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The Effect of Sex Steroids and Corticosteroids on the Content of Soluble Proteins, Nucleic Acids and Reducing Sugars in *Wolffia arrhiza* (L.) Wimm. (*Lemnaceae*)

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Abstract

Research on the influence of sex steroids: β-estradiol, testosterone and corticosteroids: cortisone, cortisole (glucocorticoids), 11-deoxycorticosterone (mineralocorticoid) and prednisolone (chemical derivative of hydrocortisone) on changes of soluble proteins, nucleic acids and reducing sugars as a content of *Wolffia arrhiza* (*Lemnaceae*) has been conducted. *Wolffia* has been cultivated in Bialystok's municipal tap water (rich in mineral but poor in organic components) during a 20-day period, in the optimal concentration of 10^{-5} to 10^{-6} M. It has been ascertained that the maximal stimulation of nucleic acids (DNA & RNA) was caused by β-estradiol in the range from 176-181%, testosterone 168-173%, cortisone 154-157%, 11-deoxycorticosterone 152-155%, cortisole 141-148% and prednisolone from 129-131%, in comparison to a 100% control. The soluble proteins content was stimulated the strongest by β-estradiol - 181%, testosterone - 170%, cortisole - 141%, cortisone - 138%, prednisolone - 137%, and weaker by 11-deoxycorticosterone - 128%. Reducing sugars content was stimulated most intensely only on the 5th day of cultivation by cortisone in 165%, 11-deoxycorticosterone - 160-164%, cortisole in 157% and prednisolone in 149%, whereas β-estradiol had a stimulatory influence of 133-138% and testosterone – 119-121% in comparison to 100% control during the whole period of 20 days of *Wolffia arrhiza* cultivation.

Keywords: *Wolffia arrhiza*, soluble proteins, nucleic acid (DNA and RNA), reducing sugars, β-estradiol, testosterone, corticosteroids: cortisone, cortisole, 11-deoxycorticosterone, prednisolone.

Introduction

Thanks to being mixotrophic, *Wolffia arrhiza* has great abilities to adapt to various environmental conditions and to detoxicate agricultural and communal sewage rich in organic substances, which can be used by *Wolffia* as an energy and carbon source in different metabolic pathways. *Wolffia's* biological characteristics show exciting prospects for widespread use in sewage treatment works, mainly in small urban or rural environments [1, 2, 3, 4, 5, 6, 7, 8, 9].

Exogenous usage of steroidal hormones in appropriate concentrations would enable selective growth control of plants, and produced biomass could be utilized as a fertilizer in agriculture and forestry, as well as in reclaiming wastelands, e.g. municipal waste dumps.

Wolffia arrhiza contains trophic components important to animals such as proteins rich in exogenous amino acids, starches, non–saturated fatty acids, vitamins and mineral components. *Wolffia's* vegetative forms contain 40-50% protein of dry weight, while its survival forms (described as turions) contain 40-50% starch of dry weight. *Wolffia* also contains various organic compounds rich in nitrogen, phosphorus and quite a large amount of magnesium, calcium, zinc and copper. Moreover, rootless *Wolffia* - mainly a phototrophic form - contains considerable amounts of chlorophyll, carot-

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enoids, flavonoids and vitamin B_{12}. The latest research on rootless *Wolffia* documented the presence of steroid sex hormones – andro-, estrogens and corticosteroids, which are characterized by high anabolic activity in animals and in a few researched plants [10, 11, 12, 1, 2, 3, 4, 5, 6, 7, 8, 9]. Thus, additional growth stimulation through exogenous and selectively used steroidal hormones seems to be advantageous in the usage of highly trophic biomass as an additive to animal feed, cultivated fish, or it can be a source of highly concentrated protein for cattle and pigs (Oron 1994).

It has been known, that some algae and fungi, and especially many different taxonomic groups of vascular plants contain steroid hormones from groups of andro- and estrogens, corticosteroids, mainly glucocorticoids, sporadically mineralocorticoids, brassinosteroids and ekdysteroids. Many typical animal steroids and their specific derivatives have been reported to be present in many different taxonomic groups of plants [12, 13, 14, 15, 16, 16a].

It is also known from a few existing fragmentary results [17, 18, 19, 20, 21, 22, 23, 24] that plants' steroid hormones are characterized by a large and very diversified biological and metabolical activity, dependent on their chemical structure, environmental conditions and genetic specification of single organisms. Typical animal steroids exogenously applied to plant organisms often undergo a chemical transformation (biotransformation) and become typical phytosteroids, which show large and varied biological activity [25, 13, 14, 17]. Empiric studies conducted on vascular plants such as beans (*Phaseolus vulgaris*), pea seedlings (*Pisum sativum*), on alga *Chlorella vulgaris* and on yeast *Saccharomyces cearevisiae* demonstrated that glucocorticoids cause a stimulative effect on elongation growth, especially in hypocotyl and lateral roots, increasing the amount of fresh and dry weight, content of RNA, proteins, photosynthetic pigments and reducing sugars [26, 27, 28, 29, 30, 31, 31a, 21, 22, 24].

On the other hand, typical mineralocorticoids such as aldosterone, 11-deoxycorticosterone and some hydroxyl and methyleno- derivatives inhibited germination of seeds, the development of generative organs, vegetative growth and biosynthesis of nucleic acids as well. Exceptions were proteins and rRNA, of which the contents were stimulated, in lateral roots. [32, 33, 34, 20, 21, 22, 29].

Estrogens demonstrated the highest biological activity in researched plants. Used in very small optimal concentrations of 10^{-7}–10^{-10} M, they caused an intensive stimulation of seed germination, growth and development process of plant embryos, especially in young plants in the beginning of the vegetation period. Estrogens stimulated the intensity of the photosynthetic process and other anabolic processes, increased the accumulation of nucleic acids (mainly RNA), the most biologically active soluble proteins, sugars (mainly monosaccharides) derived from photosynthetic process, and photosynthetic pigments, particularly chlorophylls and carotenoids.

Typical male steroid hormones (androgens), mainly testosterone and its derivatives, stimulated only the germination of seeds and the development of plants' embryos in the beginning of the heterotrophic period. Androgens promoted the formation of stamens and spermatozoid production in the process of flower development. While the vegetative development of small plants begins with its photosynthesis process, it is inhibited under the influence of androgens [35, 36, 37, 38, 39, 14, 16, 16a, 22, 24, 28, 31, 31a].

Based on the literature mentioned above, research was conducted on the influence of the main representatives of sex steroids (β-estradiol and testosterone), typical glucocorticoids (cortisone, cortisole and its synthetic derivative prednisolone) and also 11- deoxycorticosterone (mineralocorticoids) in optimal concentration of 10^{-5} – 10^{-7} M on the content changes of soluble proteins, reducing sugars and nucleic acid (DNA and RNA) during a 20 day *Wolffia* cultivation.

Selective growth control of this plant through the use of animal steroidal hormones in various concentrations, stimulating in different degrees the amount of proteins and reducing sugars in *Wollfia* cells seems to be advantageous in the quest to find new food sources; mainly inexpensive and high quality ingredients for the food industry, e.g. alcohol production, where the main ingredient is starch. The above requires more detailed empiric studies.

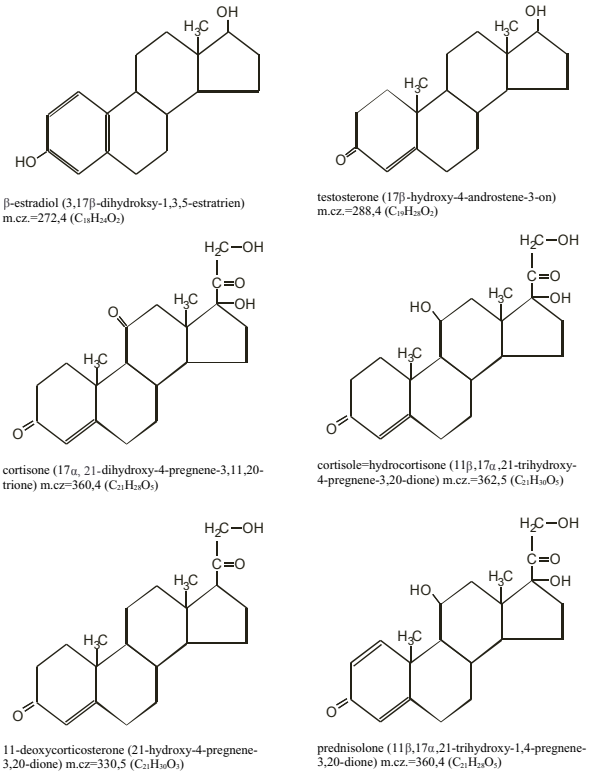

Fig.1. The structural and sumaric patterns and casual and systematic names of applied steroid hormones and their molecule mass (Sigma-Aldrich Firm).

Table 1. The influence of steroids at optimal concentrations on analyzed matabolites in *Wolffia arrhiza* in 5th and 10th day of cultivation.

Names of steroid hormones	Applied concentration (M)	Content of water-soluble proteins (mg/g biomass)		Content of nucleic acids (DNA+ RNA) (mg/g biomass)		Content of reducing sugars (µg/g biomass)	
		5th day of cultivation	10th day of cultivation	5th day of cultivation	10th day of cultivation	5th day of cultivation	10th day of cultivation
Control	0	15.51	18.68	3.99	4.11	177.65	233.14
ß- estradiol	10^{-5}	23.57	29.41	5.78	6.34	210.45	301.82
	10^{-6}	**26.84**	**33.84**	**6.81**	**7.43**	**236.82**	**321.40**
	10^{-7}	18.61	22.03	5.48	6.03	185.00	251.71
Testosterone	10^{-5}	22.42	26.38	5.64	6.26	208.78	253.20
	10^{-6}	**26.33**	**29.03**	**6.60**	**7.13**	**211.56**	**269.50**
	10^{-7}	17.22	20.92	5.53	5.95	197.40	259.22
Cortisone	10^{-4}	19.41	21.59	5.20	5.59	256.80	275.86
	10^{-5}	**20.74**	**25.69**	**6.26**	**6.35**	**292.50**	**302.67**
	10^{-6}	19.44	23.46	5.82	6.07	253.00	267.17
Cortisole (hydrocortisone)	10^{-5}	20.11	21.95	4.94	5.21	252.14	276.78
	10^{-6}	**21.92**	**23.59**	**5.52**	**6.08**	**279.14**	**312.92**
	10^{-7}	20.69	22.66	5.13	5.64	260.67	293.22
Prednisolone	10^{-5}	19.86	20.91	4.53	5.02	248.20	274.86
	10^{-6}	**21.25**	**22.51**	**4.93**	**5.26**	**264.83**	**296.13**
	10^{-7}	18.71	19.47	4.16	4.56	249.00	260.17
11-deoxycorticosterone	10^{-4}	19.18	19.80	5.86	6.09	285.00	306.50
	10^{-5}	**19.93**	**21.74**	**6.06**	**6.39**	**292.00**	**314.38**
	10^{-6}	17.99	20.42	5.64	5.67	254.33	277.38
Range of standard deviation		0.35 - 1.58	0.19 – 1.48	0.19 – 0.54	0.18 – 0.40	0.97 – 1.96	0.43 – 1.75

Material and Methods

The experiments were carried out on fresh homogenous cultures of *Wolffia arrhiza* (L.) Wimm (*Lemnaceae*). The rootless *Wolffia* was grown for 20 days under stable conditions (growth chamber), i.e. 21(±1)°C with 12 hour fluorescent light giving a photosynthetically active radiation (PAR) intensity of 50 µmol $m^{-2}s^{-1}$. *Wolffia arrhiza* was grown in 2-litre crystallizers, 19 cm in diameter, containing 1 litre of fluid medium. The containers were covered with perforated plastic film of complete light transmittance. Cultures were conducted in ten replications.

In view of this we undertook comparative biochemical studies of the effects of the main representative of sex and cortical steroids (Fig. 1) differing in chemical structure on the content of soluble proteins, nucleic acids and reducing sugars in *Wolffia arrhiza*. In the experiments the above steroid compounds were applied in concentration ranges of 10^{-4} or 10^{-5} to 10^{-7}M on the 15th day of culture, when Wolffia arrhiza had reached the maximal level of development and metabolism [40, 41, 42, 43, 3, 5, 7].

The biomass was collected and after dehydration on filter paper, samples of approximately 0.1-0.2 g were weighed, according the method of common use in laboratory practice, was applied after Hallegraef [45]. The total content of these biochemical parameters was determined as µg and mg in g of *Wolffia arrhiza* biomass [44, 45].

The fraction of water-soluble proteins was analyzed spectrophotometrically using biuretic reaction after homogenization of *Wolffia arriza* in their water extraction, as described by Ostrowski and Filipowicz [42].

A similar preparation procedure was employed for spectrophotometric determination of reducing sugars, according to Somogyi – Nelson's (Hodge and Hofreiter, [46]) method. The total content of nucleic acids (DNA + RNA) was determined spectrophotometrically using the orcine reagent, as described by Kłyszejko-Stefanowicz [44].

A Shimadzu 1201 UV-VIS spectrophotometer (Japan) was used for all measurements.

The results were evaluated using Student's t-test for unpaired data [47].

Results and Discussion

The research results on the influence of β-estradiol and testosterone and corticosteroids (cortisone, cortisole, prednisolone and 11-deoxycorticosterone) on absolute numbers of soluble proteins, nucleic acids (DNA + RNA), reducing sugars (aldohexoses) in *Wolffia arrhiza* in the optimal concentration from 10^{-4} or 10^{-5} to 10^{-6} or 10^{-7} M are contained in Tables 1-2. The percentage of content of each analyzed metabolites under the influence of steroid sex hormones and corticoids in the most effective concentration of 10^{-5} or 10^{-6} M in comparison to control value – 100% are presented in Figs. 2-4.

In general, all applied steroids (sex hormones and glucocorticoids) displayed a considerable biological activity of stimulative character, maximal concentration in the range of 10^{-5} – 10^{-6} M. The most intensive stimulation influence on proteins and nucleic acids (DNA + RNA)

Table 2. The influence of steroids in optimal concentration on analyzed matabolites in *Wolffia arrhiza* in 15th and 20th day of cultivation.

Names of steroid hormones	Applied concentration (M)	Content of water-soluble proteins (mg/g biomass)		Content of nucleic acids (DNA+ RNA) (mg/g biomass)		Content of reducing sugars (μg/g biomass)	
		15th day of cultivation	20th day of cultivation	15th day of cultivation	20th day of cultivation	15th day of cultivation	20th day of cultivation
Control	0	23.59	26.40	4.53	4.97	277.63	302.00
ß- estradiol	10^{-5}	32.18	34.86	7.23	7.99	320.25	375.10
	10^{-6}	**34.95**	**39.94**	**7.96**	**9.01**	**374.30**	**407.25**
	10^{-7}	25.69	26.97	6.47	7.11	288.67	338.29
Testosterone	10^{-5}	28.23	32.04	6.75	7.30	289.78	325.00
	10^{-6}	**30.67**	**36.24**	**7.60**	**8.10**	**321.25**	**366.33**
	10^{-7}	22.63	25.59	6.19	6.52	265.88	323.22
Cortisone	10^{-4}	22.53	25.21	5.99	6.34	302.00	330.71
	10^{-5}	**27.36**	**32.70**	**6.71**	**7.23**	**338.50**	**382.33**
	10^{-6}	24.39	27.39	6.22	6.69	286.50	307.25
Cortisole (hydrocortisone)	10^{-5}	23.54	24.59	5.66	6.04	307.86	340.25
	10^{-6}	**25.54**	**31.95**	**6.39**	**6.80**	**360.50**	**396.22**
	10^{-7}	23.98	26.52	6.00	6.34	321.14	352.14
Prednisolone	10^{-5}	23.75	25.07	5.31	5.92	299.33	339.83
	10^{-6}	**24.56**	**27.18**	**5.94**	**6.44**	**323.14**	**376.75**
	10^{-7}	21.56	23.54	4.98	5.52	281.00	316.38
11-deoxycorticosterone	10^{-4}	21.05	24.45	6.31	6.64	315.80	359.17
	10^{-5}	**22.92**	**26.84**	**6.59**	**6.96**	**345.00**	**397.00**
	10^{-6}	21.79	25.58	5.97	6.35	302.43	342.00
Range of standard deviation		0.20 – 0.95	0.25 – 1.88	0.10 – 0.29	0.36 – 0.75	0.50 – 1.87	0.65 – 1.96

showed, between the 5th and 10th days of cultivation of sex steroids, β-estradiol and weaker testosterone.

The total content of nucleic acids was stimulated the strongest by steroids (β-estradiol, testosterone) at a concentration of 10^{-6} M in the range of 180.8-181.1% in comparison to 100% control. Also, residual concentration of above mentioned steroid hormones - 10^{-5} and 10^{-7} M - had a stimulative influence and increased the content of DNA and RNA in the range of 140-150%.

Among applied corticosteroids in optimal concentration, the most intensive stimulative influence on nucleic acids caused cortisone - 157.1%, 11-deoxycorticosterone 155.3%, cortisole 147.8% and the weakest effect - prednisolone 129.4%.

Also, contents of soluble proteins were most intensively stimulated by sex steroids between the 5th and 10th days of *Wolffia* cultivation, in the concentration of 10^{-6}M: β-estradiol in the range of 173.1-181.2%, testosterone 169.8-155.4% with reference to control value of 100%. A considerable decrease of stimulation to the level of 155-130% was noted between the 10th and 15th days of Wolffia cultivation. Corticosteroids caused significantly weaker stimulatory effect on protein content, and the strongest between the 5th and 10th days of cultivation: cortisone 133.7-137.5%, prednisolone 137.0-120.5% and cortisole 141.3-126.3% and the weakest of 11-deoxycorticosterone from 128.5 to 116.4%, compared with the control 100% .

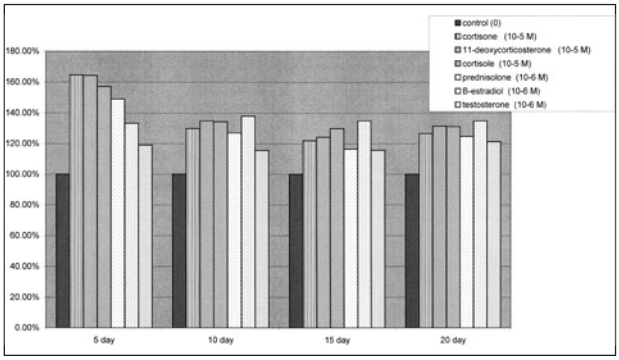

Fig.2. The maximum stimulation of reducing sugars content under the influence of applied steroids in *Wolffia arrhiza* cultivation (in % of biomass), in comparison to 100% control.

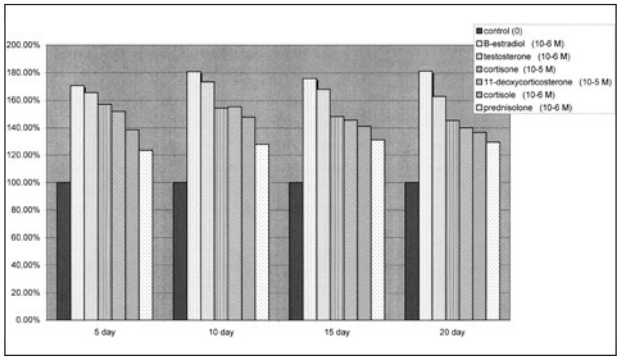

Fig.3. The maximum stimulation of nucleic acids (DNA+RNA) content under the influence of applied steroids in *Wolffia arrhiza* cultivation (in % of biomass), in comparison to 100% control.

However, the content of reducing sugars (aldohexoses) in *Wolffia arrhiza* was considerably stronger, stimulated by all applied corticosteroids, than the sex steroids β-estradiol and testosterone. The highest content of sugars was noted on the 5th day of the rootless *Wolffia* culture under the optimum concentration of 10^{-5} M cortisone: 164.7% as compared with the control (100%). Similar stimulation was documented under the influence of 11-deoxycorticosterone in the concentration 10^{-4}-10^{-5} M, also on the 5th day of cultivation in the range of 164.3-160.4%. The content of sugars was similarly stimulated on the 5th day of culture by cortisole in the optimum concentration 10^{-6} M - 157.1%, and considerable weaker at 149.1% level under the influence of 10^{-6} M prednisolone. Sex steroids caused weaker stimulation of sugar content, throughout the whole 20-days period of *Wolffia arrhiza* cultivation, mainly in the concentration of 10^{-6}M. The stimulation of sugar contents under the influence of β–estradiol reached 133.3-137.9%, and 115.6-121.3% when affected by testosterone - in comparison with 100% control.

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 [14, 19, 26, 30, 32, 36].

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, 17λ, 21-trihydroxy-4 pregnene-3,20-dione). On the other hand, cortisone (17λ, 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 1st and 2nd carbon atoms. 11-deoxycorticosterone (21-hydroxy-4-pregnene-3,20-dione) mineralocorticoid in comparison to glucocorticoids does not have two atoms of oxygen at carbon atoms C-11 and C-17. The characteristic feature of sex steroids in comparison to corticosteroids is the presence of a hydroxyl group at carbon atom C-17, instead of aliphatic double-carbon fragment with two atoms of oxygen.

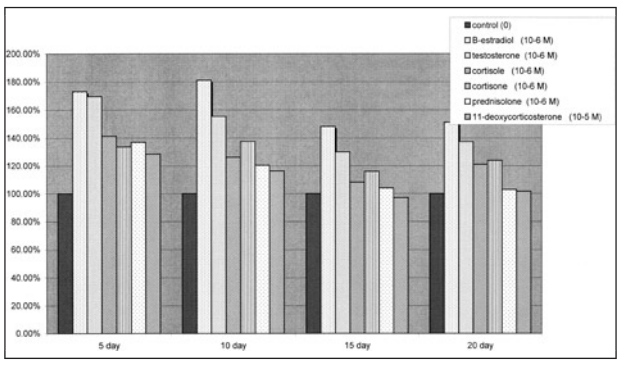

Fig.4. The maximum stimulation of soluble proteins content under the influence of applied steroids in Wolffia arrhiza cultivation (in % of biomass), in comparison to 100% control.

The characteristic features of estrogens, in this case β-estradiol, are an aromatic character of A ring, the hydroxyl group of phenolic type at the third carbon atom, and also no methyl group at carbon atom C-6. However, testosterone, which belongs to androgens, in comparison to β-estradiol, has only one double bond in A ring between carbon atom C-4 and C-5, methyl group at carbon atom C-6, kethone group instead of hydroxyl group at carbon atom C-3.

The mentioned-above differences in chemical structure of used steroid sex and corticoid hormones had a major influence over biological and biochemical activities.

In this case, the research was focused on changes of total content of water-soluble proteins, reducing sugars – aldohexoses, and nucleic acids (DNA+RNA). From among used hormones, sex steroids showed stronger stimulation (strong β-estradiol, than testosterone) of nucleic acids and proteins content in *Wolffia arrhiz*a cultivation, in comparison to analyzed glucocorticoids and mineralocorticoids. However, the content of reducing sugars - aldohexoses - was the most intensely stimulated by all researched gluco- and mineralocorticoids, in comparison to sex steroids.

The obtained results are similar to data obtained from a few publications relating to research of exogenously applied sex steroids and corticosteroids on growth and biochemical changes in vascular plants: seeds, peas, beans and lentils [46, 16, 16a, 20, 21, 22, 23, 33].

Mentioned above empirical research showed that corticosteroids and β-estradiol have a minimal stimulative effect on photosynthetic pigment content, mainly chlorophylls, and it intensively stimulates nucleic acid content and a fraction of water-soluble proteins, which are the most metabolically active.

Prior studies conducted on unicellular alga *Chlorella vulgaris* [48, 28, 29] documented that under the influence of optimal concentration of 10^{-8} – 10^{-10} M β-estradiol [28], showed stimulating activity of protein and sugar contents, the strongest between the 5th and 15th days of cultivation in the range from 110-115 to 121 and 123%, in comparison to control value - 100%. Also, biological activity of hydrocortisone in *Chlorella vulgaris* was stronger than of progesterone, in reference to protein content [48, 49]. Hydrocortisone in the optimal concentration of $5x10^{-5}$M proved the highest stimulation of the water-soluble proteins ranging from 130 to 197%, monosaccharides 179%, and soluble sugars 271%, while progesterone applied in the optimal concentration of $5x10^{-6}$M stimulated proteins content at 133-155%, sugars from 150 to 200%. Nevertheless, the research done on 11-deoxycorticosterone and prednisolone applied in maximal concentration of $5x10^{-6}$ M in *Chlorella vulgaris* cultivation [29] noted intensive stimulation of the content of water soluble proteins reaching 167-196%, reducing sugars 233-275%, and slightly nucleic acids (DNA+RNA) from 103 to 106% compared to the control 100% between the 5th and 15th cultivation day.

Moreover, based on Kopcewicz's experiments [31, 31a, 38] ran on seeding of pea (*Pisum sativum*) and pine (*Pinus silvestris*) it is known, that under the influence of

used estrogens: estrone, estradiole and estriole in concentration of 0.1-0.001 μg to one plant, the elongation growth was stimulated by 30%, and endogenous sugars and gibberelins content. It was also found from Mohsen et. al. [46] studies carried out on seeds of *Phormidium angustissimum*, that β-estradiol in concentration 10^{-6}M increased monosaccharides content by about 10%, disaccharides by about 25%, soluble proteins of 16% and total content of lipids from 8 to 12%.

Results from this presented research, in comparison to literature data mentioned above, proved that β-estradiol in *Wolffia arrhiza* cultivation showed the most intensive stimulation influence on the total content of nucleic acids (DNA+ RNA) and soluble proteins, in comparison to *Chlorella vulgaris* culture. Stimulating activity on sugars content, mainly reducing sugars, under the influence of β-estradiol was minimally stronger in *Wolffia arrhiza* than in *Chlorella vulgaris*.

Corticosteroids applied to DNA and RNA content showed several times greater activity levels in *Wolffia arrhiza* than in *Chlorella vulgaris*. However, their stimulating effect on soluble proteins and sugar contents - mainly aldohexoses - was considerably stronger in *Chlorella vulgaris* than in *Wolffia arrhiza* cultivation.

Many various metabolites and steroid components from dead plants, animal organisms and cattle farm sewages leach to the hydro- and lithosphere. Also, various products of extracellular secretion from bacteria, fungi and algae (mainly *Chlorophyceae*) pass through to aquatic environments as metabolites from photosynthesis processes. A number of those components, besides being trophic, also posses hormonal characteristics, as well as growth and metabolism regulation abilities of micro and macro organisms, e.g. algae and duckweed [1, 2, 5, 8, 14, 24, 33, 40, 41].

Therefore, the presented research, which focused on the influence of exogenous steroids on growth, biochemical and metabolic changes in mixotrophic *Wolffia arrhiza*, will also have a utilitarian meaning in the future, besides its cognitive quality.

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