-breeding of *C. aurea* and *C. porsii*, which belong to the *Iridaceae* family. The maximum height of the plant is 90 cm. Sword-shaped leaves and inflorescence shoots with a dense spike of orange flowers grow from annual tubers, which contain numerous biologically active substances, including saponins. These natural triterpene glycosides are commonly found in the plant kingdom. They affect living organisms and therefore are classified as allelopathic substances. To date, they have been identified in more than 500 species [1]. The name derives from the Latin word *sapo*—soap, as foam is produced when saponins come into contact with water. The formation of foam is possible because saponins are able to considerably reduce the surface tension due to their amphiphilic structure [1,2]. Saponins have anti-inflammatory, antifungal, antibacterial, cytotoxic and anticancer properties [3–5]. They are used to produce cosmetics, and as precursors in the synthesis of sex hormones and corticosteroids [3,6–11].

Phenolic acids are very common in the plant kingdom. They are secondary metabolites that have a positive influence on human health. They exhibit antioxidant activity; they

eliminate reactive oxygen species, block free radicals, inhibit enzymes from the oxidase group, support enzymes with antioxidant properties and chelate some metals [12]. The antioxidant properties of phenolic acids are related to the number of hydroxyl groups in the molecule and the degree of their esterification. The antioxidant activity in compounds with one hydroxyl group is intensified by the presence of one or two methoxy groups on the ring. The introduction of either an alkyl or methoxy group with electron donors in the ortho position increases the stability of the antioxidant properties of phenolic acids [13,14].

Flavonoids are very common plant secondary metabolites. These compounds are also pigments. They are biochemically active ingredients, which give colour to flowers and fruits. They are co-pigments of anthocyanidins. They also act as filters protecting plants from UV radiation and as antioxidants protecting plants from free radicals generated during photosynthesis. Flavonoid compounds are characterised by multidirectional biological activity, and are used for medicinal, therapeutic and cosmetic purposes. Some flavonoids seal blood vessels and have an influence on the circulation of blood in cardiac muscle. They have anti-inflammatory, anti-ulcer, anti-allergic, anti-aggregating, spasmolytic, and diuretic effects, and they protect the liver [10]. Flavonoids from the group of flavones, flavonols and their glycosides are characterised by antibacterial, antifungal and antiviral activity. They also inhibit the activity of some enzymes [15]. The mechanism and strength of their action depend on the chemical structure and the presence or absence of various functional groups [16]. Carotenoids have very important functions in the plant kingdom. They are responsible for the stability of lipid membranes, participate in the accumulation of light during photosynthesis, and provide protection from the photooxidation process caused by reactive oxygen species formed as a result of the excitation of chlorophyll during photosynthesis [17]. They are components of various fruits and vegetables and have considerable influence on the proper diet [18].

Growth regulators of the gibberellin and cytokinin group are increasingly often used for cultivation of ornamental plants. They positively influence the flowering of various species and improve the quality of plants—length of stems, size of flowers or inflorescences. Apart from that, they increase the content of chlorophyll and proteins [8–10] and the uptake of nutrients [19,20].

This study was conducted to assess the influence of gibberellic acid on the content of biologically active substances in *Crocosmia* × *crocosmiiflora* 'Lucifer' tubers.

2. Materials and Methods

Crocosmia × *crocosmiiflora* (Lemoine) N.E.Br. 'Lucifer' tubers (circumference 14 cm) were planted in 0.7 dm^3 pots filled with peat substrate (pH 6.2), enriched with Peters Professional multi-componentfertiliser containing macro- (20:10:20) and microelements at a concentration of 1 $g \cdot dm^{-3}$. Before planting, the tubers were soaked in water solutions of gibberellic acid (GA_3) at concentrations of 200, 400 and 600 $mg \cdot dm^{-3}$ for 24 h. Control tubers were soaked in distilled water for 24 h. There were 15 plants in each treatment—5 plants in triplicate.

The plants were grown in a greenhouse. During the growing season they were watered regularly. Every 14 days they were fertilised with Peters Professional multi-component fertiliser at a concentration of 0.2%.

After the end of the growing season, in October, the tubers were dug out and cleaned. Replacement and adventitious tubers were separated. After drying, nine replacement tubers were selected from each treatments and crushed to prepare precisely weighed samples for analysis.

The content of four groups of antioxidant biologically active substances in the tubers was measured: saponins (medicagenic acid, medicagenic acid 3-*O*-triglucoside and polygallic acid), phenolic acid (caffeic acid, *p*-coumaric acid and gallic acid), flavonoids (kaempferol, kaempferol 3-*O*-rhamnosylglucoside, quercetin, quercetin 3-*O*-glucoside) and carotenoids (crocin, β-carotene). These metabolites are glycosidic derivated to form flavonoids. In this work, determination of aglycone forms of flavonoid methods and phe-

nolic compounds are described. The standards of the chemicals analyzed were purchased for a purity of >0.95 (Sigma Aldrich, Louis, MO, USA).

2.1. Determination of Saponins

Fifty-gram samples were weighed from the crushed *Crocosmia* tubers. They were placed in 250 cm^3 conical flasks and moistened with distilled water to obtain a suspension. Next, they were incubated for 24 h at 40 °C. Then ethanol maceration was applied to extract the samples [21,22]. Ethanol (96%) (Chempur, Piekary Śląskie, Poland) was added to each sample at a ratio of 1:10 (*w*/*v*). The samples were shaken for 1 h. They were macerated in a refrigerator for 48 h. Next, the samples were filtered and evaporated to dryness in a rotary evaporator. Then, the extract was hydrolysed with 2 M methanolic hydrochloric acid by heating for 3 h under a reflux condenser. The solvent was concentrated to a volume of 2 mL in a flask. Next, distilled water (20 mL) was added and chloroform (3 × 20 mL) (Chempur, Piekary Śląskie, Poland) was used for extraction to elute saponin aglycones. The combined chloroform layers were evaporated to dryness and the residue was dissolved in ethyl alcohol (96%). The extracts were stored in a refrigerator at 4 °C until analysis.

2.2. Determination of Selected Bioactive Compounds Using UPLC

Bioactive compounds in samples were determined after alkaline and acidic hydrolysis [23,24]. To run alkaline hydrolysis, 1 mL distilled water and 4 mL 2 M aqueous sodium hydroxide were added to test tubes. Tightly sealed test tubes were heated in a water bath at 95 °C for 30 min. After cooling (approx. 20 min), the test tubes were neutralized with 2 mL 6 M aqueous hydrochloric acid solution (pH = 2). Next, samples were cooled in water with ice. Flavonoids were extracted from the inorganic phase using diethyl ether (2 × 2 mL). Formed ether extracts were continuously transferred to 8 mL vials. Next acid hydrolysis was run. For this purpose, the aqueous phase was supplemented with 3 mL 6 M aqueous hydrochloric acid solution. Tightly sealed test tubes were heated in a water bath at 95 °C for 30 min. After being cooled in water with ice the samples were extracted with diethyl ether (2 × 2 mL). Produced ether extracts were continuously transferred to 8 mL vials, after which they were evaporated to dryness in a stream of nitrogen. Prior to analyses, samples were dissolved in 1 mL methanol. Determination was performed using an Aquity H class UPLC system equipped with a Waters Acquity PDA detector (Waters, Milford, MA, USA). Chromatographic separation was performed on a Acquity UPLC® BEH C18 column (100 mm × 2.1 mm, particle size 1.7 µm) (Waters, Ireland). The elution gradient was carried out using the following mobile phase composition: A: acetonitrile with 0.1% formic acid, B: 1% aqueous formic acid mixture (pH = 2). Concentrations of phenolic compounds were determined using an internal standard at wavelengths λ = 320 nm and 280 nm. Compounds were identified based on a comparison of retention time of the analyzed peak with the retention time of the standard and by adding a specific amount of the standard (Sigma Aldrich, Louis, MO, USA) to the analyzed samples and a repeated analysis. The detection level was 1 $\mu g \cdot g^{-1}$. Retention times of assayed compounds were as follows: medicagenic acid 3.21 min, medicagenic acid 3-*O*-triglucoside 15.75 min, kaempferol 6.11 min, kaempferol 3-*O*-rhamnosylglucoside 37.26 min, gallic acid 8.85 min, polygalic acid 10.40 min, caffeic acid 26.19 min, *p*-coumaric acid 40.20 min, quercetin 39.58 min, quercetin 3-*O*-glucoside 38.90 min, crocin 44.60 min.

2.3. Identification of Carotenoids

Acquity UPLC (Waters, Milford, MA, USA) was used to isolate and quantify carotenoids in tuber samples by means of saponification. Carotenoid extracts were obtained from ground tubers (0.4 mg), which were triturated with a mixture of acetone and petroleum ether (1:1). Then, after separation of the plant tissue, the acetone and the hydrophilic fraction were removed from the extract by washing with water. As a result, an ether extract with a mixture of carotenoid pigments was obtained. Next, the extract was concentrated in a vacuum evaporator at 35 °C until an oily residue was obtained. Then it was dissolved in

2 mL of methanol (Merck, Burlington, Ma, USA) and analysed chromatographically. Lutein, zeaxanthin and β-carotene were identified with the Acquity UPLC (Waters, Milford, MA, USA) with a Waters Acquity PDA detector (Waters, Milford, MA, USA). An Acquity UPLC® BEH C18 column (100 mm × 2.1 mm, particle size 1.7 µm) (Waters, Ireland) was used for chromatographic separation. The following solvents were used for elution: A—methanol, B—water and methyl tert-butyl ether (MTBE). A gradient was applied at a flow rate of 0.4 mL/min. The column and samples were thermostated. The column temperature was 30 °C; the temperature of the samples was 10 °C. During the analysis the solutions were degassed in a Waters device. The injection volume was 10 µL. The registration was carried out at a wavelength λ = 445 nm. Compounds were identified on the basis of spectra ranging from 200 to 600 nm and retention times compared with standards.

2.4. Total Polyphenolic Content

Fifty-gram samples were collected for determination of polyphenols. The samples were ground with a laboratory mill. Total phenolic compounds were extracted with 80% MeOH. Ten-gram samples were flooded with 100 mL MeOH and placed in an ultrasound bath for 30 min. Next, the precipitate was collected into distillation flasks and the extraction process was repeated three times. The combined extracts were evaporated to dryness in an evaporator. Resulted extracts were dissolved in methanol and dried in a stream of nitrogen (N). Distilled water (0.5 mL) and 0.125 mL of Folin–Ciocalteu reagent (Fluka) were added to 0.125 mL extract. After 6 min, the mixture was supplemented with 1.25 mL of 7% aqueous Na_2CO_3 solution and 1 mL of distilled water. After 90 min, absorbance was read at a wavelength of 760 nm in relation to water (Helios spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA). The results were expressed as mg gallic acid/kg DW sample. The regression equation was y = 0.9226x − 0.0465 and R^2 = 0.9975.

2.5. $ABTS^{\bullet+}$ Method

To make $ABTS^{\bullet+}$ from ABTS salt, 3 mM of $K_2S_2O_8$ was reacted with 8 mM ABTS salt in distilled water for 16 h at room temperature in the dark. The $ABTS^{\bullet+}$ solution was then diluted with a pH 7.4 phosphate buffer solution containing 150 mM NaCl (PBS) to obtain an initial absorbance of 1.5 at 730 nm. A fresh $ABTS^{\bullet+}$ solution was prepared for each analysis. The reaction kinetics was determined over a 2 h period with readings every 15 min. The reactions were complete in 30 min. Samples and standards (100 µm) were reacted with the $ABTS^{\bullet+}$ solution (2.900 µm) for 30 min. Trolox was used as a standard. The results were expressed as $ABTS^{\bullet+}$ (µmol TROLOX·kg^{-1}) DW sample. The regression equation was y = −0.2958x + 0.6167 and R^2 = 0.9908.

2.6. Statistical Analysis

The results were processed statistically. One-way analysis of variance was used. The means were grouped with Duncan's test at a significance level α = 0.05. The program Statistica version 13.3 was used.

3. Results and Discussion

3.1. Saponins

Our research showed that *Crocosmia* tubers could be a valuable source of saponins. The following saponins were distinguished: medicagenic acid, medicagenic acid 3-*O*-triglucoside and polygalic acid. The content of medicagenic acid and polygalic acid in the tubers increased significantly after treatment with GA_3. The content of medicagenic acid and polygallic acid in the tubers treated with GA_3 increased by 42.9–57.1% and 50%, respectively. However, the treatment of the tubers with GA_3 did not result in significant differences in the content of medicagenic acid 3-*O*-triglucoside (Figure 1). It is difficult to find any information on the dependence between the saponin content in plants and the activity of GA_3 in available publications. It may be that GA_3, as a natural plant regulator that regulates plant physiological processes, can also significantly affect plant metabolism,

and modify the course of biosynthetic pathways of different types of metabolites, both primary and secondary. The effectiveness of saponins has been demonstrated in numerous studies. They inhibit the development of various fungi on plants [25,26]. Their antifungal activity results from their ability to form complexes with sterols and phospholipids in the fungal cell membrane. This causes changes in membrane permeability and cell lysis [27]. However, it is noteworthy that there are differences in the biological activity of individual compounds due to their chemical structure. This fact was confirmed by the study conducted by Martyniuk and Jurzysta [11], who compared the ability of various medicagenic acid glycosides extracted from *Medicago sativa* roots to inhibit the growth of *Gaeumannomyces graminis* var. *tritici*. The researchers observed that among the saponins of medicagenic acid found in *Medicago sativa* roots, only the monoglucoside of this acid significantly inhibited the fungal growth. Martyniuk et al. [25,26] proved the antifungal effectiveness of saponins in cereals, where they inhibited the development of pathogens. Oda et al. [28] suggested that the level of haemolytic activity of saponins was related to the type of the glycine part and the presence of a sugar chain. The replacement of chemicals with natural plant metabolites should be a priority to combat fungal and bacterial diseases because the progressive degradation of the environment, resulting from various factors, is an undeniable danger to everybody.

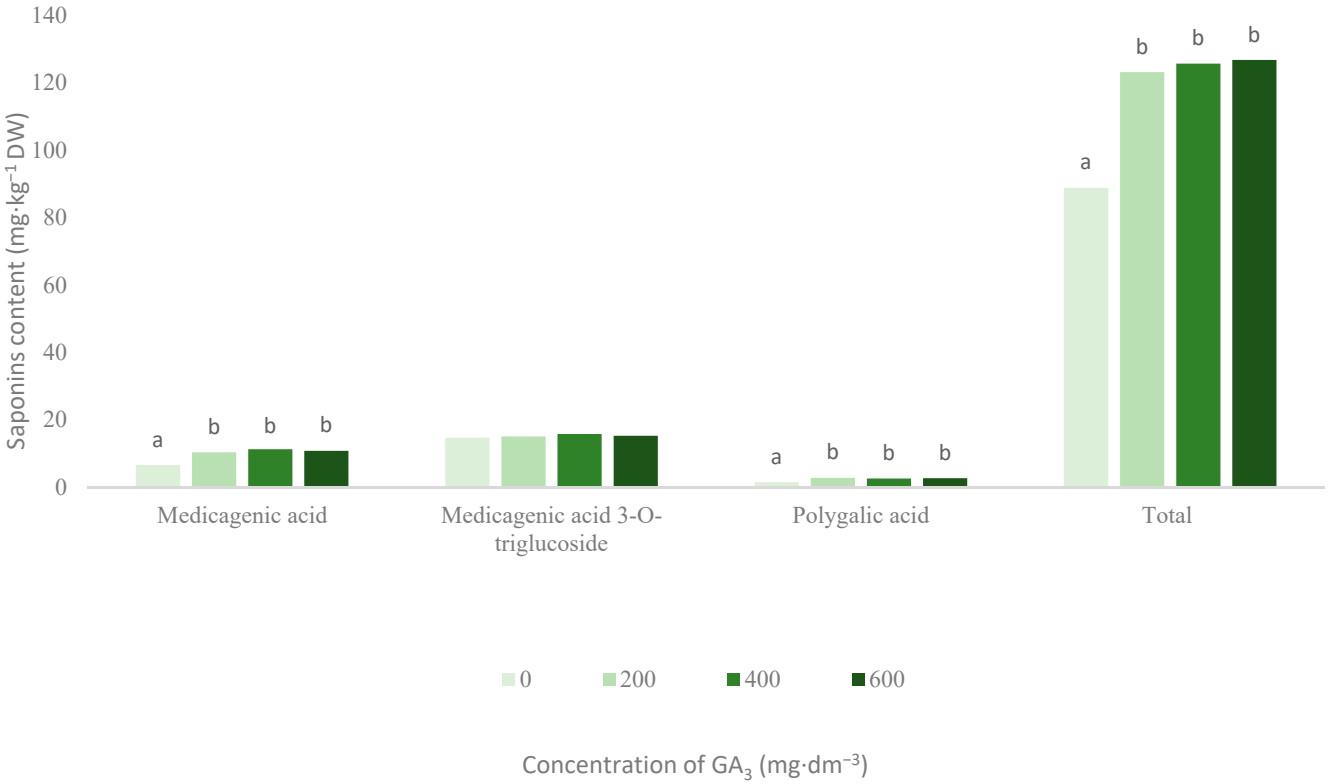

Figure 1. Saponins content of the *Crocosmia* × *crocosmiiflora* 'Lucifer' tubers after application of GA_3. Means followed by the same letter do not differ significantly at $\alpha = 0.05$.

3.2. Phenolic Acids

The following types of phenolic acids were distinguished in the tubers: caffeic acid, *p*-coumaric acid and gallic acid. The concentrations of gibberellic acid used in the experiment significantly influenced the content of the compounds under analysis in the tubers (Figure 2). The highest significant amounts of caffeic acid were found in the tubers soaked in GA_3 at concentrations of 400 and 600 mg·dm^{-3}, i.e., 5.3% and 6.7%, respectively. When GA_3 at a concentration of 200 mg·dm^{-3} was applied, the increase in the content of caffeic acid was smaller, but significantly greater than in the control tubers. GA_3 caused a

significant increase in the content of *p*-coumaric acid. Regardless of the concentration of this gibberellin, the content of *p*-coumaric acid in the tubers increased by 10.8–13.0%. GA_3 also significantly increased the content in gallic acid in the tubers. In comparison with the control tubers, the content of this compound increased by 17.6–29.4%. Phenolic compounds protect plants from bacterial and fungal diseases. Research has proved the antibacterial activity of caffeic acid, especially against Gram positive bacteria, and *p*-coumaric acid [10]. Kaempferol, which was isolated from the *Crocosmia* × *crocosmiiflora* 'Lucifer' tubers, has a comprehensive effect [29]. It is active against Gram positive and Gram negative bacteria as well as fungi of the *Candida glabata* genus [15,16]. Quercetin, which was also isolated from the tubers, is a naturally occurring polar auxin transport inhibitor [30]. Apart from that, it has anti-inflammatory, anticancer, antiviral, antioxidant and psychostimulatory effects. It is capable of inhibiting lipid peroxidation, platelet aggregation and capillary permeability and it stimulates mitochondrial biogenesis [31,32].

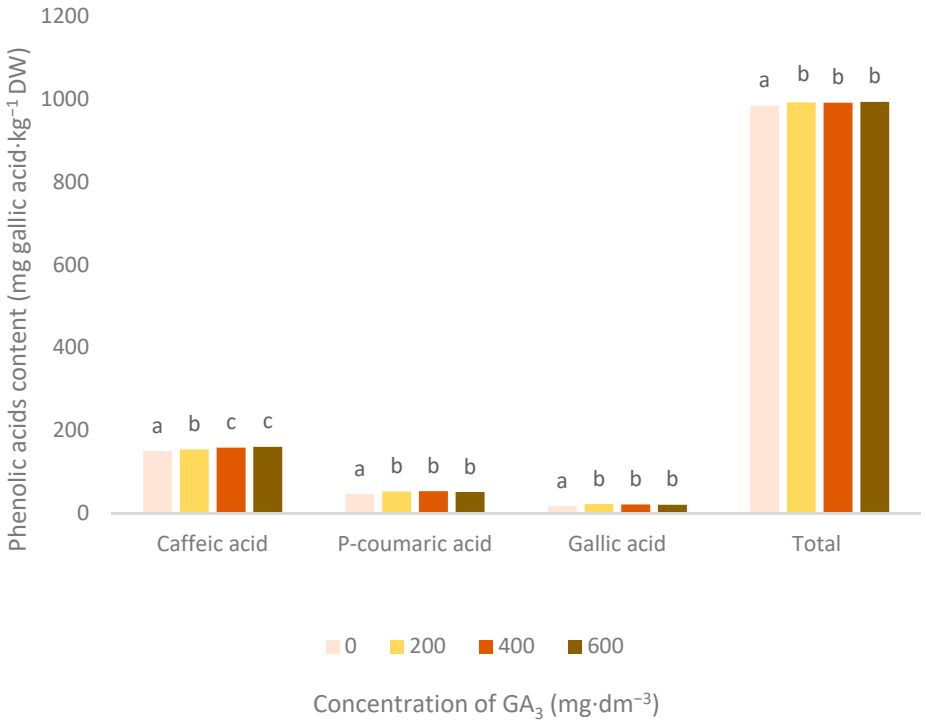

Figure 2. Phenolic acids content of the *Crocosmia* × *crocosmiiflora* 'Lucifer' tubers after application of GA_3. Means followed by the same letter do not differ significantly at $\alpha = 0.05$.

3.3. Flavonoid Pigments

In the flavonoid group, GA_3 significantly stimulated the production of four isolated compounds (Figure 3). When GA_3 was applied at a concentration of 200 mg·dm^{-3}, the content of kaempferol increased by 15%, quercetin by 7–8.2%, and quercetin 3-*O*-glucoside by 1.8%. When GA_3 was applied at concentrations of 400 and 600 mg·dm^{-3}, these values increased by 4.1% and 3.6%, whereas the content of kaempferol 3-*O*-rhamnosylglucoside increased by 1.5–3.4%.

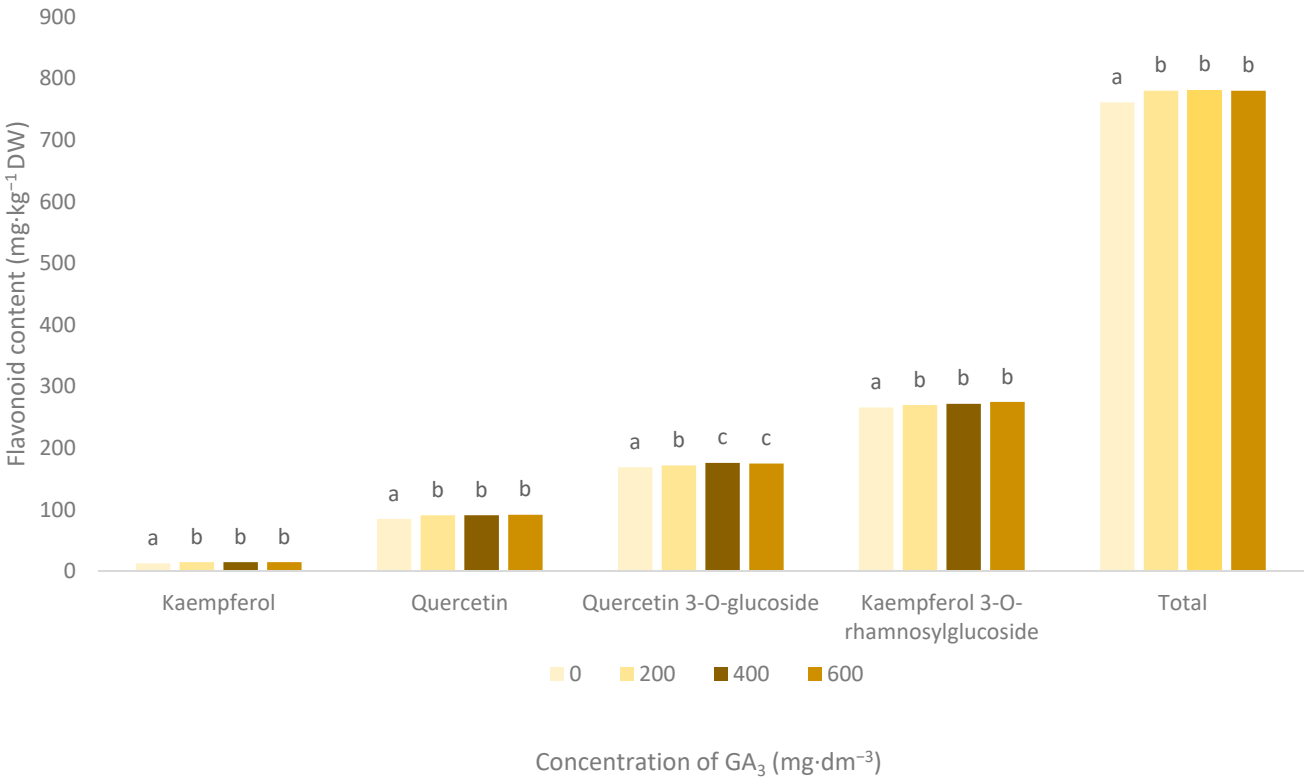

Figure 3. Flavonoid content of the *Crocosmia* × *crocosmiiflora* 'Lucifer' tubers after application of GA_3. Means followed by the same letter do not differ significantly at $\alpha = 0.05$.

3.4. Carotenoid Pigments

As far as carotenoids are concerned, GA_3 caused a significant increase in the content of β-carotene only (by 7.9%, 5.2% and 7.9%, respectively) (Figure 4).

Carotenoids were expected to be found in the *Crocosmia* × *crocosmiiflora* 'Lucifer' tubers, because the water solutions in which the tubers were soaked turned red. Naturally, these tubers are not an edible organ, so the presence of pigments is important only from the point of view of plant physiology. As results from available reference publications, treatment of the plants with gibberellin and cytokinin increases their content of pigments. Therefore, it was not surprising to observe increased values of flavonoids and carotenoids in the *Crocosmia* × *crocosmiiflora* 'Lucifer' tubers after the GA_3 treatment. Janowska and Stanecki [33] observed that when *Zantedeschia albomaculata* 'Albomaculata' rhizomes were soaked in water solutions of a GA_3 and BA mixture before planting, there were higher values of the leaf greenness index, which is correlated with the chlorophyll content. The results of this study are in line with the findings of the research by Janowska [34,35].

3.5. Antioxidant Activity

The study showed that the tubers of the *Crocosmia* × *crocosmiiflora* 'Lucifer' were a rich source of antioxidants. Bioactive antioxidant substances from four groups, i.e., saponins, phenolic acid, flavonoids, and carotenoids were determined from the *Crocosmia* tubers. When GA_3 was used to soak the tubers, the antioxidant activity of the extracts from tubers increased proportionally to the GA_3 concentrations, i.e., by 2.7%, 2.9% and 4.4%, respectively (Figure 5).

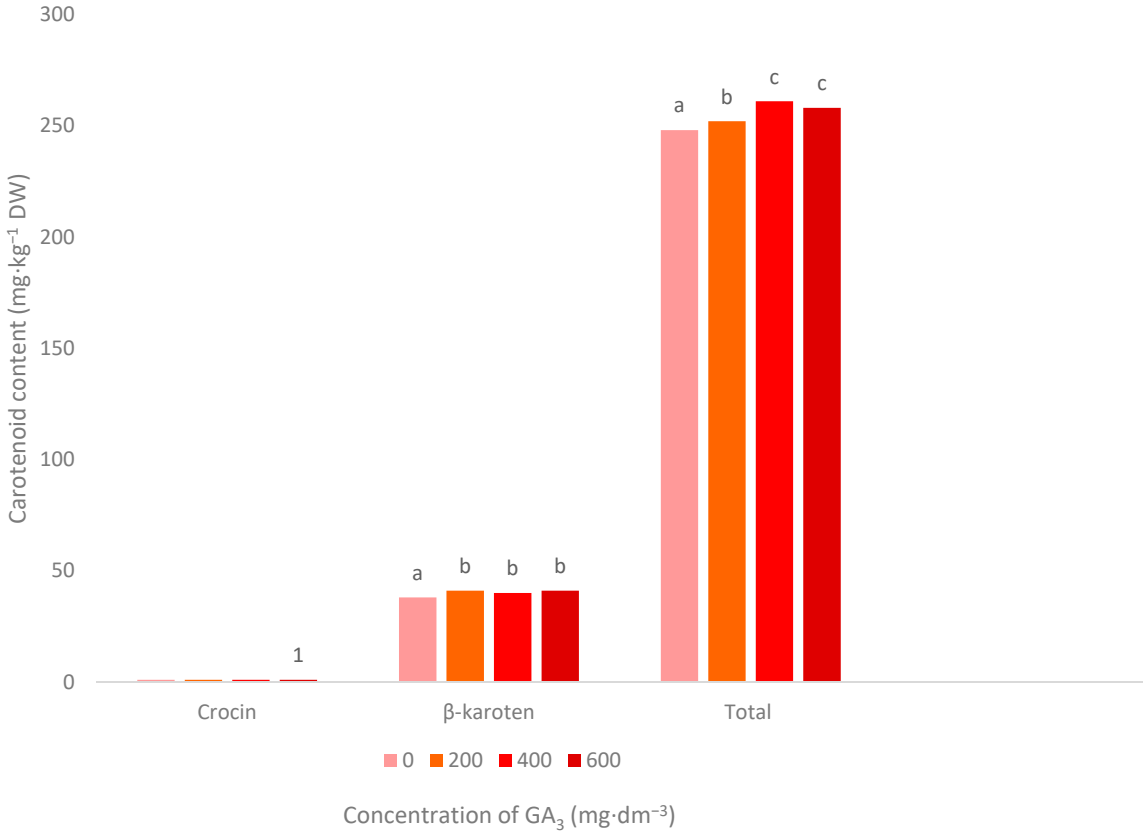

Figure 4. Carotenoid content of the *Crocosmia* × *crocosmiiflora* 'Lucifer' tubers after application of GA_3. Means followed by the same letter do not differ significantly at $\alpha = 0.05$.

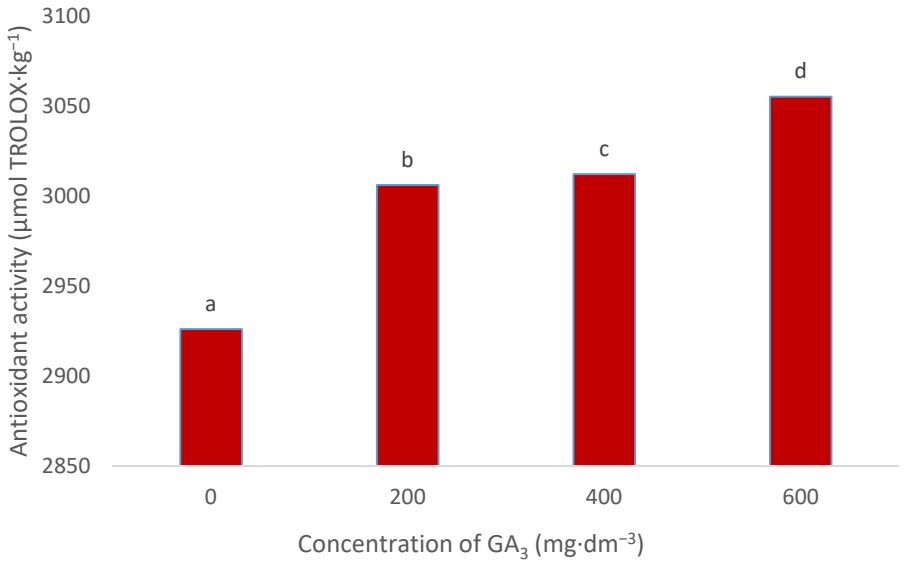

Figure 5. Antioxidant activity of the extracts from tubers of the *Crocosmia* × *crocosmiiflora* 'Lucifer' after application of GA_3. Means followed by the same letter do not differ significantly at $\alpha = 0.05$.

GA_3 effectively increases the content of biologically active substances in *Crocosmia* tubers applied at a concentration of 400–600 mg·dm^{-3}.

4. Conclusions

The tubers of the *Crocosmia* × *crocosmiiflora* 'Lucifer' are a promising source of potential antioxidants, and their extracts can be used in pharmaceutical industry, as well

as in cosmetics products and antifungal and antibacterial substances. Four groups of biologically active substances with antioxidant properties were determined from *Crocosmia* tubers: saponins (medicagenic acid, medicagenic acid 3-*O*-triglucoside and polygallic acid), phenolic acid (caffeic acid, *p*-coumaric acid and gallic acid), flavonoids (kaempferol, kaempferol 3-*O*-rhamnosylglucoside, quercetin, quercetin 3-*O*-glucoside) and carotenoids (crocin, β-carotene). When GA_3 was applied, the antioxidant activity of the extracts from tubers increased proportionally to the GA_3 concentrations used in the study. GA_3 increased the content of the substances present in the tubers, with the exception of the content of crocin and medicagenic acid 3-*O*-triglucoside. For soaking of *Crocosmia* tubers, it is recommended to use GA_3 at a concentration of 400–600 $mg \cdot dm^{-3}$.

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