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Biochemical Activity of Biochanin A in the Green Alga *Chlorella Vulgaris* Beijerinck (*Chlorophyceae*)

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Abstract

This study was conducted to investigate the influence of biochanin A (isoflavone characterised by estrogenic activity) upon the growth expressed as number of cells and the content of chlorophyll *a* and *b,* total carotenoids, reducing sugars, water-soluble proteins and nucleic acids (DNA, RNA) in the unicellular green alga *Chlorella vulgaris*, as an experimental model. Biochanin A in a concentration of 10^{-6} M exerted the greatest biological activity, principally, on the 6th day of cultivation, whereas during the 9th day of algal culure it had weak stimulating properties. Under the influence of 10^{-6} M biochanin A, an increase in the number of cells to the level of 186% and the content of water-soluble proteins to 255%, reducing sugars to 505%, in comparision with the control culture (taken to be 100%), was observed. Moreover, the content of DNA was intensively stimulated in the range of 184% and RNA content reached the value 202% in regard to the control. Among the photosynthetic pigments, stimulation of the content of chlorophyll *a* to the level of 191%, chlorophyll *b* to 180% and total carotenoids to 172%, compared with the control culture of algae devoid of biochanin A, was recorded.

Keywords: *Chlorella vulgaris,* biochanin A, growth, chlorophyll *a* and *b*, total carotenoids, water-soluble proteins, nucleic acids (DNA, RNA), reducing sugars

Introduction

Biochanin A (5,7-dihydroxy-4'-methoxyisoflavone) is a member of a class of isoflavonoid compouds characterised by estrogenic activity in humans and animals (Fig. 1). It commonly occurs in many species of vascular plants, mostly belonging to the families legumes (*Leguminoseae*), papilonaceous plants (*Papilionaceae*) and many species of grasses and cereals (*Graminae*). Biochanin A has been also isolated from pegged trees and shrubs, fruit--trees, stone-trees, in the main, from genus plum-tree. The plant species which are the richest source of biochanin A include red clover (*Trifolium pratense*), which contains 0.8% this compound in dry weight of leaves, juniper (*Genista tinctoria*), soybean (*Soja hispida*) and plum (*Prunus spinosa*) [1, 2, 3, 4, 5].

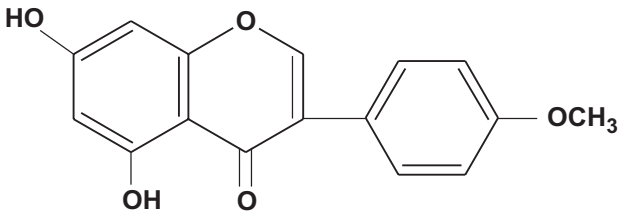

Fig. 1. Structure of biochanin A.

Apart from biochanin A, numerous isoflavonoid compounds which can act as phytoestrogenes in mammals such as formononentine, genistein, daidzein, angolensin,

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phaseolin, pisatin, gliceolin, irygenin, kewiton, kumestrol, medikarpin, prunetin and pterokarpin were identified in the above-mentioned vascular plants. Some of the isoflavone compounds, particularly phaseolin, gliceolin, kiewiton, kumestrol, medikarpin and pisatin, play the role of phytoncides or phytoalexins which, in many plant species, protect the plants from the invasion of viral, bacterial and fungal pathogens. It appears from previous research that such isoflavones as genistein, daidzein, ekwol, formononentine and biochanin A are the most known and biological active compounds with the greatest estrogenic activity in mammal organisms [5, 6, 7, 8].

Estrogenic activity of isoflavones depends mainly on the number and localization of hydroxyl and methoxyl groups in the aromatic skeleton of isoflavone, which bears analogical cyclical structure resemblance to estrone, a female endogenous steroidal hormone or to dietylostilbestrol - its chemical synthetic analogue. Therefore, in vivo biochanin A can interfere with mechanisms controlled by animal steroidal hormone trough competition for its receptors [6, 7, 8]. In addiction, research of Burda and Suchecki [4] exhibited that isoflavones, which can be found in fodder, undergo chemical biotransformation in animal organisms. For example, formononetine, which was isolated from red clover and posesses only one –OH group, undergoes in an animal organism firstly demethylation and then reduction into a compound with a higher estrogenic activity. However, genistein and biochanin A, characterised by the strongest estrogenic activity, during detoxification in liver they are degraded to *p* - ethylphenol, which doesn't posses estrogenic properties [2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13].

Empirical data obtained in studies made on animals show that genistein display the strongest biological activity, whereas biochanin A and daidzein have weak estrogenic properties [3, 4, 5]. The results of laboratory tests, conducted mainly on mice, indicate that daidzein and genistein, of which considerable amounts in the range of 1-2.25 µg/g biomass are found in soya (*Soya hispida*), used in small concentrations range of 10^{-7}-10^{-5} M induce the increase of cytochrome P-450 content and its activity in the liver microsomes. On the other hand, isoflavones at higher concentrations show growth inhibitory effect on skin cancer cells and its proliferation. Genistein has also been observed to induce apoptosis in a variety of types of tumour cell lines [6, 8].

Isoflavonoid compounds in the concentration range of 10^{-7}-10^{-5} M have been characterised as protein-tyrosine kinase (PTK) strong inhibitors in a variety animal cells. Therefore, isoflavones can play a significant physiological role in regulation of protein phosphorylation. Some results [11, 12] demonstrate that genistein as the tyrosine kinase inhibitor also had a direct inhibitory effect on glycine receptors. However, genistein in the concentration of 10^{-6} M maximally induced activity of the protein-tyrosine phosphatase.

Recent studies carried out on human hepatoma cell line suggest that genistein, which is similar to estradiol, could be responsible for the activation of the apolipoprotein (apo A–I) gene expression. Regulation of apo A–I gene expression by genistein is a major mechanism for the induction processes of apoptosis in cancer cells [13].

The former investigations into estrogenic activity of isoflavones are slender and concerned mainly with their antioxidative and anticancerogenic bioactivity in both animals and humans [6, 7, 8, 11, 12, 13]. There is no empirical data in relation to the physiological and metabolic activity of isoflavones in vascular plants in which these compounds commonly occur as natural secondary metabolites and considerable amounts of these isoflavones are present in the cells of thallophyte plants such as fungi and algae.

In view of the above, the ability of biochanin A, characterised by estrogenic activity, at optimal concentration range of 10^{-8}-10^{-5} M to influence upon the growth and the change of content of some metabolically important components like water-soluble proteins, reducing sugars, chlorophyll *a* and *b*, total carotenoids and nucleic acids (DNA, RNA) in cells of green alga *Chlorella vulgaris* - one of the principal species in freshwater ecosystems [14, 15], was studied.

Material and Methods

The green alga *Chlorella vulgaris (Chlorophyceae)* was grown under controlled conditions at 25±0.5°C. Ellumination was supplied during a 16h photoperiod (8h dark period) by a bank of fluorescent lights yelding a photon flux of 50 µmol $\cdot m^{-2} \cdot s^{-1}$ at the surface of the tubes. Permament synchronous growth was established according to the method of Pirson and Lorenzen [16] in the conditions developed by Sayegh and Greppin [17]. The culture medium used was modified Knop's medium. The pH of the medium was adjusted to 6.8 with 1N NaOH. The *C. vulgaris* cells were cultured in Erlenmayer flasks (500 ml) containing 250 ml medium and shaken at 150 rpm in a rotary shaker.

The number of cells was determined by direct counting of cells in the growth medium using a Bürker chamber. pH was measured in the medium using a Cole–Parmer pH Benchtop Meter.

Determination of soluble proteins content was done by extracting the algal pellet overnight in 0.1N NaOH at 4°C. Concentration of protein was determined by the method of Lowry *et al*. [18] with a protein kit calibrated with bovine serum albumin (Sigma, St. Louis MO USA) as the standard.

The content of the nucleic acid DNA and RNA was determined spectrophotometrically, according to the method described by Rogers and Bendich [19] and Szczegielniak [20]. All reagents used for nucleic acid determination were purchased from Sigma Chemicals Co. (St. Louis, MO, USA).

The reducing sugars concentration was determined spectophotometrically, according to method as described by Samogyi and Nelson's [21, 22].

Determination of the content of chlorophyll *a* and *b* as well as total carotenoids [23] in cells of algae followed homogenization of algal fresh weight in methanol. The absorbance of the extract was measured with Shimadzu 1201 spectrophotometer at 470, 653 and 666 nm. The amounts of chlorophyll *a* and *b* and total carotenoids present in the extract were calculated according to the equation of Wellburn [24].

Statistical analysis treatment consisted of five replicates and each experiment was carried out at least twice at different times, a Minitab statistical package was used to carry out a one-way ANOVA. Significance was determined using t-test LSD values based on the ANOVA data.

Results

Numeric results regarding content of analysed biochemical parameters in cells of the green alga *Chlorella vulgaris* under the influence of the most optimal concentration 10^{-6} M of biochanin A are presented in Table 1. Whereas the average percentage content of individual analysed results of examinations are presented graphically in Figures 2-9.

Under the influence of the optimal concentration 10^{-6} M of biochanin A in cells of *C. vulgaris*, all analysed biochemical parameters underwent the strongest stimulation on the 6th day of algal culture in comparison to the control group, considered to have the value 100%. For example, the number of *C. vulgaris* cells on the 6th day of cultivation, under the influence of 10^{-6} M of biochanin A, was stimulated up to the value of 186% and to the value of 177% on the 9th day of the experiment. Contents of water-soluble proteins and reducing sugars were stimulated

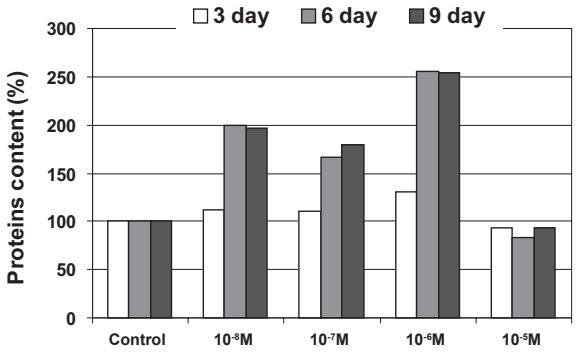

Fig. 2. The percentage content of water-soluble proteins in *C. vulgaris* cells under the influence of 10^{-8}-10^{-5} M biochanin A between 3-9 days of culture, compared to control, (SE < 5 %).

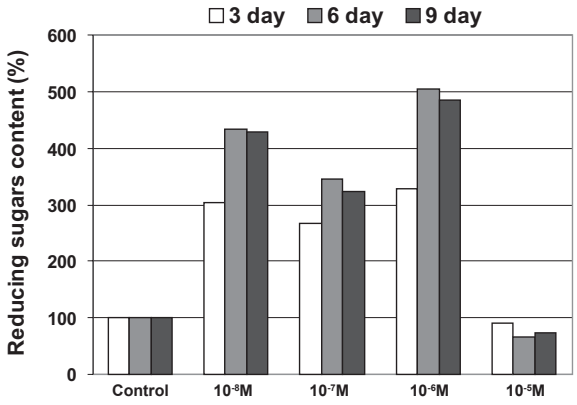

Fig. 3. The percentage content of reducing sugars in *C. vulgaris* cells under the influence of 10^{-8}-10^{-5} M biochanin A between 3-9 days of culture, compared to control, (SE < 5%).

Table 1. Effect of optimal concentration 10^{-6} M biochanin A on the content of biochemical parameters in *Chlorella vulgaris*.

Analyzed parameters	Biochanin A and control	Time of culture in days		
		3	6	9
Number of cells (10^{5} ·ml^{-1})	Biochanin A Control	21 15	41 22	48 27
Soluble proteins (10^{-8} µg/cell)	Biochanin A Control	7.48 5.76	20.60 8.08	21.96 8.64
DNA (10^{-8} µg/cell)	Biochanin A Control	0.014 0.009	0.035 0.019	0.043 0.028
RNA (10^{-8} µg/cell)	Biochanin A Control	0.063 0.041	0.105 0.052	0.132 0.077
Chlorophyll *a* (10^{-8} µg/cell)	Biochanin A Control	2.41 1.89	7.70 4.04	8.14 6.01
Chlorophyll *b* (10^{-8} µg/cell)	Biochanin A Control	1.16 0.87	2.81 1.57	3.51 2.52
Chlorophyll *a* + *b* (10^{-8} µg/cell)	Biochanin A Control	3.57 2.76	10.51 5.61	11.65 8.53
Total carotenoids (10^{-8} µg/cell)	Biochanin A Control	0.61 0.48	1.83 1.06	2.25 1.61
Reducing sugars (10^{-8} µg/cell)	Biochanin A Control	4.48 1.36	11.92 2.36	12.76 2.69

SE<5%

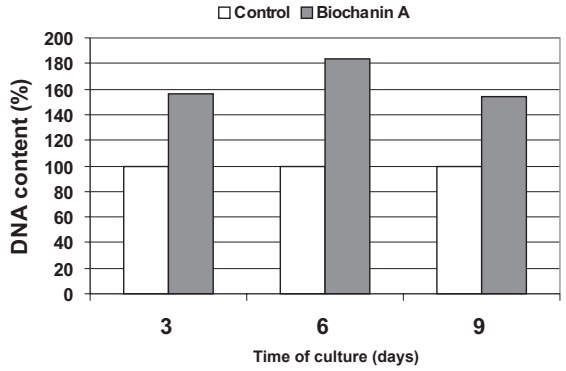

Fig. 4. The percentage content of DNA in *C. vulgaris* cells under the influence of 10^{-8}-10^{-5} M biochanin A between 3-9 days of culture, compared to control, (SE < 5%).

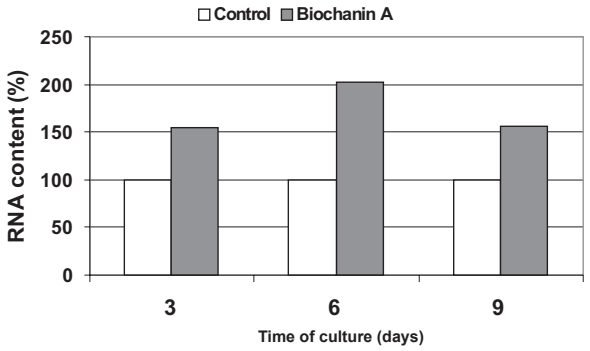

Fig. 5. The percentage content of RNA in *C. vulgaris* cells under the influence of 10^{-8}-10^{-5} M biochanin A between 3-9 days of culture, compared to control, (SE < 5%).

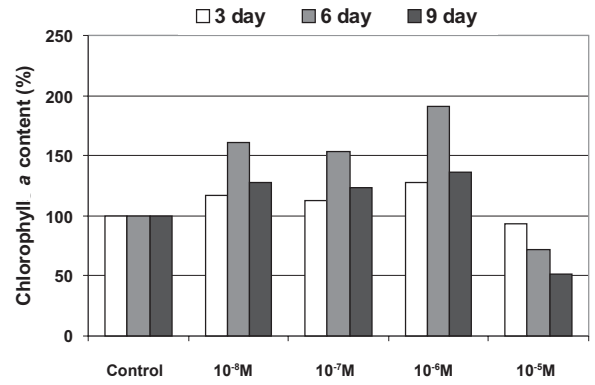

Fig. 6. The percentage content of chlorophyll *a* in *C. vulgaris* cells under the influence of 10^{-8}-10^{-5} M biochanin A between 3-9 days of culture, compared to control, (SE < 5%).

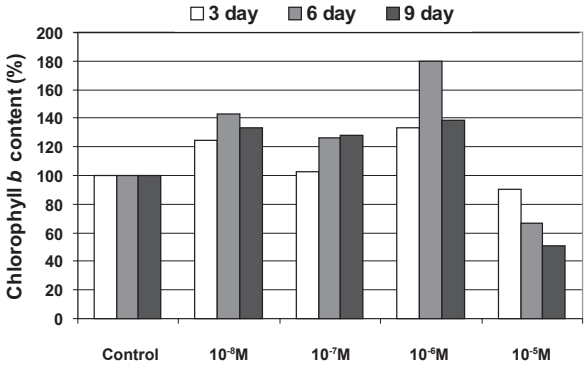

Fig. 7. The percentage content of chlorophyll *b* in *C. vulgaris* cells under the influence of 10^{-8}-10^{-5} M biochanin A between 3-9 days of culture, compared to control, (SE < 5%).

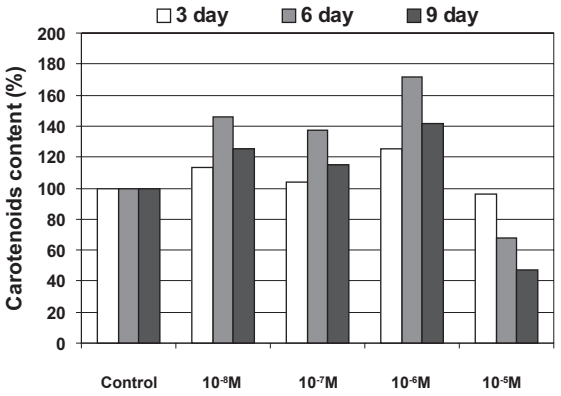

Fig. 8. The percentage content of total carotenoids in *C. vulgaris* cells under the influence of 10^{-8}-10^{-5} M biochanin A between 3-9 days of culture, compared to control, (SE < 5%).

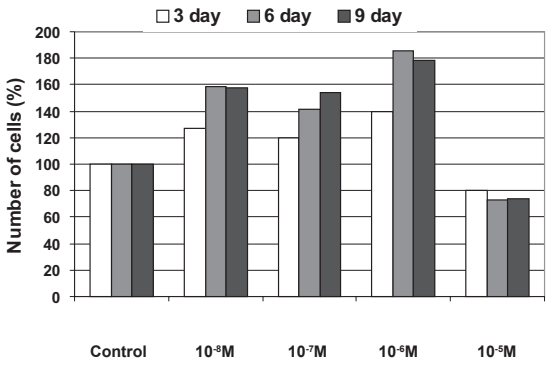

Fig. 9. The percentage number of *C. vulgaris* cells under the influence of 10^{-8}-10^{-5} M biochanin A between 3-9 days of culture, compared to control, (SE < 5%).

most effectively, reaching the values 255% and 505%, respectively. Stimulation of the content of nucleic acids can be characterized as DNA was stimulated to the value of 184%, RNA-202% in relation to the control. However, the content of chlorophyll *a* reached the value of 191% while for chlorophyll *b* it reached the value of 180% and for the total pool of carotenoids it reached the value of 172% in comparison to the control culture of *C. vulgaris* (100%).

On the other hand, the same compound in the highest studied concentration of 10^{-5} M exhibited inhibitory effects on the intensity of growth and biochemism of *C. vulgaris* during the 9 days' algae culture. Under 10^{-5} M of biochanin A, a maximall fall in the number of algae cells of 73%, of water-soluble proteins 83%, reducing sugars 66%, chlorophyll *a* 52%, chlorophyll *b* 51% and total carotenoids 47% in relation to the control (100%), occurred.

The growth of eucaryotic green algae and content of examined biochemical metabolites in its cells was slightly less effective on the 9th day of cultivation of *C. vulgaris* and significantly less effective on the 3rd day of the experiment.

Discussion

Biochanin A is the representative of isoflavones and it possesses a chemical structure quite similar to genistein and daidzein. In relation to humans and animals, it is characterized by estrogenic properties, while its physiological-metabolic activity in plants, particularly thallophyte plants such as algae, has not yet been examined [2, 3, 10].

The empirical data obtained in these studies demonstrate, that biochanin A at concentrations ranging from 10^{-6} to 10^{-8} M has a strongly stimulating effect on intensity of growth of *Chlorella vulgaris* cells, which is expressed by their number. The maximal effect of its activity was reached on the 6th day of the algal culture, under the concentration of 10^{-6} M. Among analysed biochemical parameters, the following indicated the strongest stimulating effect on the content of: reducing sugars, nucleic acids (DNA and RNA) and also water-soluble proteins. However, the content of the photosynthetic pigments chlorophyll *a* and *b* as well as total carotenoids was stimulated much less effectively.

The earlier results from conducted experiments regarding metabolic activity of estradiol in the cells of *C. vulgaris* [25] indicated that this estrogen under very small concentrations of 10^{-9}-10^{-11} M has the strongest stimulating activity on the content of chlorophylls (in the range of 121-150%) and the pool of carotenoids (in the range of 140-200%) in comparison with control group (100%). However, biochanin A exhibits the strongest stimulating influence on metabolism of the algae cells while used under concentrations ranging from 10^{-5} to 10^{-8} M. Estradiol, therefore is a compound with superior biological activity in comparison to biochanin A, because its smaller concentrations cause quite strong stimulation of the growth intensity of *C. vulgaris* cells and biochemical parameters which were examined in them and which include the following: photosynthetic pigments, proteins and monosaccharides.

It is known from the literature [9, 10, 26-31], that in germinating seeds of pea (*Pisum sativum*), pine (*Pinus silvestris*), spruce (*Picea excelsa*) and other vascular plants, estradiol - used under concentrations ranging from 0.1 to 0.001 μg per a plant - stimulated growth of plants mentioned above ranging from 40 to 50%. It also increased the content of auxins, gibberellins and cytokinins while it also significantly decreased the amount of abscisic acid (ABA). The influence of estrogens on vascular plants, especially on germination of seeds and development of flowers and their physiological and metabolic activity, depends on gibberellins to a large extent, which they interact with synergic reactions [26, 27].

According to literature data [3, 9, 26, 28, 30, 31], it is well known that estrogens and gibberellins have a partially similar molecular mechanism of influencing processes of growth, development and metabolism of a plant cell. The basic role in this mechanism is played by peculiar receptors and common, secondary chemical transmitters of cellular signals. So far, no individual cellular receptors for isoflavonoids were detected and perhaps that is why it can be suspected that they interact directly with receptors peculiar for estrogenic hormones. The receptors of estrogens and gibberellins were detected mainly in the cytoplasm, nucleoplasm and nuclear chromatin and probably that is an explanation why they induce the most universal influence on cell division and all processes connected with them, i.e. replication, transcription and translation.

High increase in the content of reducing sugars - mainly monosaccharides - in the cells of green alga *C. vulgaris* under the influence of biochanin A may be explained by the increased intensity of the process of photosynthesis. It is known from previous examinations performed on *C. vulgaris* [25] that estradiol significantly increases the intensity of the photosynthesis process and also the content of chlorophyll pigments. In algae cells biochanin A also causes significant growth of quantity of chlorophylls *a* and *b* as well as carotenoids, which take part in the photosynthesis process. On the 6th day of cultivation of *C. vulgaris*, the content of xanthophylls with 2 oxygen atoms in a molecule (oxygen-poor xanthophylls), increased reaching the value of 224% - this especially regards zeaxanthin, while for xanthophylls with 3 or 4 oxygen atoms in a molecule (oxygen-rich xanthophylls) stimulation reached the level of 180%. As it was earlier proved [1], xanthophylls, which take part in the xanthophyll cycle, disperse excess solar energy absorbed by the photosynthetic apparatus and this way they protect it against damage.

From earlier research also carried out on *C. vulgaris* [32], results show that corticosteroids such as 11-deoxycorticosterone and prednisolone, just like estradiol and biochanin A, intensively stimulate the growth of cells expressed by content of fresh and dry weight as well as by the content of water-soluble proteins and reducing

sugars, while their influence on DNA and RNA content was negligible.

Studies performed on liver cells of mice [8] indicate that daidzein and genistein induce specific changes in the structure of cytochorme P-450, which takes part in the transportation of electrons between flavoproteins and cytochrome oxidase. Under higher concentrations of these isoflavonoid compounds, interruptions in the course of reactions of the respiratory chain are also possible.

Genistein under the concentration of 10^{-5} M in animal cells reduces activity of protein-tyrosine kinase while under the concentration of 10^{-6} M this compound, the stimulation of protein-tyrosine phosphatase activity, was also discovered [11]. Activating the influence of genistein on activity of both enzymes in animal cells is analogous to the quantity of optimal concentrations of biochanin A, which exhibits the maximum activating effect on growth and other analysed biochemical parameters in the alga *C. vulgaris*.

It is known from the literature [5, 6, 7, 8, 13] that biological activity of flavonoids, including that of isoflavones, depends mainly on the number of hydroxyl groups (-OH) or methoxyl groups ($-OCH_3$) and their distribution in aromatic rings A and B. Mainly, the distribution of hydroxyl groups in the B ring determines the type of biological activity. For example, one sole -OH group in the *para* position activates the synthesis of IAA oxidase, which in turn decomposes auxins. However, two –OH groups in the *orto* and *para* positions cause an opposite effect because they bring about an increase in auxins content in plant cells.

Mostly probably, the presence of one methoxyl group ($-OCH_3$) in the B ring in biochanin A in relation to daidzein and genistein, which include hydroxyl groups, significantly decreases its biological activity.

What's more, research of Burda and Suchecki [4] – which were conducted on animal organisms - indicated that isoflavonoid compounds, which can be found in fodder, undergo chemical biotransformation. For example formononetine, which can be found in red clover and has one –OH group, undergoes first a demethylation and then a reduction into a compound with a higher estrogenic activity in mammals.

Moreover, experiments performed on vascular plants also indicated that steroidal, animal hormones introduced into them, could undergo minor chemical modification and may become proper phytosteroids or undergo biological inactivation or even degradation. It is suspected that in vascular plants - including algae and fungi - there are peculiar enzymes which have a catalytic effect on oxidation--reduction, demethylation or methylation of proper atoms of carbon in steroid rings, leading to the creation of proper endogenic or modified phytosteroids [3, 33, 34].

The efficiency of stimulating activity of biochanin A on analysed biochemical parameters in the green alga *C. vulgaris* is significant in comparison with the control group lacking exogenous isoflavones. It should be expected that daidzein and genistein – which do not have the methoxyl group ($-OCH_3$) in the B ring of the isoflavonoid structure - both in algae and vascular plants can be more active biologically in comparison to biochanin A. Apart from that, the effect of stimulating activity depends mainly on the value of concentration of hormonal or growth factor, the period of its activity, environmental conditions and development phase of individual cells or the whole plant as well.

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