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The Effect of Flavonoids on Hydra – Alga Symbiosis and the Implementation of the Given Experiment in Schools

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Abstract

Hydra (Cnidaria) is a cosmopolite freshwater invertebrate that establishes symbiosis with unicellular algae. Its simple body is comprised of three layers: epidermis, mesoglea and gastrodermis. The goal of this research was to determine the effect of flavonoids quercetin and naringenin upon hydra – alga symbiosis, to determine how motivated teachers are to perform such a mode of teaching and whether this motivation depends on their age, years of working experience, the school they work in, available time, space and material. An anonymous survey on a sample of 100 biology teachers was used in the research. Hydras and algae were treated with quercetin and naringenin in concentrations of 0.2, 0.25 and 0.3 gL^{-1} *and compared to the control, and Chlorella bioassay was used. Morphological changes included migration, reaction to mechanical stimuli, asexual reproduction, secretion of mucus and mortality. Cyto-histological analysis revealed changes of all three layers and growth inhibition. Naringenin exerted altogether higher impact than quercetin. Survey results indicated that teachers are willing to use this experiment in school, and that most schools have the minimum required equipment. Because of the possibility of adapting this experiment in different temporal and spatial conditions, its performance in school is suggested.*

Key words: *learning; motivation; naringenin; quercetin; teaching.*

Introduction

Passive learning results in students acquiring incomplete knowledge prone to degradation and also less applicable in everyday life (Mazur, 1997). Therefore, it is necessary to employ methods of active learning and to educate teachers in organizing extracurricular activities for students. Although teachers accept that methods of active teaching are good, only a limited number of teachers actually use them in class. It is very important for students that the concepts of natural fields are adopted through original reality and with the help of practical work because it provides them with easier and faster knowledge acquisition and experimental learning (Hofstein & Mamlok-Naaman, 2007; Hofstein & Kind, 2012; Lewthwaite, 2014). It is also essential that teaching of natural sciences is properly designed as active learning and observation of natural reality so that students can apply them in everyday life (Cranton & Taylor, 2012; Meyer, 2005).

Genus *Hydra* (Cnidaria) belongs to freshwater invertebrates. The body of hydra is comprised of epidermis, mesoglea and gastrodermis. Green hydra establishes a symbiotic relationship with algae of the genus *Chlorella* (Douglas, 1994). Since green algae are a basis of many food chains, it is necessary to be acquainted with the effect of xenobiotics on algae and other components of a food chain. Hydras can be very useful organisms for teachers because brown hydra is a free-living organism, and green hydra represents an excellent example of an endosymbiotic relationship. Endosymbiosis is a form of symbiotic relationship in which one cell inhabits the other, i.e. at least two genomes of different evolutionary origin exist within the same cytoplasm (Ebringer & Krajčovič, 1994). Because of the cosmopolite availability, hydra represents an organism desirable for practical work in schools. In addition, different concepts can be adopted with the help of hydra – alga symbiotic system, offering a valuable basis for many teaching units.

Quercetin and naringenin belong to the most common bioflavonoids used in the human nutrition, but because of the differences in the chemical structures they are separated into different groups. Quercetin belongs to flavonols and naringenin belongs to flavanones. They are readily available and can be purchased at pharmacies and supplements stores. Although quercetin seems to be the first logical choice to induce positive or negative effects because of its chemical structure, it has been shown that naringenin is also a very effective flavonoid (Sirovina, Oršolić, Zovko Končić, & Gregorović, 2016), so it is well suited to compare their actions under the same conditions. Well-described properties of quercetin and naringenin are their abilities to act as antioxidants, but they can also produce a pro-oxidative effect, depending on their concentration and the source of free radicals within the cell (Kovačević & Matulić, 2013; Lee, Kim, Park, Chung, & Jang, 2003).

This paper aims to determine which of these organisms, symbiotic or free-living, have a greater ability to survive under changed environmental conditions i.e. treatment with quercetin and naringenin, and which of the two flavonoids has more deleterious effect. Previous findings show a pronounced effect of different xenobiotics (antibiotics, metals) upon hydra – alga symbiosis, and that symbiotic hydra survives better in an unfavourable environment than non-symbiotic species (Kalafatić, Kovačević, Ljubešić, & Šunjić, 2001; Kovačević, Želježić, Kalafatić, & Horvatin, 2007). The effect of quercetin has so far shown to be deleterious on hydra – alga symbiosis, including morphological and behavioural changes such as depigmentations and deformations (Kovačević & Matulić, 2013). In this follow-up research we investigated the effect of naringenin on green hydra and the effect of quercetin and naringenin on free-living brown hydra, two endosymbiotic algal species isolated from green hydra and one free-living algal species. The goal of this research was to determine and compare the effect and possible extent of action of the flavonoids quercetin and naringenin upon hydra – alga symbiosis, i.e. upon freshwater cnidarians, the isolated endosymbiotic algae and their free-living relative, as well as to determine the morphological, cyto-histological and Chlorella bioassay effects and any specificities that might occur.

Since the adaptation of a particular experiment requires significant amount of work and feedback from the teacher, and its performance requires great motivation, the goal of this research was also to determine how motivated teachers are to perform such a mode of teaching during extracurricular activities, and whether this motivation depends on their age, years of work experience and the school they work in. The aim of this study was also to determine whether schools fulfil the minimal requirements for teachers to perform this or similar experiment, especially the availability of materials and equipment, space and time for its application.

Materials and Methods

Experimental organisms used in these experiments were green (*Hydra viridissima* Pallas, 1766) and brown hydra (*Hydra oligactis* Pallas, 1766), green algae isolated from green hydra host (*Desmodesmus subspicatus* (Chlorophyceae) (Chodat) Hegewald and Schmidt, and *Mychonastes homosphaera* (Chlorophyceae) (Skuja) Kalina and Punčochářová), and a free-living related alga *Chlorella vulgaris* Beij [K&H, 1992], strain SAG 211-11b.

Comparative toxicity test of quercetin and naringenin was performed. Four groups of 10 unfed green hydras were used in a 72-hour experiment with naringenin and four groups of 10 unfed brown hydras were used in a 72-hour experiment with quercetin and naringenin each, in 60 mL glass dishes (concentrations of 0.2, 0.25 and 0.3 gL^{-1} for each flavonoid and a control, 50 mL of each). So far, the experiments on the effect of quercetin upon green hydra have already been performed (Kovačević & Matulić, 2013), and in this paper those results are compared with the obtained results. Morphological changes in hydras included mortality, excitability, presence of mucus, migration and asexual reproduction, and cyto-histological changes included damage to the cellular layers ectodermis and gastrodermis, and non-cellular layer mesoglea. Hydra specimens were taken for standard histological analysis after the 72-hour experiment. For all three species of algae a 21-day Chlorella bioassay was applied (Kovačević, Jelenčić, Kalafatić, & Ljubešić, 2008), using also four groups for each algal species, 6 tubes per species and per concentration (the same concentrations of quercetin, naringenin and a control). Algal cells were analysed after the 96-hour treatment. Parameters of cytological changes in algae included growth inhibition, fragmentation of chloroplast, bleaching and cellular irregularities. The experiments were performed in duplicate. The samples were observed using a stereo-microscope and the analysis of hydras and algae was performed by using a Nikon Eclipse E600 light microscope. Micrographs were recorded by using a Nikon D7200 camera.

The method of anonymous survey was used for contacting biology teachers; the sample comprised 100 teachers, 65 % of whom were primary and 35 % high school teachers. The survey analysed the level of teacher motivation depending on school type, their age and years of work experience. The motivation of teachers to perform the suggested experiment in class was determined on a 1 to 5 scale, with 1 representing complete lack of motivation, and 5 representing high motivation. The second part of the survey was composed to examine whether a particular school offered extracurricular activities, and whether teachers have time, space and equipment necessary for the performance of the presented experiment. The surveys were analysed and statistically processed by 'Microsoft Office Excel 2013'. Statistical significance of the results obtained in this research was tested using the analysis of variance (ANOVA) and Student's t-test ($p<0.01$).

Results and Discussion

During the experimental work it was established that quercetin and naringenin caused morphological and cyto-histological changes in hydras and cytological changes in endosymbiotic and related free-living algae. Higher concentrations caused greater changes on experimental animals, but the most intensive morphological changes were observed in 0.25 gL^{-1} of naringenin. It is known that already lower concentration of toxicants may cause greater effect on the test organisms (Pavlica, Klobučar, Vetma, Erben, & Papeš, 2000). This was manifested by migration of hydras in the experimental dish, slower reaction to mechanical stimuli, excreting larger volumes of mucus, and occurrence of mortality after a 72-hour treatment (Tables 1 and 2).

Table 1
Mortality of green and brown hydra treated with naringenin (%)

hydra species	green hydra	brown hydra	green hydra	brown hydra	green hydra	brown hydra
period of treatment / hs concentration of naringenin / gL^{-1}	24	24	48	48	72	72
control	0	0	0	0	0	0
0.2	0	0	0	10	0	15
0.25	0	0	25	70	100	100
0.3	0	0	0	10	0	75

Table 2

Mortality of brown hydra treated with quercetin (%)

hydra species	brown hydra		
concentration of quercetin / gL^{-1} \ period of treatment / hs	24	48	72
control	0	0	0
0.2	0	10	20
0.25	10	10	20
0.3	10	10	30

Depending on the concentration, xenobiotics may cause morphological changes and changes in behaviour and cellular structure (Kalafatić et al., 2001). Migration of brown hydra was the first visible change, and by the increase of concentration, brown hydra reacted to mechanical stimulus proportionally slower. Migrations are the possible result of searching for better micro-environmental conditions (Kovačević et al., 2007). Secretion of mucus was observed in the less viable specimens, i.e. those that did not migrate towards the water surface and that reacted less to mechanical stimuli. Secretion of the increased amount of mucus is a result of damage to the ectodermal myoepithelial cells, and serves as a mechanism of detoxification (Kovačević, Gregorović, Kalafatić, & Jaklinović, 2009). Green hydras went through the process of asexual reproduction and their number increased up to 35 % during the experiment, while brown hydras showed no signs of budding. Green hydra treated with quercetin showed a higher rate of migration in higher concentrations, but the highest rate of mucus production in the middle concentration (0.25 gL^{-1}) and irregular reactions to mechanical stimuli. In lower concentrations mortality was lower than in brown hydra, but in the highest concentration mortality of green hydra was higher (46.6 %) (Kovačević & Matulić, 2013) than in brown hydra (30 %). Naringenin exerted higher mortality of hydras than quercetin, and higher mortality rate was present in brown hydra.

Besides morphological changes, cyto-histological changes in hydras were also monitored. Changes and damages to all layers of brown hydra in treatment with quercetin were evident. Higher concentration caused the migration of zymogen cells and I-cells towards mesoglea and their dedifferentiation in other cell types in order to regenerate the hydra body. Mesoglea was wrinkled and partially changed in thickness (Figure 1). In green hydra treated with quercetin the same trends are present, and degradation and damage of algal symbionts is noticed (Kovačević & Matulić, 2013). Only a small amount of changes in treatment with naringenin occurred. In the treatment of green hydra with naringenin only the highest concentration caused migration of I-cells towards mesoglea, pointing out the regeneration processes, and mesoglea was thicker than in the control. In brown hydra even the lowest concentration caused the appearance of thicker mesoglea. In the highest concentration I-cells were in the process of dedifferentiation, thus supporting the regeneration, and mesoglea was thinned.

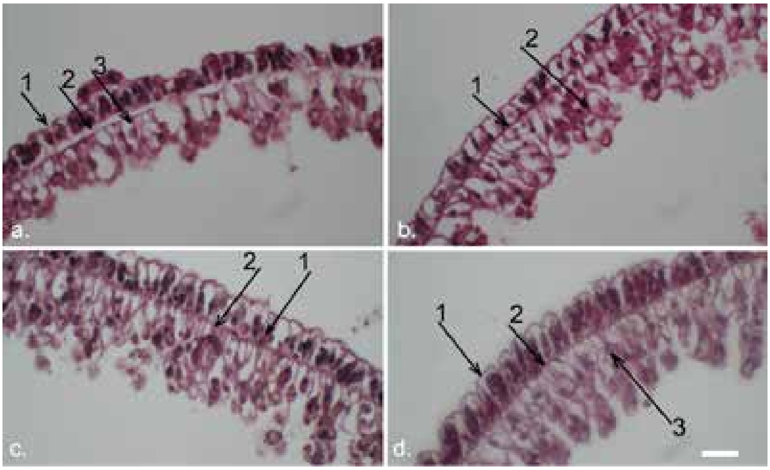


Figure 1. Brown hydra treated with quercetin for 72 hs in comparison to the control. Bar 25 μm

(a. Control. 1 – ectodermis, 2 – mesoglea, 3 – gastrodermis; b. Brown hydra treated with 0.2 gL^{-1} of quercetin. 1 – thinned mesoglea, 2 – migrating zymogen cell; c. Brown hydra treated with 0.25 gL^{-1} of quercetin. 1 – migrating ectodermal interstitial cell, 2 – damaged mesoglea; d. Brown hydra treated with 0.3 gL^{-1} of quercetin. 1 – regenerating ectodermis, 2 – damaged mesoglea, 3 – migration of cells in gastrodermis.)

It is known that zymogen cells contribute in hydra's regeneration by dedifferentiating into gastrodermal interstitial cells, which differentiate into other types of cells (Žnidarić, 1971). Interstitial cells, which serve as a reserve of somatic embryonic elements, and which enable the transformation and differentiation into other cell types, were more active in hydras treated with higher concentrations of quercetin, confirming higher degree of damage. Brown hydra proved to be more susceptible to the unfavourable environmental effects of naringenin than green hydra, confirming the advantage of symbiotic relationship.

Free-living algal species *Chlorella vulgaris* demonstrated higher flexibility in exposure to naringenin than the endosymbiotic algae *Mychonastes homosphaera* and *Desmodesmus subspicatus*. With higher concentration it had partially damaged chloroplasts and empty cells. It was established that there is the highest growth inhibition in the species *D. subspicatus*, including the occurrence of small cells with empty lumen, fragmentation of chloroplast and the disappearance of green colour of algae (Figures 2 and 3). In species *M. homosphaera* the same trends, but in much narrower range were observed. Exposure to quercetin showed the same, but much weaker trend: the most viable was the free-living *C. vulgaris*, followed by the isolated *M. homosphaera* and *D. subspicatus*.

Figure 2. Chlorella bioassay. Desmodesmus subspicatus treated with naringenin for 96 hs in comparison to the control

(a. Green control; b.- d. *D. subspicatus* treated with 0.2, 0.25 and 0.3 gL^{-1} of naringenin. Growth inhibition present in all tubes and concentrations.)

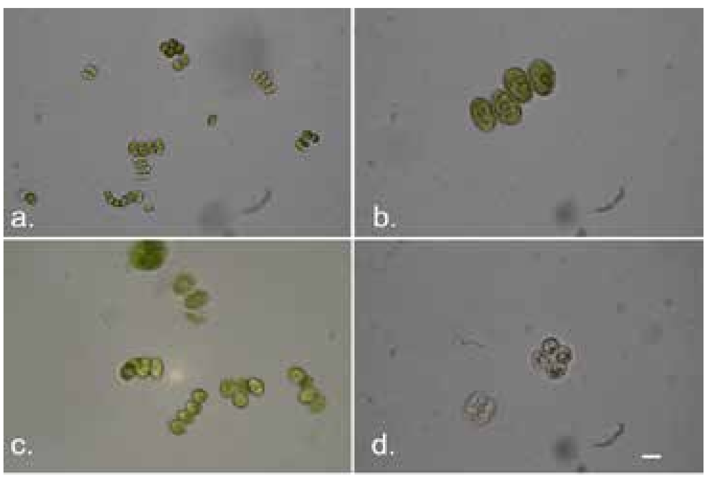


Figure 3. Desmodesmus subspicatus treated with naringenin for 96 hs in comparison to the control. Bar 5 µm

(a. Control; b.- c. *D. subspicatus* treated with 0.2 and 0.25 gL^{-1} of naringenin. Irregularities in cell formations; d. *D. subspicatus* treated with 0.3 gL^{-1} of naringenin. Non-viable cells, and growth inhibition.)

By increasing the concentration of quercetin, the viability of isolated algae was reduced. In the species *D. subspicatus* intensive growth inhibition, and degradation and loss of chloroplasts was present. In the species *M. homosphaera* only partial growth inhibition was noticed in the 0.25 gL^{-1} concentration. In the free-living alga *C. vulgaris* the concentration of 0.25 gL^{-1} of quercetin caused a hormetic effect, i.e. the increase of biomass, denoting stimulation of growth by low levels of potentially toxic agents (Stebbing, 1982).

Teachers showed to be highly motivated to conduct this experiment in the classroom with their students, both in regular classes, as part of extracurricular activities or a research project. The results showed that median value of ratings given for motivation of the tested teachers to perform such a type of an experiment during extracurricular activities was 3.77 (Figure 4). The scale was 1-5 (1 – the lowest values, 5 – the highest value).

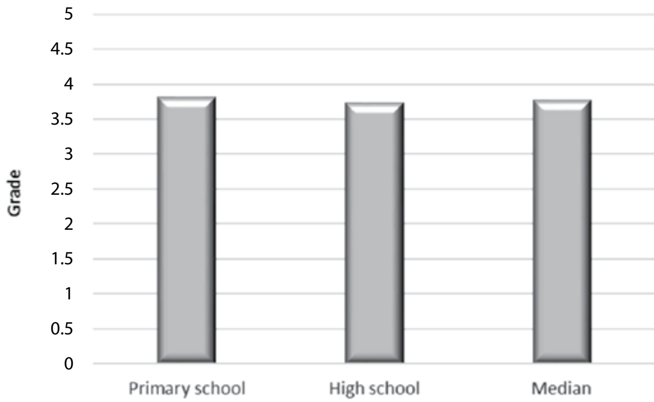
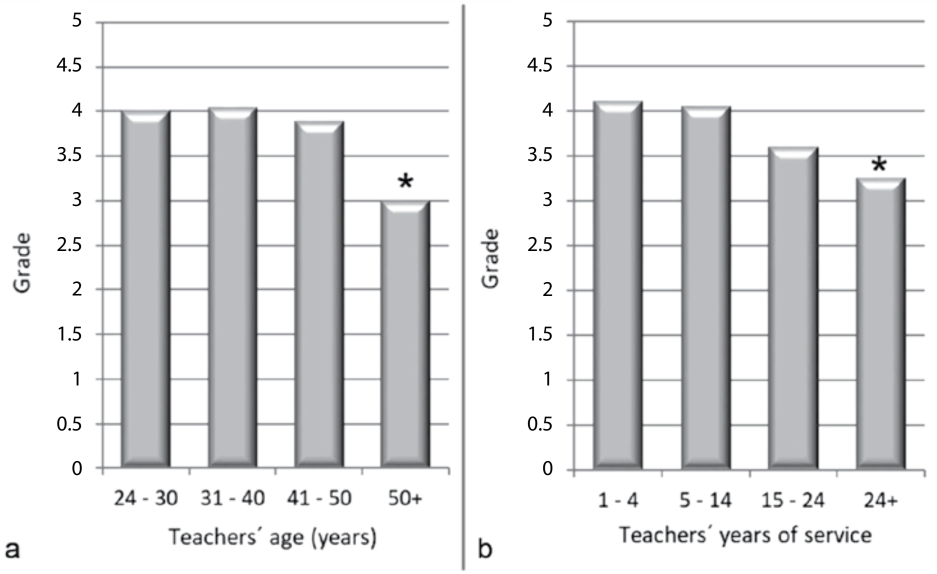


Figure 4. Value of ratings given for motivation of the tested teachers to perform such a type of an experiment during extracurricular activities in primary and high schools

Primary school teachers were found to be slightly more motivated for conducting this experiment than high school teachers but the differences can be neglected. Motivation was higher among teachers in the 31–40 age group with up to 4 years of teaching experience, and lower in the over 50 age group, with over 15 years of teaching experience, especially in over 24 years of teaching experience (Figure 5). Since many of these teachers have worked for 10 or more years, it would be useful to investigate the reasons for the decline in motivation, and to motivate teachers from that group to carry out experimental teaching.

Availability of equipment and accessories has been investigated as one of the possible limiting factors. It was found that 96.43 % of high schools and 94.04 % of primary schools possess a microscope, without which it would not be possible to carry out this experiment. Small supplies for the experiment were shown to be available both in high schools (89.29 %) and primary schools (87.04 %). Primary schools were found to have more needed glassware for the experiment (98.15 %) in comparison to high schools (89.29 %). It was found that 70 % of teachers in primary, and 79.30 % in high schools have all that is needed, i.e. microscopes, small supplies and glassware, to perform this or similar experiments, which is satisfactory (Table 3). Since the required material is cheap and easy to obtain, and considering the mentioned results, it can be concluded that the equipment in schools is not a limiting factor for carrying out experimental work of this type.



(*Statistically significant difference; a. between the group 50+ and every other group individually; b. between the group 24+ and every other group individually (*$p<0.01$, Student's t-test)

Figure 5. Total motivation of teachers: a. depending on the age of teachers; b. depending on the years of teachers' working experience

Limiting factors for performing the experiments are often space and time. Survey showed that primary schools have more space available for the performance of extracurricular activities (77 %) than high schools (58.62 %). Furthermore, the survey showed that primary school teachers have more time for the implementation of extracurricular activities (55.55 %) while high school teachers have less time to perform such activities (44.83 %) (Table 3). Based on these results, it can be concluded that space and time are indeed limiting factors for carrying out experimental work of this type. It can be assumed that the reasons for this lie in a saturated schedule and a small number of schools in some areas, most of which must operate in two and, sometimes, in three shifts.

A high number of primary schools reportedly offer extracurricular activities (81.81 %) and this number was lower for high schools (55.17 %), so the current situation is not bad, although it could be improved, especially in high schools. It is interesting to note that high school teachers are equally highly motivated as their primary school colleagues (Figure 4) despite poorer working conditions, i.e. lack of time and space.

This is a good indicator of the teacher's desire to implement practical work although it has been shown that teachers prefer frontal work, which is due to lack of time, easier student control as well as desire to teach (Meyer, 2002). Our results are in accordance with the results described by Bognar (2011), who showed that teachers hesitate to use active teaching methods, mostly because of lack of self-confidence and support. Therefore, it would be desirable to provide more support for teachers and help them to improve their teaching by offering them elaborated experiments of this type.

Table 3

Possible limiting factors for carrying out experimental work

school \ possible limiting factors	equipment and accessories				available space	available time
	microscope	small supplies	glassware	all		
primary school	94.04 %	87.04 %	98.15 %	70.00 %	77.00 %	55.55 %
high school	96.43 %	89.29 %	89.29 %	79.30 %	58.62 %	44.83 %

Conclusions

Naringenin showed altogether more deleterious effect than quercetin as it had altogether stronger impact on brown hydra and exerted altogether higher morphological effects upon green and brown hydra than quercetin. Quercetin showed a similar effect on symbiotic green and free-living brown hydra. Furthermore, quercetin exerted altogether higher impact on cyto-histological changes in green and brown hydra than naringenin, which on the other hand had a stronger impact on cytological changes in algae than quercetin. Free-living alga was less susceptible to environmental changes than the isolated endosymbiotic algae, *M. homosphaera* being less susceptible than *D. homosphaera*.

The survey showed that this or similar experiments, adapted to the students' level, could be conducted both in primary and high schools. Using this kind of approach can contribute to the use of contemporary teaching strategies, active learning in biology classes and better conceptual understanding (Abrahams, Reiss, & Sharpe, 2014). Based on these results, we have decided to provide teachers with several versions of this experiment and clear instructions how to adapt it to the working environment in their schools. Primary school teachers were found to be more motivated to perform this type of experiment within the extracurricular activities than high school teachers, but it was also found that in primary and high schools the biggest problem is lack of time which teachers could devote to such an experiment. Analysis of the results showed that there are differences in school equipment and the availability of space and time, depending on the county in which schools are located. It would be useful to perform another survey on the availability of equipment, space and time depending on the cities and counties where schools are located.

The overall results pointed out the need for implementing and adapting this and similar experiments for extracurricular activities, research projects and teaching biology in schools, so teachers could be provided with better support with the aim of introducing more active teaching/learning.

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Učinak flavonoida na simbiozu hidre i alge i primjena navedenog eksperimenta u školama

Sažetak

Hydra (Cnidaria) je kozmopolitski slatkovodni beskralježnjak koji uspostavlja simbiozu s jednostaničnim algama. Njezino jednostavno tijelo sastoji se od tri sloja: epiderma, mezogleje i gastroderma. Cilj ovoga istraživanja bio je utvrditi utjecaj flavonoida kvercetina i naringenina na simbiozu hidre i alge, odrediti koliko su nastavnici motivirani za primjenu takvog načina poučavanja te ovisi li navedena motivacija o njihovoj dobi, godinama radnog iskustva, školi u kojoj rade, raspoloživom vremenu, prostoru i materijalima. U istraživanju je upotrijebljena anonimna anketa na uzorku od 100 nastavnika biologije. Hidre i alge tretirane su kvercetinom i naringeninom pri koncentracijama od 0,2, 0,25 i 0,3 gL^{-1} i uspoređene su s kontrolnim uzorkom, a koristio se biološki test Chlorella test. Morfološke promjene uključivale su migraciju, reakciju na mehaničke podražaje, nespolno razmnožavanje, izlučivanje sluzi i smrtnost. Citološko-histološkom analizom utvrđene su promjene svih triju slojeva, kao i inhibicije rasta. Naringenin je imao ukupno veći učinak od kvercetina. Rezultati dobiveni anketom pokazali su da su nastavnici spremni izvoditi navedeni eksperiment u školi te da većina škola ima minimalnu potrebnu opremu. Zbog mogućnosti prilagodbe opisanog eksperimenta različitim vremenskim i prostornim uvjetima, predlaže se njegova primjena u školi.

Ključne riječi*: učenje; motivacija; naringenin; kvercetin; nastava.*

Uvod

Posljedice pasivnog učenja su stjecanje nepotpunog znanja koje je podložno zaboravljanju i manje primjenjivo u svakodnevnom životu (Mazur, 1997). Stoga se potrebno koristiti metodama aktivnog učenja i obrazovati nastavnike da bi mogli organizirati izvannastavne aktivnosti za učenike. Iako nastavnici prihvaćaju da su metode aktivnog poučavanja dobre, samo ih ograničen broj nastavnika primjenjuje u nastavi. Vrlo je važno za učenike da se koncepti prirodnih znanosti usvajaju putem izvorne stvarnosti i kroz praktičan rad jer im omogućuje lakše i brže usvajanje znanja i eksperimentalno učenje (Hofstein i Mamlok-Naaman, 2007; Hofstein i Kind, 2012; Lewthwaite, 2014). Također je važno da poučavanje u području prirodnih znanosti bude pravilno osmišljeno kao aktivno učenje i promatranje prirodne stvarnosti da bi učenici usvojena znanja mogli primijeniti u svakodnevnom životu (Cranton i Taylor, 2012; Meyer, 2005).

Rod *Hydra* (Cnidaria) pripada slatkovodnim beskralježnjacima. Tijelo hidre sastoji se od epiderma, mezogleje i gastroderma. Zelena hidra uspostavlja simbiotski odnos s algama roda *Chlorella* (Douglas, 1994). Budući da su zelene alge temelj mnogih hranidbenih lanaca, potrebno je upoznati se s djelovanjem ksenobiotika na alge i druge organizme u hranidbenom lancu. Hidre mogu biti vrlo korisni organizmi za nastavnike jer je smeđa hidra slobodnoživući organizam, a zelena hidra je izvrstan primjer endosimbiotskog odnosa. Endosimbioza je oblik simbiotskog odnosa u kojemu jedna stanica nastanjuje drugu, tj. unutar iste citoplazme postoje najmanje dva genoma različitog evolucijskog podrijetla (Ebringer i Krajčovič, 1994). Zbog dostupnosti kozmopolita, hidra predstavlja organizam poželjan za praktičan rad u školama. Osim toga, različiti koncepti mogu se usvojiti uz pomoć simbiotskog sustava hidre i alge, što je vrijedan temelj za mnoge nastavne jedinice.

Kvercetin i naringenin pripadaju bioflavonoidima koji su najzastupljeniji u ljudskoj prehrani, a zbog razlika u kemijskim strukturama oni pripadaju različitim skupinama. Kvercetin pripada flavonolima, a naringenin flavanonima. Lako su dostupni i mogu se kupiti u ljekarnama i trgovinama kao dodatci prehrani. Iako se čini da je kvercetin prvi logičan izbor za induciranje pozitivnih ili negativnih učinaka zbog svoje kemijske strukture, pokazalo se da je naringenin također vrlo učinkovit flavonoid (Sirovina, Oršolić, Zovko Končić i Gregorović, 2016), pa je prikladan za usporedbu aktivnosti pod istim uvjetima. Dobro opisana svojstva kvercetina i naringenina jesu njihove sposobnosti da djeluju kao antioksidanti, ali također mogu imati prooksidacijski učinak, ovisno o njegovoj koncentraciji i izvoru slobodnih radikala unutar stanice (Kovačević i Matulić, 2013; Lee, Kim, Park, Chung i Jang, 2003).

U ovome se radu želi odrediti koji od navedenih organizama, simbiotski ili slobodnoživući, imaju veću sposobnost preživljavanja u promijenjenim uvjetima okoliša, tj. nakon tretmana s kvercetinom i naringeninom i koji od dvaju flavonoida ima štetniji učinak. Prethodni rezultati ukazuju na izražen učinak različitih ksenobiotika (antibiotika, metala) na simbiozu hidre i alge, te da simbiotska hidra bolje opstaje u nepovoljnom okruženju u usporedbi s nesimbiotskim vrstama (Kalafatić, Kovačević, Ljubešić i Šunjić, 2001; Kovačević, Želježić, Kalafatić i Horvatin, 2007). Učinak kvercetina na simbiozu hidre i alge do sada je uglavnom bio štetan, i uključivao je morfološke promjene i promjene u ponašanju kao što su depigmentacije i deformacije (Kovačević i Matulić, 2013). U daljnjem istraživanju ispitivali smo učinak naringenina na zelenu hidru i učinak kvercetina i naringenina na slobodnoživuću smeđu hidru, dvije endosimbiotske vrste algi izolirane iz zelene hidre i jednu slobodnoživuću vrstu algi. Cilj ovoga istraživanja bio je utvrditi i usporediti učinak i mogući stupanj djelovanja flavonoida kvercetina i naringenina na simbiozu hidre i alge, tj. na slatkovodne žarnjake, izolirane endosimbiotske alge i njihova slobodnoživućeg srodnika, odrediti morfološke, citološko-histološke i učinke biološkog Chlorella testa, kao i dodatne specifičnosti koje se mogu pojaviti.

Budući da prilagodba određenog eksperimenta zahtijeva znatnu količinu rada i povratnu informaciju od nastavnika, a njegova provedba veliku motivaciju, cilj ovoga istraživanja bio je i utvrditi koliko su nastavnici motivirani poučavati na ovaj način tijekom izvannastavnih aktivnosti, te ovisi li motivacija o njihovoj dobi, godinama radnog iskustva i školi u kojoj rade. Cilj ovoga istraživanja bio je i utvrditi ispunjavaju li škole minimalne zahtjeve potrebne da bi nastavnici izvodili ovaj ili sličan eksperiment, s posebnim naglaskom na raspoloživost materijala i opreme, prostora i vremena za njegovu provedbu.

Materijali i metode

Eksperimentalni organizmi koji su se koristili u ovim pokusima bili su zelena (*Hydra viridissima* Pallas, 1766) i smeđa hidra (*Hydra oligactis* Pallas, 1766), zelene alge izolirane iz domaćina zelene hidre (*Desmodesmus subspicatus* (Chlorophyceae) (Chodat) Hegewald i Schmidt, i *Mychonastes homosphaera* (Chlorophyceae) (Skuja) Kalina i Punčochářová) i slobodnoživuća srodna alga *Chlorella vulgaris* Beij [K i H, 1992], soj SAG 211-11b.

Proveden je usporedni test toksičnosti kvercetina i naringenina. Četiri skupine od 10 nehranjenih zelenih hidri koristile su se u 72-satnom eksperimentu s naringeninom, a četiri skupine od 10 nehranjenih smeđih hidri koristile su se u 72-satnom eksperimentu s kvercetinom i naringeninom, u staklenim posudama od 60 ml (koncentracije 0,2, 0,25 i 0,3 gL^{-1} za svaki flavonoid i kontrolni uzorak, svaki od 50 mL). Do sada su provedeni pokusi o učinku kvercetina na zelenu hidru (Kovačević i Matulić, 2013), a u ovom su istraživanju prethodni rezultati uspoređeni s dobivenim rezultatima. Morfološke promjene hidri uključivale su smrtnost, podražljivost, prisutnost sluzi, migraciju i nespolno razmnožavanje, a citološko-histološke promjene podrazumijevale su oštećenje staničnih slojeva ektoderma i gastroderma, kao i nestanični sloj mezogleju. Uzorci hidre uzeti su za standardnu histološku analizu nakon 72-satnog eksperimenta. Za sve tri vrste algi primijenjen je 21-dnevni biološki Chlorella test (Kovačević, Jelenčić, Kalafatić i Ljubešić, 2008), a koristile su se i četiri skupine za svaku vrstu algi, 6 epruveta za svaku vrstu i koncentraciju (iste koncentracije kvercetina, naringenina i kontrolnog uzorka). Stanice algi analizirane su nakon 96-satnog tretmana. Parametri citoloških promjena u algama uključivali su inhibiciju rasta, fragmentaciju kloroplasta, izbjeljivanje i stanične nepravilnosti. Eksperimenti su ponovljeni dva puta. Uzorci su promatrani s pomoću stereo lupe, a analiza hidri i algi provedena je s pomoću Nikon Eclipse E600 svjetlosnog mikroskopa. Mikrografije su snimljene s pomoću Nikon D7200 fotoaparata.

Za prikupljanje podataka od nastavnika biologije koristila se metoda anonimne ankete, na uzorku od 100 nastavnika, od kojih su 65 % bili osnovnoškolski, a 35 % srednjoškolski nastavnici biologije. U istraživanju je analizirana razina motiviranosti nastavnika ovisno o vrsti škole, dobi nastavnika i godinama radnog iskustva. Motivacija nastavnika za izvođenje predloženog eksperimenta u razredu određena je na skali od 1 do 5, pri čemu 1 predstavlja potpuni nedostatak motivacije, a 5 visoku motivaciju. Drugi dio ankete sastavljen je kako bi se ispitalo nudi li određena škola izvannastavne aktivnosti te imaju li nastavnici vrijeme, prostor i opremu koji su potrebni za izvođenje opisanoga eksperimenta. Ankete su analizirane i statistički obrađene u 'Microsoft Office Excel 2013' računalnom programu. Statistička značajnost rezultata dobivenih u ovom istraživanju ispitana je s pomoću analize varijance (ANOVA) i t-testa ($p<0,01$).

Rezultati i rasprava

Tijekom eksperimentalnog rada utvrđeno je da su kvercetin i naringenin uzrokovali morfološke i citološko-histološke promjene u hidrama i citološke promjene u endosimbiotskim i srodnim slobodnoživućim algama. Veće koncentracije uzrokovale su veće promjene na pokusnim životinjama, ali su najintenzivnije morfološke promjene zabilježene pri koncentraciji od 0,25 gL^{-1} naringenina. Poznato je da već niža koncentracija toksikanata može izazvati značajniji učinak na ispitivane organizme (Pavlica, Klobučar, Vetma, Erben, i Papeš, 2000). Navedeno se očitovalo migracijom hidri u eksperimentalnoj posudi, sporijom reakcijom na mehaničke podražaje, izlučivanjem većih količina sluzi i pojavom smrtnosti nakon 72-satnog tretmana (tablice 1 i 2).

Tablice 1 i 2

Ovisno o koncentraciji, ksenobiotici mogu uzrokovati morfološke promjene i promjene u ponašanju i staničnoj strukturi (Kalafatić i sur., 2001). Migracija smeđe hidre bila je prva vidljiva promjena, a povećanjem koncentracije, smeđa hidra reagirala je proporcionalno sporije na mehanički podražaj. Migracije su mogući rezultat traženja boljih mikroekoloških uvjeta (Kovačević i sur., 2007). Izlučivanje sluzi opaženo je u manje održivim uzorcima, tj. onima koji nisu migrirali prema površini vode i koji su manje reagirali na mehaničke podražaje. Izlučivanje povećane količine sluzi posljedica je oštećenja ektodermalnih mioepitelnih stanica i služi kao mehanizam detoksikacije (Kovačević, Gregorović, Kalafatić i Jaklinović, 2009). Zelene hidre prošle su proces nespolnog razmnožavanja, njihov se broj povećao za 35 % tijekom pokusa, a smeđe hidre nisu pokazivale znakove pupanja. Zelena hidra tretirana kvercetinom pokazala je višu stopu migracije u višim koncentracijama, ali najvišu stopu proizvodnje sluzi u srednjoj koncentraciji (0,25 gL^{-1}) i nepravilne reakcije na mehaničke podražaje. U nižim koncentracijama smrtnost je bila niža nego u smeđe hidre, ali je u najvišoj koncentraciji smrtnost zelene hidre bila viša (46,6 %) (Kovačević i Matulić, 2013) nego u smeđe hidre (30 %). Naringenin je utjecao na višu smrtnost hidri nego kvercetin, a viša je smrtnost utvrđena u smeđe hidre.

Osim morfoloških promjena promatrane su i citološko-histološke promjene na hidrama. Uočene su evidentne promjene i oštećenja svih slojeva smeđe hidre u tretmanu kvercetinom. Viša koncentracija uzrokovala je migraciju zimogenih stanica i I-stanica prema mezogleji i njihovu dediferencijaciju u druge tipove stanica kako bi se regeneriralo tijelo hidre. Mezogleja je bila naborana i djelomično je promijenila debljinu (prikaz 1). U zelenoj hidri tretiranoj kvercetinom prisutni su isti trendovi, te su uočeni degradacija i oštećenje simbionata algi (Kovačević i Matulić, 2013). Primjena naringenina uzrokovala je tek manje promjene. Pri tretiranju zelene hidre naringeninom samo je najviša koncentracija uzrokovala migraciju I-stanica prema mezogleji, ukazujući na procese regeneracije, a mezogleja je bila deblja nego u kontrolnom uzorku. U smeđe je hidre čak i najniža koncentracija uzrokovala pojavu zadebljanja mezogleje. U najvišoj su koncentraciji I-stanice bile u procesu dediferencijacije, čime su podržavale regeneraciju, a mezogleja se stanjila.

Prikaz 1

Poznato je da zimogene stanice pridonose regeneraciji hidre tako što se dediferenciraju u gastrodermalne intersticijske stanice koje se diferenciraju u druge tipove stanica (Žnidarić, 1971). Intersticijske stanice, koje služe kao rezerva somatskih embrionalnih elemenata i koje omogućuju transformaciju i diferencijaciju u druge tipove stanica, bile su aktivnije u hidrama tretiranim višim koncentracijama kvercetina, time potvrđujući viši stupanj oštećenja. Smeđa hidra pokazala se osjetljivijom na nepovoljne učinke naringenina u odnosu na zelenu hidru, što potvrđuje prednost simbiotskog odnosa.

Slobodnoživuća vrsta alge *Chlorella vulgaris* pokazala je veću fleksibilnost u izlaganju naringeninu nego endosimbiotske alge *Mychonastes homosphaera* i *Desmodesmus subspicatus*. Pri višoj koncentraciji djelomično su oštećeni kloroplasti i prazne stanice. Utvrđeno je da postoji najveća inhibicija rasta u vrste *D. subspicatus*, uključujući pojavu malih stanica s praznim lumenom, fragmentaciju kloroplasta i nestanak zelene boje algi (prikazi 2 i 3). U vrste *M. homosphaera* uočeni su isti trendovi, ali u znatno užem rasponu. Izloženost kvercetinu pokazala je isti, ali znatno slabiji trend: najživotniji je bio slobodnoživući *C. vulgaris*, a zatim izolirane *M. homosphaera* i *D. subspicatus*.

Prikaz 2 i 3

Povišenjem koncentracije kvercetina smanjena je održivost izoliranih algi. U vrste *D. subspicatus* bila je prisutna intenzivna inhibicija rasta, te razgradnja i gubitak kloroplasta. U vrste *M. homosphaera* uočena je samo djelomična inhibicija rasta pri koncentraciji 0,25 gL^{-1}. U slobodnoživućoj algi *C. vulgaris* koncentracija kvercetina od 0,25 gL^{-1} uzrokovala je hormestički učinak, tj. povećanje biomase, što označava stimulaciju rasta niskim razinama potencijalno toksičnih agensa (Stebbing, 1982).

Nastavnici su pokazali da su visoko motivirani za provođenje ovog eksperimenta u učionici sa svojim učenicima, kako u redovitoj nastavi tako i u izvannastavnim aktivnostima ili kroz istraživački projekt. Rezultati su pokazali da je srednja vrijednost procijenjene motivacije ispitanih nastavnika za obavljanje takvog tipa eksperimenta tijekom izvannastavnih aktivnosti iznosila 3,77 (prikaz 4). Skala je bila 1 – 5 (1 – najniža vrijednost, 5 – najviša vrijednost).

Prikaz 4

Utvrđeno je da su osnovnoškolski nastavnici nešto motiviraniji za provođenje ovoga eksperimenta od srednjoškolskih nastavnika, ali razlike se mogu zanemariti. Motivacija je bila viša među nastavnicima u dobnoj skupini od 31 do 40 godina i kod onih koji su imali do 4 godine iskustva u poučavanju, a niža u dobnoj skupini više od 50 godina, s više od 15 godina iskustva u nastavi, posebno među nastavnicima s više od 24 godine iskustva u poučavanju (prikaz 5). Budući da mnogi od tih nastavnika rade 10 ili više godina, bilo bi korisno istražiti razloge za smanjenje motivacije i motivirati nastavnike iz te skupine da primjenjuju eksperimente u poučavanju.

Prikaz 5

Dostupnost opreme i pribora istražena je kao jedan od mogućih ograničavajućih čimbenika. Utvrđeno je da 96,43 % srednjih i 94,04 % osnovnih škola posjeduju mikroskop, bez kojega ne bi bilo moguće provesti ovaj eksperiment. Pokazalo se da je sitni pribor potreban za eksperiment dostupan i u srednjim (89,29 %) i osnovnim školama (87,04 %). Utvrđeno je da osnovne škole imaju više staklenog posuđa potrebnog za pokus (98,15 %), za razliku od srednjih škola (89,29 %). Utvrđeno je da 70 % nastavnika u osnovnim i 79,30 % u srednjim školama imaju sve što je potrebno za provedbu ovog ili sličnih eksperimenata, tj. mikroskope, sitni pribor i stakleno posuđe, što je zadovoljavajući rezultat (tablica 3). Budući da je potreban materijal jeftin i da se može jednostavno nabaviti te s obzirom na navedene rezultate, može se zaključiti da oprema u školama nije ograničavajući čimbenik za provođenje eksperimentalnog rada tog tipa.

Ograničavajući čimbenici za izvođenje pokusa često su prostor i vrijeme. Anketa je pokazala da u osnovnim školama ima više prostora za provedbu izvannastavnih aktivnosti (77 %) nego u srednjim školama (58,62 %). Nadalje, istraživanje je pokazalo i da nastavnici u osnovnim školama imaju više vremena za provedbu izvannastavnih aktivnosti (55,55 %), a da nastavnici srednjih škola imaju manje vremena za obavljanje takvih aktivnosti (44,83 %) (tablica 3). Na temelju opisanih rezultata može se zaključiti da su prostor i vrijeme uistinu ograničavajući čimbenici za provođenje eksperimentalnog rada toga tipa. Može se pretpostaviti da razlozi tomu leže u zasićenom rasporedu i malom broju škola u nekim područjima, od kojih većina mora organizirati nastavu u dvije, a ponekad i u tri smjene.

Tablica 3

Prema dobivenim podatcima, značajan broj osnovnih škola nudi izvannastavne aktivnosti (81,81 %), u odnosu na srednje škole (55,17 %), tako da se može utvrditi kako trenutno stanje nije loše, iako bi bilo dobro poboljšati ga, posebno u srednjim školama. Zanimljivo je napomenuti da su nastavnici srednjih škola jednako visoko motivirani kao i njihovi kolege u osnovnoj školi (prikaz 4), unatoč lošijim uvjetima rada, tj. nedostatku vremena i prostora. To je dobar pokazatelj želje nastavnika da provedu praktičan rad, iako se pokazalo da nastavnici preferiraju frontalni rad zbog nedostatka vremena, lakše kontrole učenika i želje za poučavanjem (Meyer, 2002). Naši su rezultati u skladu s rezultatima koje je opisao Bognar (2011), a koji su pokazali da nastavnici oklijevaju u primjeni aktivnih metoda poučavanja, uglavnom zbog nedostatka samopouzdanja i podrške. Stoga bi bilo poželjno pružiti veću podršku nastavnicima i pomoći im da poboljšaju svoje poučavanje dajući im razrađene eksperimente.

Zaključci

Naringenin je pokazao općenito štetniji učinak od kvercetina jer je imao ukupno snažniji učinak na smeđu hidru i imao je značajnije morfološke učinke na zelenu i smeđu hidru u odnosu na kvercetin. Kvercetin je pokazao sličan učinak na simbiotsku zelenu i slobodnoživuću smeđu hidru. Nadalje, kvercetin je imao općenito značajniji učinak na citološko-histološke promjene u zelene i smeđe hidre od naringenina, koji je s druge strane imao jači učinak na citološke promjene u algama u odnosu na kvercetin. Slobodnoživuće alge bile su manje podložne promjenama u okolišu u odnosu na izolirane endosimbiotske alge, *M. homosphaera* koje su manje osjetljive nego *D. homosphaera*.

Istraživanje je pokazalo da se ovaj ili sličan eksperiment, prilagođen razini učenika, može provoditi i u osnovnim i srednjim školama. Primjena takvog pristupa može doprinijeti upotrebi suvremenih nastavnih strategija, aktivnom učenju u nastavi biologije i boljem konceptualnom razumijevanju (Abrahams, Reiss, i Sharpe, 2014). Na temelju prikazanih rezultata odlučili smo ponuditi nastavnicima nekoliko inačica ovog eksperimenta i jasne upute kako ga prilagoditi radnom okruženju u njihovim školama. Utvrđeno je da su osnovnoškolski nastavnici motiviraniji za provedbu te vrste eksperimenta u izvannastavnim aktivnostima od srednjoškolskih nastavnika, ali je također utvrđeno da je u osnovnim i srednjim školama najveći problem nedostatak vremena koje bi nastavnici mogli posvetiti takvom eksperimentu. Analiza rezultata pokazala je da postoje razlike u opremljenosti škola i dostupnosti prostora i vremena, ovisno o županiji u kojoj se škole nalaze. Bilo bi korisno provesti dodatno istraživanje o dostupnosti opreme, prostora i vremena s obzirom na gradove i županije u kojima se škole nalaze.

Sveukupni rezultati ukazali su na potrebu provedbe i prilagođavanja opisanoga i sličnih eksperimenata za izvannastavne aktivnosti, istraživačke projekte i poučavanje biologije u školama, da bi nastavnici dobili bolju podršku s ciljem uvođenja aktivnijeg poučavanja/učenja.