Original Research

The Effect of β-estradiol and Corticosteroids on Chlorophylls and Carotenoids Content in *Wolffia arrhiza* (L.) Wimm. (*Lemnaceae*) Growing in Municipal Bialystok Tap Water

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Abstract

It is well known that many steroid compounds, mainly from large stock-raising farms, more frequently end up in rural or communal sewage systems. It is also known that the duckweed family (Lemnaceae), especially Wolffia arrhiza, is more and more commonly used in the biotechnology of purifying the above-mentioned sewage systems due to its heterotrophic and detoxication ability, as well as its ease of adoption to unfavorable environmental conditions. Therefore, our research analyzes the influence of β-estradiol and chemically and functionally diversified corticosteroids: cortisone, cortisole (glucocorticoids), 11-deoxycorticosterone (mineralocorticoids) and prednisolone (chemical derivative of hydrocortisone) on chlorophylls and carotenoids content in photoautotrophic *Wolffia arrhiza* (*Lemnaceae*), growing in municipal Białystok tap water (rich in minerals but poor in organic components). From the applied steroid hormones in optimal concentration of 10^{-6}M β-estradiol caused the strongest stimulatory effect on photosynthetic pigments, a little less strong - cortisone, slight stimulative - cortisole, and weak 11-deoxycorticosterone. Prednisolone showed a weak inhibitory influence on all types of chlorophylls and carotenoids in comparison with the control culture without exogenous hormones. Applied steroid hormones had a weak stimulative influence over chlorophylls a and b in *Wolffia*; the strongest was β-estradiol between the 5^{th} and the 10^{th} day of cultivation, in the range of 116.5-121.3% in comparison to the control value (100%). The researched steroids had a much stronger influence on carotenoid content, especially β-carotene, alloxanthin (oxygen - poor xanthophylls) and violaxanthin (oxygen - rich xanthophylls). Under the influence of β-estradiol the amount of β-carotene rose by the maximum 160.6%, alloxanthin by 187.9% and violaxanthin by 154.3% in comparison to the control. Our research results demonstrated that β-estradiol and - from applied corticosteroids - cortisone and hydrocorticosterone, had more stimulatory influence on carotenoid content in *Wolffia arrhiza*, but less stimulatory effect on unicellular *Chlorella vulgaris*.

Keywords: *Wolffia arrhiza*, chlorophylls, carotenoids, β-estradiol, corticosteroids, cortisone, cortisole, 11-deoxycorticosterone, prednisolone.

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Introduction

The duckweed family (*Lemnaceae*), mainly the rootless *Wolffia arrhiza*, which lives in aquatic environments rich in organic components, can change its nutrition from phototrophic to mixotrophic and in extreme conditions - with no light - to entirely heterotrophic. These plants are also able to rapid vegetative reproduction, easily adapt to unfavorable environmental conditions, and they have detoxication ability. Thanks to these biological properties and a pleustonic kind of life, the duckweed family is more and more commonly used in biotechnology of purifying agricultural and communal sewage. In the nearest future, *Wolffia* shows exciting prospects for widespread use in small urban or rural bloomeries [1, 2, 3, 4, 5, 6, 7,].

Wolffia arrhiza contains trophic components important to animals such as protein, which constitutes 40-50% of dry weight and is rich in exogenous amino acids: arginine, lizyne, methionine, aspartic acid and glutamic acid. *Wolffia* is also a rich source of carbohydrates, mainly starch, which consist of 40-50% of dry weight of its survival form described as turions. Besides these facts, *Wolffia* contains many various organic compounds rich in nitrogen and phosphorus, and also quite a large amount of magnesium, calcium, zinc and copper. Moreover, *Wolffia* contains a considerable amount of lipids wealthy in non-saturated fatty acids, chlorophylls, carotenoids, flavonoids and vitamins, mainly B_{12} [1, 3, 4, 5, 6, 7]. The latest research [8] documented the presence of steroid hormones - andro- and estrogens - in rootless *Wolffia*. They are characterized by high anabolic activity, which is important in animal rearing.

Therefore, in many countries (chiefly in Eastern Asia, Africa and Southern America) *Wolffia arrhiza* has been used for many years as food and feed additives. In some countries, *Wolffia* biomass is used in cattle breeding, mainly ducks, herbivarous fish, and as a supplement in cattle and swine feed as well. Because of high trophic value and containment of very active metabolites, the rootless *Wolffia* is used in many countries of Africa and Asia in treatment of some diseases. The USA conducts tests using *Wolffia* in space ships as a food addition and oxygen source [3, 6, 7, 8].

It has been known for a long time that some algae and fungi contain steroid hormones from groups of andro- and estrogens, corticosteroids, mainly glucocorticoids, brassinosteroids and ekdysteroids. Many typical animal steroids and their specific derivatives have been reported to be present in many different taxonomic groups of plants [9, 10, 11, 12, 13, 14, 15, 16]. It is known from a few existing fragmentary results [15, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27] that plants' steroid hormones are characterized by a large and diversified biological activity, dependent on their chemical structure, environmental conditions and genetic specification of single organisms. Typical animal steroids exogenously applied to plant organisms often undergo chemical transformation - biotransformation, and become typical phytosteroids, which show large and varied biological activity [14, 15, 16, 17].

Empirical studies conducted on vascular plants such as beans (*Phaseolum vulgaris*), pea seedlings (*Pisum satinum*), *Digitalis lanata* and others (i.e. algae, yeast) demonstrated that glucocorticoids caused a stimulative effect on elongation growth, especially in hypocotyl and lateral roots. Under the influence of optimum concentration, mainly in the range of 10^{-6}-10^{-8}M, the amount of fresh and dry weight significantly increased, especially content of all nucleic acids, proteins, photosynthetic pigments and reducing sugars [15, 19, 20, 22, 26, 28, 29, 30, 31, 32].

On the other hand, typical mineralocorticoids such as aldosterone, 11-deoxycorticosterone and some hydroxyl and methyleno- derivatives in some plant species like peas, beans and lentils inhibited germination of seeds, development of generative organs, vegetative growth and biosynthesis of nucleic acids. Exceptions were proteins and rRNA, of which content were stimulated, and formation of the roots [13, 15, 19, 20, 22, 28, 33].

On the other side, phytoestrogens and typical animal estrogens in the range of optimal concentration of 10^{-6}-10^{-10}M caused intensive stimulation of seed germination, growth and development process of plant embryos, especially in young plants in the beginning of vegetation period. Estrogens stimulated the intensity of the photosynthetic process, increased the accumulation of nucleic acids (mainly RNA), the most biologically active soluble proteins, sugars (mainly monosacharides) and photosynthetic pigments, particularly chlorophylls and carotenoids [12, 13, 15, 21, 23, 24, 25, 34, 35, 36, 37, 38].

Based on these facts, this research focused on comparative analyses of biochemical activity depending on chemical structures of steroid hormones: β-estradiol, cortisone, cortisole, 11-deoxycorticosterone and prednisolone in the range of optimal physiological concentrations.

A fundamental aim of the research was to determine the changes of levels of each photosynthetic pigment under the influence of chemically and functionally diversified steroid hormones taken from groups of females (β-estradiol), glucocorticoids (cortisone, cortisole, prednisolone) and mineralocorticoids - (11- deoxycorticosterone).

Materials and Methods

Rootless *Wolffia arrhiza* was grown for 15 days under stable conditions (growth chamber), i.e. 21 (±1)°C with 12-hour fluorescent light giving a photosynthetically active radiation intensity of 50 µmol $m^{-2}s^{-1}$. The experiments were carried out in 2-litre crystallizers, 19 cm in diameter, containing 1 liter of fluid medium. The containers were covered with perforated plastic film for complete light transmittance.

The *Wolffia arrhiza* was grown in tap water poor in organic components. The mean resource of mineral substances was a generally phototrophic organism. Cultures were conducted in ten replications.

We undertook comparative biochemical studies of the effect of steroid hormones: β-estradiol, cortisone, cortisole, 11-deoxycorticosterone, prednisolone (products of Sigma) differing in chemical structure on the changes of chlorophylls and carotenoids in the *Wolffia arrhiza*, growing in tap water. In the experiments, the above compounds were applied in the optimal concentration ranges of 10^{-4} or 10^{-5} to 10^{-7}M the culture of the *Wolffia arrhiza* [3, 7].

The biomass after dehydration was collected on paper filter, samples of approximately 1g were weighed immediately and covered with 2ml of spectrally pure acetone. After homogenization of the samples, extraction in a nitrogen atmosphere was complete after a period of 12 hours in darkness and a temperature of 20°C.

Pigments were determined by ion-pairing, reverse-phase HPLC. According to Mantoura, 300 μl of ion-pairing reagent (1.5 g tetrabuthylammonium, acetate and 7.7g ammonium acetate, made up to 100 ml with water) was added to 1 ml of the clear extract, Llewellyn [39]. The HPLC equipment consisted of a Shimadzu LC-6A double system pump, driven by a gradient programmer Shimadzu SCL-6B and Rheodyne 7125 injector equipped with a 20 μl loop. Detection was done by a Shimadzu SPD-6AV UV-VIS spectrophotometric detector set on 440nm and Simadzu RF-535 fluorescence detector (λ_{ex}= 430 nm, λ_{em}= 670nm).

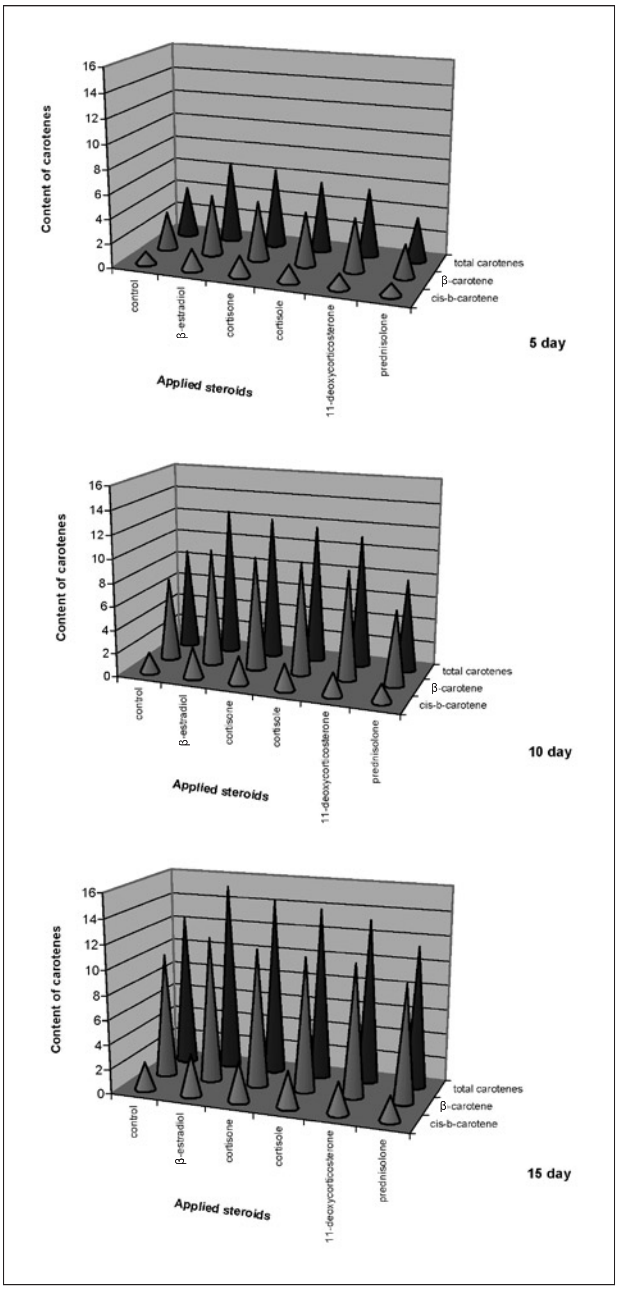

Fig. 1. The effect of steroids applied in the optimal concentration 10^{-6}M on carotenes content (in μg/g biomass).

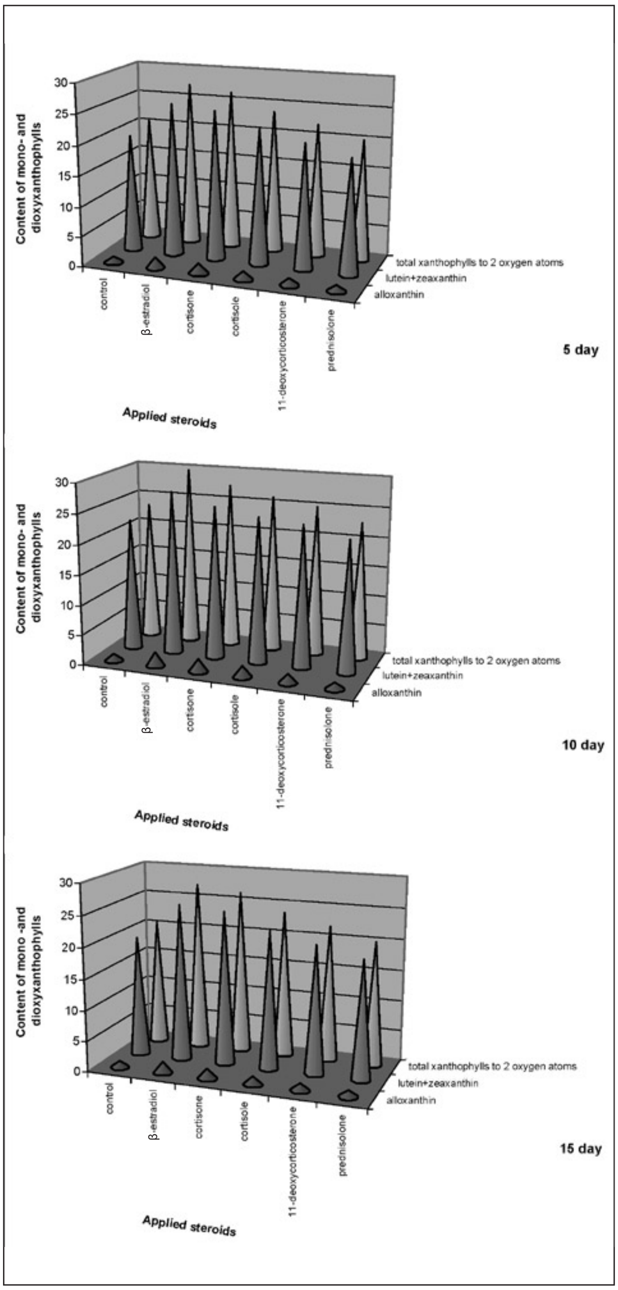

Fig. 2. The effect of steroids applied in the optimal concentration 10^{-6}M on xanthophylls content from 1 to 2 oxygen atoms in molecule (in μg/g biomass).

Chromatographic separation was carried out with a Waters Spherisorb C_{18} ODS 2 column (250×4.6 mm, 5µm particles). The solvents for the HPLC gradient (Baker) used were as follows: solvent A was ion-pairing reagent: water : methanol (10:10:80); solvent B was acetone: methanol (20:80) according to Mantoura, Llewellyn [39].

Pigment standards: chlorophylls a and b and β-carotene were obtained from Sigma Co., whereas derivatives were prepared by methods of Sartory [40]. Other pigment standards were obtained from the International Agency for ^{14}C Determinations, Denmark. The results were evaluated using Student's t-test for unpaired data.

Results and Discussion

The research results on the influence of β-estradiol and corticosteroids: cortisone, cortisole, 11-deoxycorticosterone and prednisolone on photosynthetic pigments in Wolffia arrhiza, in the optimal concentration 10^{-6} M, are presented in Figs. 1-6. The percentage of content of each kind of chlorophylls and carotenoids under the influence of applied steroids in comparison to control treatment as 100%, are contained in Table 1.

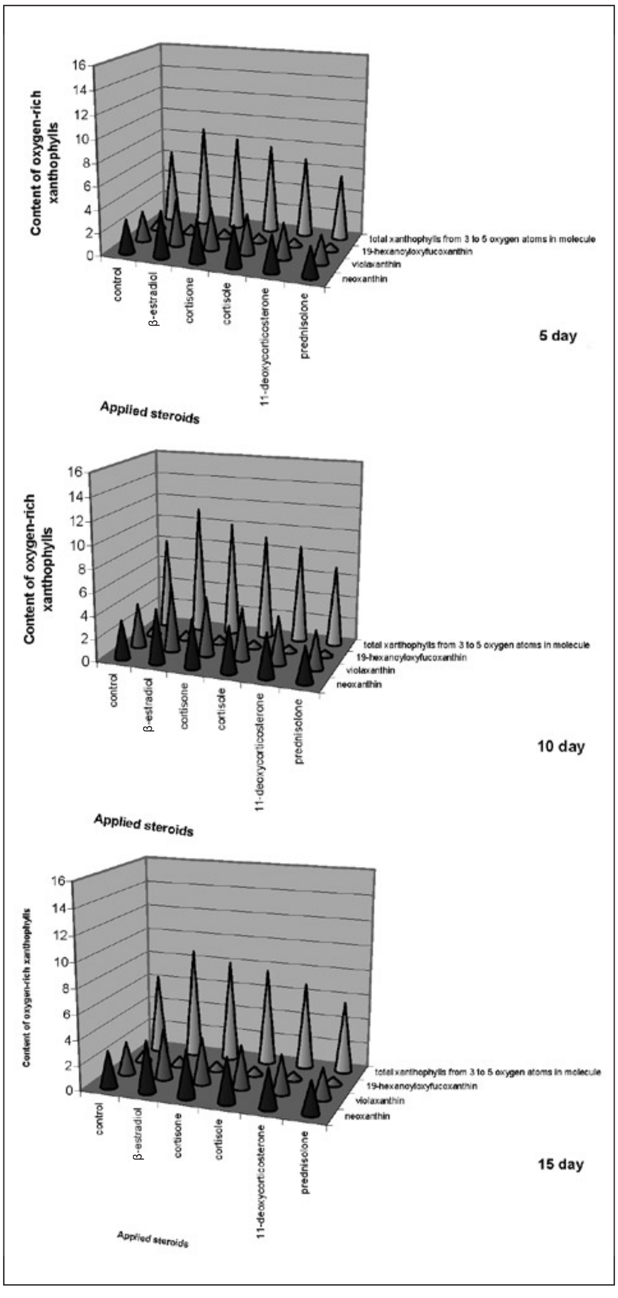

Fig. 3. The effect of steroids applied in the optimal concentration 10^{-6}M on oxygen -rich xanthophylls content (in µg/g biomass).

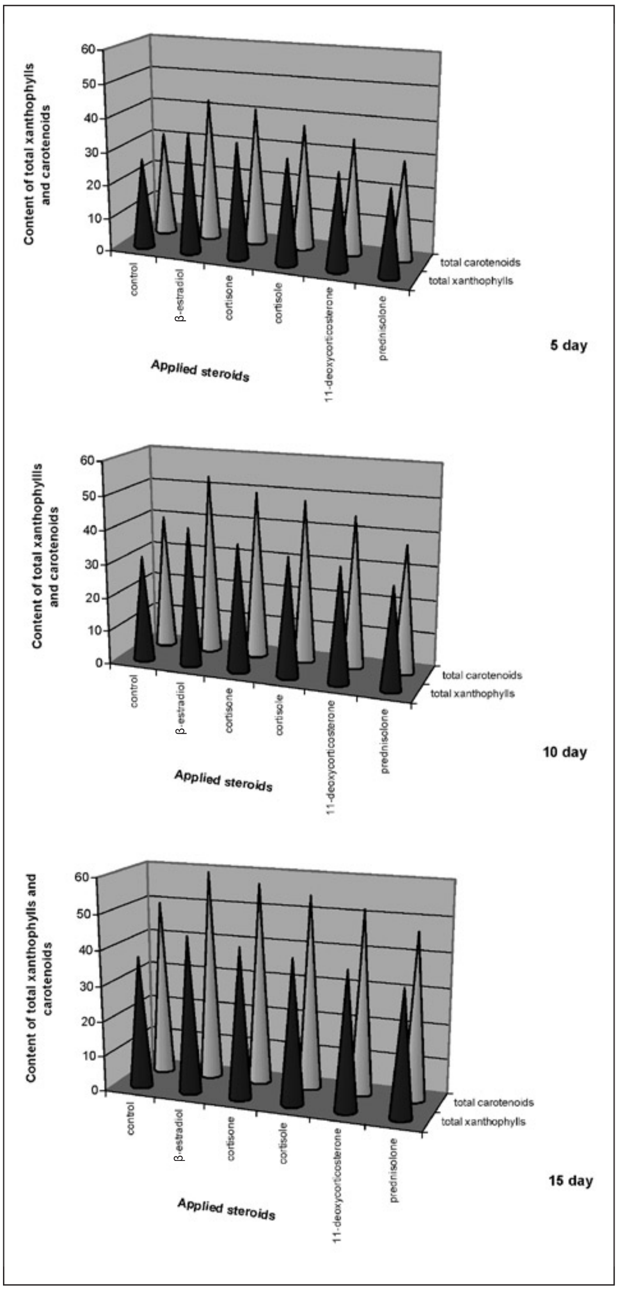

Fig. 4. The effect of steroids applied in the optimal concentration 10^{-6}M on total xanthophylls and carotenoids content (in µg/g biomass).

In general, the content of chlorophyll a and b, β-carotene, in particular xanthophylls, were most intensively stimulated in concentration of 10^{-6}M by β-estradiol, a little less by cortisone, much less by cortisole and in a small degree by 11-deoxycorticosterone. However, prednisolone - synthetic chemical derivatives of hydrocortisone - barely influenced inhibitionally the content of all kinds of chlorophylls and carotenoids in comparison to control cultivation of rootless *Wolffia* as 100%.

Steroid hormones used in research were a derivative of pregnane and their work mechanics affected mainly the nucleus cell and ribosomes, chiefly the process of transcription and translation [15, 18, 30, 31, 32, 37].

The characteristic feature of glucocorticoids is the oxygen atom at carbon C-11 of the cyklopentanoperhydrophenanthrenic skeleton. The main representative of glucocorticoids is a hydrocortisone called cortisole (11β, 17α, 21-trihydroxy- 4 pregnene - 3,20 - dione). On the other hand, cortisone (17α, 21-dihydroxy-4-pregnene-3,11,20-trione) is a dehydrogenated form of cortisole at the C-11 atom, and prednisolone is a synthetic glucocorticoid, which differs from hydrocortisone in possessing the double bond between the 1[st] and the 2[nd] carbon's atom. 11-deoxycorticosterone (21-hydroxy-4-pregnene-3,20-dione), in comparison to glucocorticoids, does not have two atoms of oxygen

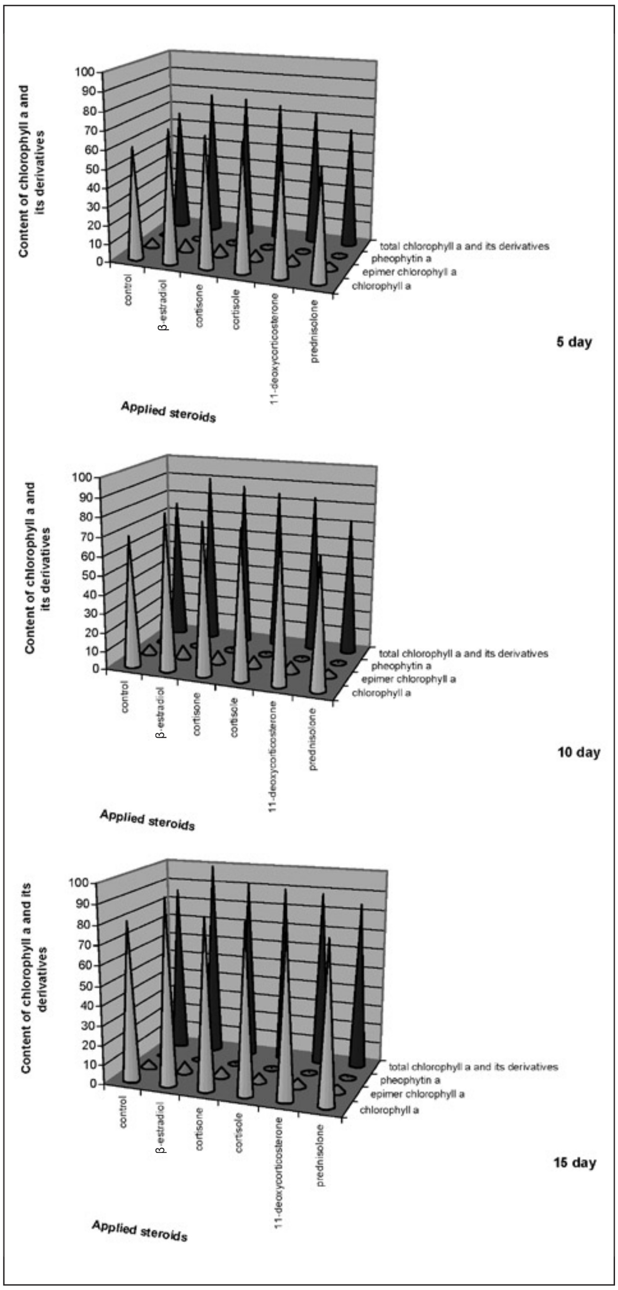

Fig. 5. The effect of steroids applied in the optimal concentration 10^{-6}M on total xanthophylls and its derivatives content (in μg/g biomass).

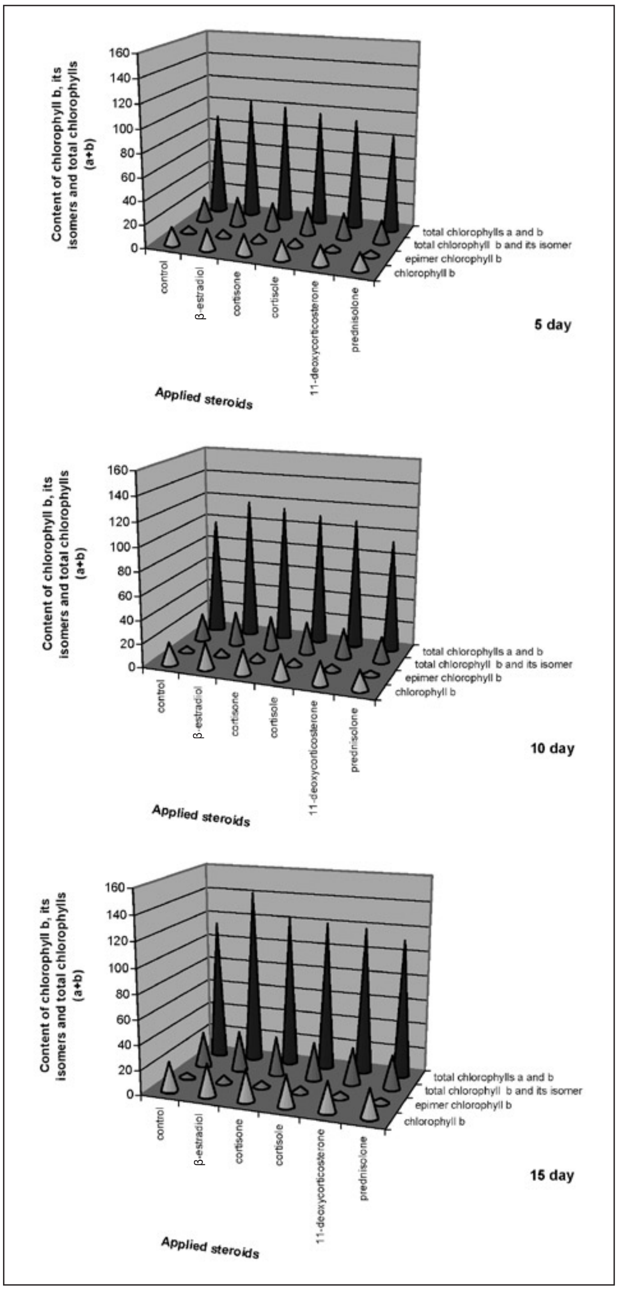

Fig. 6. The effect of steroids applied in the optimal concentration 10^{-6}M on chlorophyll its isomers and total chlorophylls (a+b) content (in μg/g biomass).

Table 1. The percentage amount of principal groups of photosynthetic pigments in the biomass of *Wolffia arrhiza* in relation to the control culture (100%) under the optimal influence of 10^{-6}M β-estradiol and corticosteroids.

Time of culture (days)	Main groups of pigments / Names of steroid hormones	Carotenes	Oxygen-poor xanthophylls	Oxygen rich xanthophylls	Pool xanthophylls	Pool carotenoids	Chlorophyll a and its derivatives	Chlorophyll b and its isomers	Pool chlorophylls (a+b) and their isomers
5	β-estradiol	160.6	132.6	138.7	134.0	137.5	118.3	116.5	117.8
	Cortisone	156.2	128.5	127.6	128.3	132.0	116.2	111.8	115.1
	Cortisole	139.9	115.6	121.2	117.0	120.0	113.1	108.6	112.0
	11-deoxycorticosterone	133.7	107.2	108.6	107.5	111.0	109.3	103.5	107.9
	Prednisolone	88.1	97.9	89.1	95.8	94.8	97.2	91.2	95.8
10	β-estradiol	144.7	127.2	140.2	130.5	133.6	120.2	121.3	120.5
	Cortisone	139.4	117.6	125.8	119.7	123.9	116.1	117.5	116.5
	Cortisole	135.3	112.1	114.6	112.7	120.1	113.3	112.7	113.0
	11-deoxycorticosterone	129.0	107.5	107.0	107.4	112.1	111.5	106.5	110.3
	Prednisolone	90.1	98.1	87.3	95.4	94.2	96.8	91.0	95.5
15	β-estradiol	123.6	121.7	119.9	118.5	119.8	116.0	113.5	124.2
	Cortisone	116.6	113.5	116.2	114.2	114.8	106.9	110.7	107.8
	Cortisole	112.7	109.4	109.3	109.5	110.2	105.2	108.4	106.0
	11-deoxycorticosterone	108.1	104.1	103.9	104.0	105.0	103.9	103.3	103.7
	Prednisolone	93.4	96.2	91.2	94.9	94.5	98.9	95.2	98.0

x-SE less than 5% (n=5); significantly different from the representative value in the control
$p < 0.05 - 0.001$

at carbon atoms C-11 and C-17. The characteristic features of estrogens, in this case β-estradiol, are a quite aromatic character of A ring, the hydroxyl group of phenolic type at the third carbon's atom, and also no methyl group at carbon atom C-6 and double carbon aliphatic part at C-17 [9, 10, 11, 13, 15, 20, 21, 22, 23, 24].

These differences in chemical structure of used steroid sex hormones and corticoid hormones influenced mainly biological and biochemical activities. In this case, they play a basic role in the photosynthetic process.

β-estradiol showed the most intensitive stimulation influence on chlorophyll a and b in *Wolffia*, when used in the concentration 10^{-6} M between the 5^{th} and the 10^{th} day of cultivation and in the range of 116.5-121.3% in comparison to the control value (100%). Cortisone in the range of 116.1-117.5% caused a slightly weaker stimulatory effect, and significantly weaker stimulation was done by 11- deoxycorticosterone from 109.3 to 112.1%. However, prednisolone influenced inhibitionally - on the 91.2-97.2% level in comparison to 100% control.

Under the influence of β-estradiol and applied corticoids, the accumulation of each carotenoid was more strongly stimulated, particularly of β-carotene, which was over 2-3 times more intensive than the stimulation of chlorophyll a and b, also between the 5^{th} and the 10^{th} day of *Wolffia* cultivation. The content of carotenoids were mostly stimulated by β-estradiol in the range of 144.7 - 160.6% and cortisone 139.4-156.2%, a little weaker by cortisole 135.3-139.9% and the weakest by 11-deoxycorticosterone - 129.1-133.7%. However prednisolone - quite inhibitionally - 88.1-90.2%, in comparison to the control cultivation (100%).

The oxygen poor xanthophylls, which contains up to 2 oxygen atoms in a molecule, stimulated the strongest by β-estradiol in maximum of 132.6% and cortisone of 128.5%, weaker by cortisole 115.6% and 11-deoxycorticosterone 107.2%, and a little inhibitionally by prednisolone of 97.9%. From among those xanthophylls the alloxanthin was stimulated the most significantly by β-estradiol at the maximum of 187.9%, cortisone 166.1%, cortisole 147.6% and 11-deoxycorticosterone 125.8%.

Also, the amount of oxygen-rich xanthophylls, which contains 3 to 5 oxygen atoms in a molecule, was the most intensively stimulated by β-estradiol, at a maximum of 140.2%, weaker by cortisone at 127.6% and cortisole at 121.2% and the weakest by 11-deoxycorticosterone at 108.5%. Prednisolone influenced inhibitionally at 87.3% in comparison to control (100%). Among the xanthophylls group, violaxanthin was stimulated the strongest, maximum under β-estradiol influence of 154.3%, cortisone 142.3%, cortisole 133.7% and 11-deoxycorticosterone 119.4%, in comparison to 100% of control.

It has been shown in earlier research made on the unicellular alga *Chlorella vulgaris* [29], that hydrocortisone in the optimum concentration of 5×10^{-5}M worked very stimulative on chlorophylls a and b, in the range of 184-200%, all carotenoids at 170-190%, including carotene at 193-208%, and mainly poor-oxygen xanthophylls from 171 to 191%. Studies conducted on *Chlorella vulgaris* [38] proved that β-estradiol had a stimulative influence in a very small concentration of 10^{-8}-10^{-10}M, at strongest between the 5^{th} and the 15^{th} day of algae cultivation, and caused the highest rate of increase of the amount of chlorophylls in the range of 126-135% and all carotenoids to 175%, in comparison to control cultivation 100%.

At the same time the cultivation of 11-deoxycorticosterone and prednisolone [28] applied to *Chlorella vulgaris* had a stimulatory influence also at the highest in concentration of 5×10^{-6}M of dry weight 110-134%, DNA and RNA in 103-106%. Both applied steroids caused the greatest stimulatory effect on the content of water soluble proteins in 267-296% and reducing sugars in the range 333-375% - compared to control cultivation - (100%). The results obtained from this research demonstrate that β-estradiol and applied corticosteroids, mainly glucocorticosteroids (for example hydrocorticosterone), had more stimulatory influence on chlorophyll and carotenoid contents, mainly carotene in algae *Chlorella vulgaris*, than in *Wolffia arrhiza*.

Many countries use hormonal steroid compounds in animal breeding. They activate the anabolic processes and are passed through excrement and urine to aquatic environments. Various chemical metabolites and products of their modification, steroid components, get to the water ecosystem from algae, fungi, vascular plants and animals, mainly arthropods and vertebrates. Besides, many products of extracellural secretions from bacteria, fungi and algae, mainly green algae, percolate to aquatic environments. Extracellural excretion may reach about 25% of metabolites from photosynthetic processes. From among compounds which go to aquatic ecosystems, a large part of them has trophic characteristics and quite a large group of them has hormonal and vitamin abilities, which regulate growth and metabolism: stimulatively, inhibitionally, modificationally and even toxically for micro- and macro- plant organisms, for example algae and duckweed plants - mainly rootless *Wolffia* [3, 5, 6, 7, 13, 15, 23, 28].

Therefore, the presented research will also have an utilitarian meaning in the future beside its cognitive quality.

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