ENHANCEMENT OF CHEMOPREVENTIVE COMPOUNDS CONTENT IN CABBAGE BY TREATMENT WITH A SELENIUM BASED PLANT BIOSTIMULANT

Anca Oancea[1], Alexandra Gaspar[1], Ana-Maria Seciu[1], Laura Mihaela Stefan[1], Elena Utoiu[1], Lucia Moldovan[1], Danut Turcu[2], Dumitru Cristinel Badiu[3,4], Oana Craciunescu[1]

[1]National Institute of Research and Development for Biological Sciences, 296, Splaiul Independentei, 060031, Bucharest, Romania

[2]Faculty of Veterinary Medicine, "Spiru Haret" University, 256, Basarabia Blvd, 030352, Bucharest, Romania

[3]Carol Davila University of Medicine and Pharmacy, 37, Dionisie Lupu street, 020021 Bucharest, Romania

[4]Bagdasar Arseni Emergency Clinic Hospital, 12, Berceni street, 041915, Bucharest, Romania

Abstract

We have obtained cabbage as functional food with high content of chemopreventive compounds by treatment with a new plant biostimulant mixture containing selenium salt, betaine and spraying adjuvants. Such treatment determined an increased accumulation of selenium in cabbage plants, while maintaining a high content of glucosinolates. We have studied the in vitro antitumoral effect of selenium biofortified cabbage extracts using a tumor cell line and we observed a significant inhibition of cell proliferation, after 72 h of in cultivation. We did not observe modifications on the health status of laboratory rabbits fed with cabbage treated with the selenium-based biocomposition.

Key words: *Brassica oleracea, water stressed plants, selenium-based composition, sulforaphane, HPLC, in vitro antitumoral activity*

1. INTRODUCTION

Brassica vegetables crops (*Brassica* spp.) are known to possess chemopreventive properties (Jahangir et al. 2009, Avato & Argentieri 2015), having the potential to inhibit the development of certain cancers (Spitz et al. 2000, Giovannucci et al. 2003, Ambrosone et al. 2004). The beneficial effects of cruciferous vegetables on human health is due to the high content of bioactive compounds, such as vitamins, minerals, carotenoids and a special group of chemicals named glucosinolates (Jahangir et al. 2009). The anticarcinogenic properties of *Brassica* vegetables are often correlated to glucosinolates breakdown products, such as isothiocyanates. Sulforaphane, the isothiocyanate derived from glucoraphanin is the most studied chemopreventive agent in carcinogenesis (Pawlik et al. 2013, Li et al. 2014, Rudolf, Cervinka & Rudolf 2014, Wu et al. 2016).

Brassica vegetables are also known to be selenium hyperaccumulator plants, when they are cultivated in selenium rich soils (Çakır, Turgut-Kara & Arı 2012). Selenium is recognized as an essential microelement for animals, including humans (Duntas & Benvenga 2015) and bacteria (Stock & Rother 2009), being present as selenoamino acids into more than 25 classes of selenoproteins (Roman et al. 2014), including important enzymes such as glutathione peroxidases, thyroid hormones deiodinase and thioredoxin reductases (Papp et al. 2007). Such functional selenoproteins were not identified in cultivated plants (Van Hoewyk 2013). However, selenium induces beneficial effects on plants, such as: plant growth stimulation (Hartikainen & Xue 1999), reduction of UV-induced oxidative damage (Hartikainen, Xue & Piironen 2000), regulation of the water status of plants under drought conditions and increased antioxidative capacity of senescing plants (Kuznetsov et al. 2003) or plant protection against fungal infections and phloem-feeding aphids (Hanson et al. 2004).

Selenium has a very narrow physiological window. Ingestion of selenium doses lower than the recommended dietary allowance (RDA), which is of 50 µg/day for women and 70 µg/day for men,

increases the risk of diseases related to non-detoxified reactive oxygen species, i.e. Keshan disease or weakened immune system (Tapiero et al. 2003) or to thyroid metabolism/hypothyroidism (Zimmermann & Köhrle 2002). Doses of selenium up to 200% RDA protect from cancer and type 2 diabetes (Rayman 2012). Excess of selenium intake, >200% RDA, significantly increases the risk of type 2 diabetes and cancer (Jablonska & Vinceti 2015). In plants, low doses of selenium protect them against abiotic stress (Feng et al. 2013), including drought (Ahmad et al. 2016). High doses of selenium are toxic for plants, inducing oxidative stress and distortions of protein function (Gupta & Gupta 2016).

Different selenium effects are related to selenium status (Rayman 2012) and selenium status is determined by the selenium level in soil (Winkel et al. 2013). In soils with lower selenium content, supplementation is needed and it is done mainly by agronomic biofortification, i.e. treatments with selenium applied to plants (Broadley et al. 2006). In the soils from Romania, selenium is at the deficit limit. Diseases caused by selenium deficiency have been reported in animals from different regions of Romania (Serdaru et al. 2003; Serdaru & Giurgiu 2007). Compared to the global value, considered as being non-affected by deficit or excess, selenium content is reduced by: 30% in Fagaras Depression (Lacatusu et al. 2012), 40% in Romanian Plain and 63% in Dobrogea (Lacatusu et al. 2010). The low content of mobile selenium enhances the selenium deficit in soils from Romanian Plain and Dobrogea and requires biofortification (Lacatusu et al. 2010).

Various biofortification approaches based on selenate or selenite have been proposed for Brassica crops, aiming to increase not only the content of selenoamino acids/selenoproteins, but also the level of glucosinolates (Abdulah et al. 2009, Chan, Afton & Caruso 2010, Sepulveda et al. 2013, Avila et al. 2014). A peculiarity of selenium deficit in Romanian soil is its direct correlation with the aridity index (Oancea et al. 2014). This leads us to the formulation of the concept of protective biofortification, i.e. a selenium treatment of Brassica crops, which intends both to supplement the food chains with bioactives ingredients and to protect treated plants against abiotic stress (Oancea et al. 2015). The protective biofortification of plants aims to exploit the biostimulant potential of selenium, which presents several characteristics specific to this new class of agricultural inputs, i.e. increased plant tolerance to abiotic stress and higher quality of edible yield (du Jardin 2015). The objective of this study was to obtain cabbage crops (*B. oleracea* var. *capitata* L.) with high content of chemopreventive compounds by treatment with a new plant biostimulant composition, based on sodium selenate, betaine and spraying adjuvants and to analyze their biological activity *in vitro* and *in vivo*.

2. MATERIAL AND METHODS

2.1. Plant material and treatment

White cabbage (*Brassica oleracea* var. *capitata* L.) seedlings were transplanted, in late spring, in an experimental field located on Ştefan cel Mare, Călăraşi county, Romania. The plants were cultivated at a density equivalent to 55,000 plants x ha^{-1}, according to the recommended cultivation technology (Seciu et al. 2016). In the field, cabbage plants were treated with a mixture of 10 µM Na_2SeO_4, 5 mM betaine and 1% spraying adjuvant, by foliar spraying, twice, at 4 and 8 weeks after cultivation. The crops were grown in normal watering conditions (watered daily) (C1) and in water stress conditions (watered once every two days) (C2). Cabbage was harvested at 9 weeks after transplantation and used to analyze sulforaphane and total selenium content. Plants treated with Se-based composition were compared with controls (plants cultivated without selenium treatments) cultivated in normal watering conditions (C3) and water stress conditions (C4).

2.2. Analysis of total selenium content

Total selenium content was measured using an atomic absorption spectrometer (Agilent AA-1475, with Vapor Generation Accessory, VGA 76, and Agilent Se-hollow cathode lamp), according to SR EN ISO 15586:2004. The results were reported in µg/g dry weight (d.w.).

2.3. Analysis of sulforaphane content

Sulforaphane extraction and analysis were performed according to Campas-Baypoli et al. (2010). Briefly, 1 g fresh sample was mixed with 4 mL acidic water (pH 6). After incubation at 45 °C, for 2.5 h, the mixture was extracted with 20 mL dichloromethane. The dichloromethane fraction was loaded on SPE silica gel (SiOH) disposable cartridges and the obtained extracts were evaporated to dryness and dissolved in acetonitrile. Before HPLC analysis, the samples were filtered through a 0.45 μm membrane and injected in a HPLC Agilent 1200 system. Chromatographic separation was carried out on a Zorbax XDB C18 column (150x4.6 mm i.d.), with 70% acetonitrile as mobile phase, at a flow rate of 0.6 mL/min. Sulforaphane was detected at a wavelength of 202 nm. DL-sulforaphane was used as standard for quantification. The results were reported in μg/g dry weight (d.w.).

2.4. In vitro cell culture experiments

For cell culture experiments, fresh *Brassica* plants (10 g) were extracted in 20 mL deionized water, at 4 °C, for 24 h. The cabbage extracts were centrifuged at 2500 rpm, for 10 min and the supernatant was filtered through 0.2 μm membrane filter. The resulting solutions were stored at -20 °C until cell culture analysis. Treated and control plants cultivated in both normal watered and water stressed conditions (C1-C4) were used for *in vitro* experiments.

The antiproliferative activity of cabbage extracts was evaluated in Caco-2 tumor cell line derived from human colorectal adenocarcinoma (ECACC, Sigma-Aldrich) using MTT assay (Craciunescu et al. 2013). Briefly, $5x10^4$ Caco-2 cells/mL were seeded in 96-well culture plates, in MEM culture medium supplemented with 10% fetal bovine serum and 1% antibiotics (penicillin, streptomycin and neomycin) and incubated in humidified atmosphere with 5% CO_2, at 37 °C, for 24 h. After the incubation period, the culture medium was discarded and replaced with fresh medium containing different concentrations (100, 500, 1000 and 2000 μg/mL) of cabbage extracts. After 72 h of cultivation in the presence of the samples, the culture medium was removed, the cells were washed with phosphate buffered saline, pH 7.4, and 100 μL MTT working solution (0.25 mg/mL) was added to each well. After 3 h of incubation in 5% CO_2 atmosphere, at 37 °C, 100 μL isopropanol was added to each well, the plates were gently shaken at room temperature, for 15 min. The optical density of the colored solution was measured at 570 nm using a Mithras LB 940 microplate reader (Berthold Technologies) and was directly correlated to the number of metabolically active cells in the culture. The results were reported as cell viability percent from control sample (cells incubated without plant extracts), considered 100% viable. All samples were tested in triplicate.

2.5. Flow cytometry analysis of cell cycle

For cell cycle analysis, Caco-2 cells were seeded in 6-well plates, at a density of $5x10^4$ cells/mL and allowed to adhere in a CO_2 incubator, at 37 °C, for 24 h. Then, the cells were treated with cabbage extracts (C1-C4, 2000 μg/mL) and cultivated in standard conditions, for 72 h. At the end of the incubation period, analysis of cell cycle was performed as previously described (Wang et al. 2010). Briefly, cells were trypsinized, washed with PBS, fixed in 70% ethanol, overnight and treated with RNAse A (100 μg/mL) at 37 °C, for 30 min. Then, cells were stained with propidium iodide (50 μg/mL) at 4 °C, for 30 min. Cellular DNA content was analyzed using a BD LSR II flow cytometer (Becton Dickinson, USA) and the percentage of cells in each phase of the cell cycle (G0/G1, S and G2/M) was determined using ModFit™ LT 3.0 software (Becton Dickinson, USA).

2.6. In vivo experiments

Rabbits from New Zealand breed were used for *in vivo* tests, according to the code of ethics and animal welfare. Groups of five animals received cabbage from each experimental variant (C1-C4) in their food, for 14 days, in a quantity equivalent to the cabbage consumption of a man weighing 70 kg. Blood samples were collected from each animal, at the beginning and at the end of the experiment, to analyze the hematological parameters using an Autohematology Analyzer equipment, Mindray model BC-2800 VET. The biochemical analyses for hepatic and renal function were performed using a Reflovet Plus equipment. A control group of animals received their regular concentrated food.

2.7. Statistical analysis

Results were expressed as mean of 3 values ± standard deviation (SD). The statistical analysis of the results was performed on control-sample pairs using Student's *t*-test. Significant differences were considered at values of $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Effect of selenium treatment on sulforaphane and selenium content in B. oleracea

Mature cabbage plants contain glucoraphanin, sinigrin and glucobrassicin as main glucosinolate compounds (Park et al. 2014). In our study, we have examined the effect of selenium-based composition treatment of *Brassica* crops on the glucosinolates level by detection of sulforaphane, the hydrolysis product of glucoraphanin, in cabbage extracts. HPLC profiles of C1-C4 extracts are shown in Figure 1, where sulforaphane peak was present at 4.8-4.9 min. The content of sulforaphane in cabbage plants ranged from 45.114 to 73.039 µg/g d.w. (Table 1).

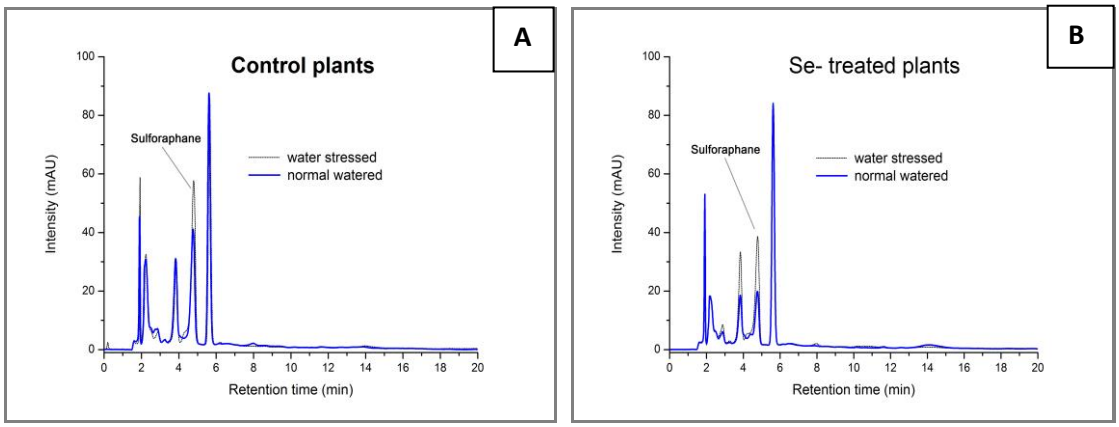


Figure 1. HPLC chromatograms of sulforaphane isolated from control (A) and selenium-treated (B) cabbage plants, cultivated in normal watering and water stressed conditions.

The results showed that the treated plants, cultivated in water stressed conditions, presented higher values of sulforaphane (53.38 µg/g d.w.), compared to normal watered plants (45.11 µg/g d.w.). The highest concentration of sulforaphane was found in control plants cultivated in water stressed conditions (73.04 µg/g d.w.). It was observed a decrease of sulforaphane in treated plants cultivated in water stressed conditions, but the level was still higher than in treated plants cultivated in normal watering conditions. No differences were detected in extracts obtained from plants cultivated in normal watering conditions (45 µg/g d.w.).

Table 1. Sulforaphane and total selenium content in *B. oleracea* plants

Experimental treatment	**Sulforaphane (µg/g d.w.)**	**Total Se content (µg/g d.w.)**
10 µM sodium selenate, 5 mM betaine, 1% spraying adjuvant, normal watered (C1)	45.11 ± 2.48	4.41 ± 0.26*
control, normal watered (C3)	45.24 ± 4.26	3.68 ± 0.33
10 µM sodium selenate, 5 mM betaine, 1% spraying adjuvant, water stressed (C2)	53.38 ± 1.85*	4.20 ± 0.35*
control, water stressed (C4)	73.04 ± 2.89	3.37 ± 0.32

*$p<0.05$, compared to each control

The treatment of cabbage plants with the selenium-based composition induced significant differences in total selenium content analysis. *Brassica* crops accumulated more selenium when they were treated with the new biostimulant formula based on sodium selenate (4.20-4.41 µg/g d.w.), compared to control plants (3.37-3.68 µg/g d.w.) (Table 1). It is known that selenium competes with sulfur within uptake and assimilation processes developed in plants and several studies reported that selenium biofortication of Brassica crops induced a decrease of sulfur containing metabolites (Charron et al. 2001, Toler et al. 2007). In our study, biofortification of cabbage plants with the selenium-based composition maintained a high level of sulforaphane and induced a slight increase of total selenium content.

3.2. Crop yield of cabbage field plants

In order to assess the plant crop yield variation, cabbage plants cultured in normal watering and water stress conditions were separately harvested after nine weeks of cultivation and weighted. Similar crop yields were obtained for cabbage plants treated with the selenium-based composition, in both normal watering conditions (28.19±1.24 tones/ha) and water stressed conditions (27.82±1.15 tones/ha). In turn, the corresponding controls presented a lower yield in water stressed conditions (21.36±2.15 tones/ha), compared to normal watering conditions (30.28±1.42 tones/ha). These results indicated that the treatment with the selenium-based composition has presented a protective effect of cabbage plants cultivated under water stress conditions.

3.3. In vitro antiproliferative activity of cabbage extracts

In this study we have tested the effect of selenium-based biofortification treatment applied to cabbage field plants on the cell viability of adenocarcinoma cells. The results for normally watered plants showed that the cell viability decreased below 80% at concentrations higher than 500 µg/mL for selenium-treated plant extract, while the control cabbage extract started to alter the cell viability at a concentration of 1000 µg/mL (Figure 2A). In the case of tumor cells grown in the presence of water stressed cabbage extracts, the treated plants showed a higher antiproliferative activity, the cell viability decreasing to 72.89 % at the concentration of 100 µg/mL. In turn, the control plants induced a decrease of cell viability below 80 % at a concentration of 500 µg/mL (Figure 2).

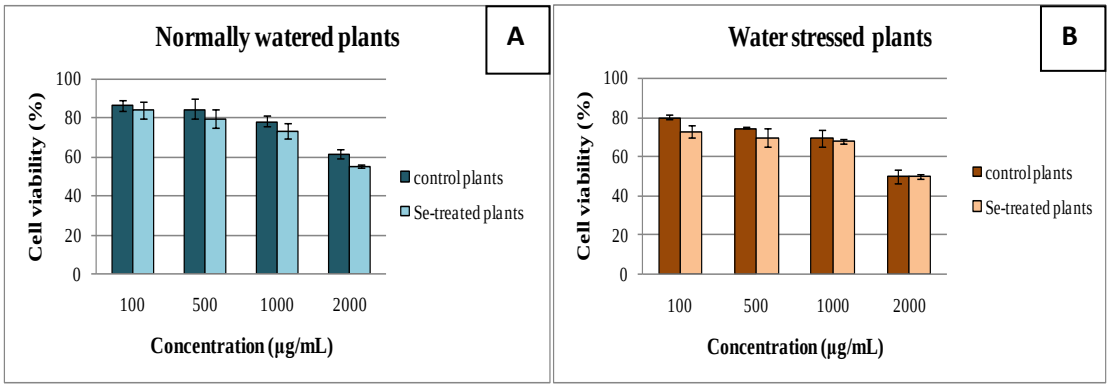


Figure 2. Cell viability of Caco-2 adenocarcinoma cells cultivated with different concentrations of plant extracts from cabbage grown in normal watering (A) and water stressed (B) conditions, evaluated by MTT assay, after 72h of cultivation. Values are expressed as mean of three determinations ± SD and reported to the control culture, considered 100% viable.

Similar results were reported for selenium biofortified cabbage seedlings, which showed an enhanced antiproliferative activity on Caco-2 human carcinoma cell line, compared to water-treated seedlings (Oancea et al. 2015). All these results demonstrated that extracts of cabbage plants treated with the selenium-based composition and cultivated in normally watered or water stressed conditions exhibited high antiproliferative activity on adenocarcinoma cells.

3.4. Effect of selenium-based biofortification treatment on cell cycle

Colorectal cancer cell lines were frequently used in cell proliferation studies and presented changes of the cell cycle due to different regulatory mechanisms. Cell cycle analysis by flow cytometry is a method for the study of cellular proliferation. The cell cycle consists of two phases: interphase and mitosis. Interphase has three distinct, succesive periods: G0/G1, which is the growth and preparation phase; S, which is the DNA replication phase; G2, which is the preparation phase for mitosis (M). Cell arrest in one of these phases demonstrates a slow progression of cell cycle, lock of DNA synthesis and a delay of cell proliferation.

In our study, the changes observed in cell cycle dynamics (between G0/G1, S and G2/M phases) were based on DNA content measurement by flow cytometry after cell treatment of Caco-2 cells with C1-C4 cabbage extracts. The histogram indicating the percent of cells in each phase of the cell cycle, after 72 h of treatment, are presented in Figure 3.

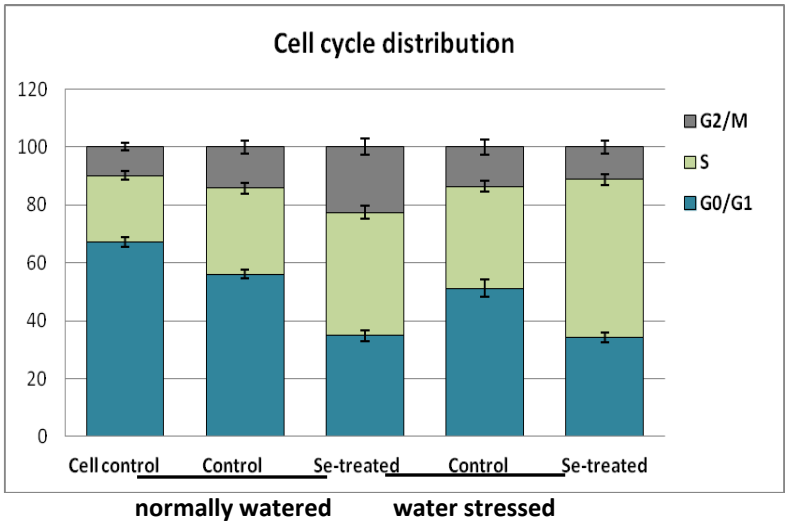


Figure 3. Effect of selenium-treated cabbage extracts cultivated in normally watered and water stressed conditions on cell cycle distribution of Caco-2 cells.

The results have showed that Caco-2 cell treatment with cabbage extracts determined a decrease of cell number in G0/G1 phase, while a higher proportion of cells were recorded in S and G2/M phases (Figure 3). All these data demonstrated that selenium-treated cabbage extracts were responsible for induction of S phase arrest, preventing progression of cell cycle to mitosis and, therefore, Caco-2 cell proliferation. These observations were in accordance with cell viability results. Similar cell culture studies indicated that curcumin extract blocked human osteosarcoma cells in S and G2/M phases and induced their apoptosis (Lee et al. 2009).

3.5. In vivo experiments

In order to analyze the functional food characteristics of cabbage crops biofortified with the selenium-based composition, we have performed reduction-refinement tests on lab animals. The hematological parameters determined in blood samples collected at the beginning and the end of the experiment were the number of white blood cell (WBC), lymphocytes (Lymph), monocytes (Mon), granulocytes (Gran), total hemoglobin (HGB) and hematocrit (HCT). The results obtained for animal groups fed with selenium-treated (C1) and control cabbage (C3) cultivated in normal watering conditions are presented in Table 2. These data showed higher values of WBC, including Lymph, Mon and Gran, after 14 days of feeding with selenium-treated cabbage (C1), indicating an increase of immune system activity. Moreover, HGB and HCT values were higher, demonstrating an improvement of red blood cell hematopoiesis and, consequently, a better tissue oxygenation. The values of hematological parameters analyzed for the control group (C3) ranged within normal limits (Table 2).

Table 2. Hematological and biochemical parameters of lab animals fed with selenium-treated (C1) and control (C3) cabbage plants cultivated in normal watering conditions

Hematological/ Biochemical parameters	Reference range	Animal group fed with C1 (selenium-treated plants)		Animal group fed with C3 (control plants)	
		day 0	day 14	day 0	day 14
WBC (x10^9/L)	5.2-13.5	5.25	8.47	6.55	7.65
Lymph (x10^9/L)	3.2-9.0	1.90	3.90	2.50	2.45
Mon (x10^9/L)	0.1-0.6	0.35	0.50	0.20	0.25
Gran (x10^9/L)	2.0-7.5	3.20	4.20	4.15	5.15
HGB (g/L)	105-170	107	147	106.5	114.5
HCT (%)	31-46	32	39	37	41
GOT (U/L)	6.7-54.2	24.95	26.15	26.80	18.55
GPT (U/L)	8.2-32.2	12.90	13.40	12.75	12.20
Creat (mg/dL)	≤ 1.8	0.99	0.81	1.27	1.19
Urea (U/L)	≤ 54	43.20	45.10	34.35	33.50

Values represent mean of three determinations for each animal.

Biochemical tests for liver consisted in analysis of transaminases GOT and GPT and those for kidneys in analysis of creatinine (Creat) and urea. The results obtained for animal groups fed with C1 and C3 indicated values within the reference range of the species (Table 2), demonstrating that the liver and the kidneys had normal function during the experiment. Similar results were obtained for the hematological and biochemical parameters of animal groups fed with selenium-treated (C2) and control (C4) cabbage plants cultivated in water stressed conditions (Table 3).

Table 3. Hematological and biochemical parameters of lab animals fed with selenium-treated (C2) and control (C4) cabbage plants cultivated in water stressed conditions

Hematological/ Biochemical parameters	Reference range	Animal group fed with C2 (selenium-treated plants)		Animal group fed with C4 (control plants)	
		day 0	day 14	day 0	day 14
WBC (x10^9/L)	5.2-13.5	4.05	4.75	5.65	6.15
Lymph (x10^9/L)	3.2-9.0	2.40	2.95	2.85	3.10
Mon (x10^9/L)	0.1-0.6	0.15	0.25	0.10	0.15
Gran (x10^9/L)	2.0-7.5	3.20	4.65	4.35	5.45
HGB (g/L)	105-170	109	119	108	112
HCT (%)	31-46	36.7	42.1	38.2	43.8
GOT (U/L)	6.7-54.2	19.25	20.75	23.80	24.50
GPT (U/L)	8.2-32.2	14.55	14.40	13.95	13.70
Creat (mg/dL)	≤ 1.8	1.29	1.49	1.40	1.58
Urea (U/L)	≤ 54	34.35	38.75	28.15	25.35

Values represent mean of three determinations for each animal.

All the results of *in vivo* testing of the functional food selenium biofortified cabbage showed a good tolerance in rabbits. All the rabbits used in experiment have survived (100% survival) and their health condition was improved.

4. CONCLUSIONS

Our results demonstrated that the new biostimulant formula based on selenium, betaine and spraying adjuvant increased the accumulation of selenium compounds without negative effects on sulforaphane content in normal watered plants. Also, this biofortification treatment, applied on Brassica crops, protected the plants against water stressed cultivation conditions. Extracts of cabbage plants treated with selenium-based composition exhibited high antiproliferative activity in Caco-2 adenocarcinoma cell line. The rabbits fed with selenium biofortified cabbage presented values of hematological and biochemical parameters within normal range of the species and improved the activity of their immune system.

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