From the Research Laboratories
В изследователските лаборатории

ALLELOPATHIC AND *IN VITRO* ANTICANCER ACTIVITY OF STEVIA AND CHIA

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Abstract. *Stevia rebaudiana* (Bertoni) Bertoni (Asteraceae) and *Salvia hispanica* L. (Lamiaceae) (chia) are *functional* foods used worldwide today. Additionally, aerial parts of both plants contain valuable secondary metabolites. Chemical composition of stevia and chia exhibits wide variations depending on geographic region, climatic conditions, time of plant collection etc. Nowadays there is a renewed interest on plants bioactive compounds concerning two important *health* problems: contamination of the environment *with agrochemicals and* inability to treat cancer. The aim of this study was to evaluate allelopathic and *in vitro* anticancer activity of water extracts made from the aerial parts of micropropagated stevia and chia grown in Bulgaria in laboratory conditions. The allelopathic potential was evaluated using germination and root/shoot elongation assays and *Allium cepa*-test. Water extracts of plants tested had no significant influence on seed germination, but affected seedling growth. Chia was *more effective* in suppressing *growth*, as *compared* to stevia. Effective Concentration causing 50% growth inhibition (EC50) value was determined: 11.12 g/l (stevia) and 6.17 g/l (chia). After exposure to extracts (EC50, for 24 h) mitotic indices in root *meristem* cells were extremely low. In such a case it is not recommended to score chromosomal aberrations. The results of the study revealed that water extract of chia possess much stronger allelopathic activity than stevia. Cytotoxic effect *in vitro* was evaluated using cell proliferation/viability of human hepatoma cell line SK-HEP-1. In the present study, stevia and chia water extracts had no influence on cancer cell line tested.

Keywords: chia; stevia; allelopathy; cytotoxicity; *Allium cepa*-test; SK-HEP-1 cell line

Introduction

Stevia rebaudiana (Bertoni) Bertoni (Asteraceae) and *Salvia hispanica* L. (Lamiaceae) are functional foods used worldwide. *Stevia rebaudiana* (stevia) is a natural sweetener and seeds of *Salvia hispanica* (chia) – a valuable food ingredient. Additionally, aerial parts of both plants contain different secondary

metabolites and are used in traditional medicine (Gupta et al., 2013; Ali et al., 2012).

Nowadays there is a renewed interest on plant bioactive compounds concerning two important problems *affecting human health*: *environmental pollution and* inability to treat cancer. *Urgent needs of the world today* are to reduce the use of agrochemicals and to find new effective anticancer drugs. *Scientific studies have proved* that herbal secondary metabolites could be useful tool for both problems solving. Some of them have allelopathic potential and could be used to control pests (Fujii et al., 2003; Gilani et al., 2010; Devkota et al., 2013; Kakati & Baruah, 2013). Additionally, various plant products possess promising anticancer properties (Gautam et al., 2014) and have shown cytotoxic effects on tumor cell lines (Reddy et al., 2003).

Some representatives of stevia and chia have been shown to contain allelochemicals in aerial parts (Taware et al., 2010; Ahmed et al., 1994). There are reports on antitumor activities of *Stevia rebaudiana* leaves (Jayaraman et al., 2008). Valuable metabolities were found in chia leaves (Amato et al., 2015). Chemical composition of stevia (Montoro et al., 2013) and chia (Ahmed et al., 1994) exhibits significant variations depending on the geographic region, climatic conditions, time of plant collection etc.

The aim of this study was to evaluate allelopathic and *in vitro* anticancer activity of water extracts made from the aerial parts of micropropagated *Stevia rebaudiana* and *Salvia hispanica* L. grown in Bulgaria in laboratory conditions. For this purpose, the following experimental approaches have been used: (1) germination and root/shoot elongation; (2) *Allium cepa*-test and (3) cell proliferation/viability of human hepatoma cell line SK-HEP-1.

Materials and methods

Plant extracts

Micropropagated plants (Zayova et al., 2013; 2016) were grown in the experimental field of Institute of plant physiology and genetics near Sofia, Bulgaria. Aerial parts of plants cut about 30 cm from the top were collected in the summer of 2014 and dried at room temperature.

Hot water extracts of air-*dried* and finely ground *aerial parts* were prepared and then allowed to cool to room temperature: stevia was boiled for 15 min and chia was covered with boiling deionized water for 60 min. Extracts were then filtered through filter paper. Based on our previous experiments (data not shown) stevia extracts were prepared at concentrations 2, 4, 6, 8, 10, 12 and 14 g/l and chia extracts – at concentrations 5, 6, 7, 8, 9 and 10 g/l.

Phytotoxicity testing

For germination and root/shoot elongation, seeds of *Triticum aestivum* L. were used. Seeds were thoroughly rinsed with tap water and deionized water. After that

seeds were placed between two sheets of filter paper and dried at 25 ˚C. Twenty seeds were placed on filter paper in each of ten Petri dishes (11 cm in diameter). Five ml of each extract or deionized water as a control were applied to the seeds. The dishes were sealed and incubated at 25 ˚C ± 1 ˚C for 72 h. Three replications of each treatment were done.

Germination assay

Germination was determined by counting the number of germinated seeds. Final germination was expressed as percentage after statistical analyses performed on the raw data.

Root/shoot elongation assay. The length of the roots and shoots of germinated seeds was measured. The percentage of root and shoot growth inhibition in relation to the control for each extract was determined. Seeds that did not germinate were not included in the root/shoot elongation test.

Determination of the effective concentration causing 50% growth inhibition (EC50) value

A growth curve was drawn: root length as percent of control (ordinate) against test concentrations (abscissa). From the growth curve EC values were obtained: EC50 = the effective concentration that decreased root growth about 50% when compared to the negative control group (deionized water, 100%) (Fiskesjö, 1985; Rank, 2003).

Allium cepa L.-test

Potential cytotoxicity and genotoxicity of water extracts were estimated using *Allium cepa* L. as test object. The onion bulbs were purchased from a biofarm certified to the BCS Öko Garantie; GLOBAL G.A.P and IFS Food. The outer scales of the bulbs and the old dry roots were carefully removed without destroying the root primordia. The bulbs were kept for root germination in deionized water for 24 h. Bulbs with new roots with length of 1.5 cm were placed in water extracts tested and were allowed to root for 24 h at 25±1 ºC. Then the root tips were washed thoroughly with deionized water, fixed in a Clarke's fixative (95% ethanol: acetic acid glacial, 3:1) for 90 minutes and hydrolyzed in 1N HCl for 8 min and in 45% acetic acid for 60 min at room temperature. Then they were stained for 90 min in 1% aceto-orcein and the terminal root tips (1-2 mm) were excised and squashed in 45% CH_3COOH. Each sample and control group consisted of 9 meristems from 3 bulbs. At least 1000 cells of each root meristem were analyzed. The microscopic analysis included assessment of the mitotic index and aberrant cells. The mitotic index was determined as a ratio between the number of cells in mitosis and the total number of analysed cells.

In vitro cytotoxicity assay

Solutions of plant extracts at concentration corresponding to determined EC50 value were used in order to evaluate their effects on cancer cell proliferation/viability. The SK-HEP-1 was obtained from National Bank for industrial Microorganisms and Cell Cultures (Bulgaria). The cells were maintained as adherent in controlled environment: DMEM medium, supplemented by 10% heat-inactivated fetal calf serum, in incubator at 37 ºC, 5% CO_2 and humidified atmosphere. In order to keep cells in log phase, the cultures were refed with fresh medium two or three times/week.

Cell proliferation/viability was assessed using Premixed WST-1 Cell Proliferation Reagent. The assay principle is based upon the reduction of the tetrazolium salt WST-1 to formazan in the mitochondria of living cells. Exponentially growing cells were seeded in 96-well flat-bottomed microplates (100 µl/well) at a density of 1×10^5 cells per ml. Time of treatment was 24 hours. Solutions were freshly prepared in DMEM and filtered using 0.22 µm filter. Four hours before the end of incubation time, cell proliferating reagent WST-1 (10 µl/well) was added to the culture media. Microplates were further incubated for 4 hours at 37 ºC. The absorbance of formazan product was quantitated at 450 nm using an ELISA reader. The cell survival fractions were calculated as a percentage of the untreated control (untreated control = 100%).

Statistical analysis

Experimental data were processed by Student's t-test.

Results

Germination assay

Allelopathic effect of water extracts on germination percentage of *T. aestivum* L. is shown in Tables 1 and 2. Stevia inhibited seed *germination* only after treatment with concentrations above 10 g/l. There was no clear dose-dependent effect. Similarly, chia extracts had no significant effect on germination. A nonlinear effect has also been observed: for example, treatment with 5 g/l and 10 g/l had the same effect (inhibition by 5.36%).

Table 1. Effect of water extracts of *Stevia rebaudiana* on germination of *T. aestivum* L. seeds

Concentration, g/l	Germination	
	means ± SD	%
0	17.67±1.53	100.00
2	17.67±1.53	99.98
4	18.67±1.15	105.64
6	18.67±1.53	105.64
8	16.33±2.31	92.44
10	16.00±1.00	90.55
12	14.33±2.08	81.12
14	12.33±3.79	69.80

Data are expressed as means ± SD (standard deviation); *P ≤ 0.05.

Table 2. Effect of water extracts of *Salvia hispanica* on germination of *T. aestivum* L. seeds

Concentration, g/l	Germination	
	means ± SD	%
0	18.50±1.00	100.00
5	17.75±0.50	95.95
6	18.75±0.50	101.35
7	17.75±0.96	95.95
8	18.00±0.82	97.30
9	17.25±2.22	93.24
10	17.25±1.89	93.24

Data are expressed as means ± SD (standard deviation); *P ≤ 0.05.

Root/shoot elongation assay

Notable inhibition of wheat growth by stevia was observed after treatment with concentrations above 10 g/l (Table 3). Unlike the germination, seedling growth was affected in dose dependent manner. Treatment affected root length of seedlings much more than shoot length. From the curve based on the obtained values of root length as percent of control against test concentrations EC50 value was obtained – 11.12 g/l (Fig. 1).

Table 3. Effect of water extracts of *Stevia rebaudiana* on root and shoot lenght of *T. aestivum* L.

	root			shoot	
Concentration, g/l	Average length (mm)±SD	Growth (%)		Average length (mm)±SD	Growth (%)
0	51.58±10.95	100		18.55±4.16	100
2	46.29±11.97*	89.74		18.47±4.24	99.98
4	45.78±9.66*	88.76		18.94±4.15	102.12
6	40.62±10.36***	78.75		18.16±4.14	97.90
8	36.60±9.72***	70.96		15.55±3.81***	83.80
10	27.67±8.79***	53.64		14.54±3.41***	78.39
12	24.34±6.38***	47.19		13.48±2.97***	72.68
14	19.87±5.84***	38.52		10.38±3.07***	55.96

Data are expressed as means ± SD (standard deviation); *P ≤ 0.05, ***P ≤ 0.001

Table 4. Effect of water extracts of *Salvia hispanica* on root and shoot lenght of *T. aestivum* L.

Concentration, g/l	root			shoot	
	Average length (mm)±SD	Growth (%)		Average length (mm)±SD	Growth (%)
0	57.76±12.55	100.00		17.12±3.31	100.00
5	30.94±10.32***	53.57		17.10±3.69 NS	99.89
6	30.77±8.95***	53.27		13.85±4.14***	80.88
7	19.19±7.05***	33.22		13.09±3.46***	76.46
8	18.96±6.75***	32.82		12.42±2.73***	72.53
9	18.37±7.15***	31.80		12.42±3.66***	72.57
10	19.71±6.12***	34.12		12.42±3.66***	72.57

Data are expressed as means ± SD (standard deviation); ***P ≤ 0.001

Table 4 *summarizes* the results about effects of chia extracts on wheat root and shoot growth. As can be seen, chia exerted a stronger inhibitory effect in comparison with stevia: influence of 10 g/l chia extract was similar to influence of 14 g/l stevia extract. Respectively, EC50 value of chia is two fold lower than EC50 value of stevia – 6.17 g/l (Fig. 2).

Allium cepa-test

Using *Allim cepa*-test the influence of plant extracts tested at concentration equal to EC50 values was determined. Both plants showed significant mitodepressive effect – mitotic indices were extremely low (Table 5). In such a case it is not recommended to score chromosomal aberrations (Rank, 2003).

Table 5. Mitotic index and phase indices in root meristematic cells of *Allium cepa* L. exposed to water extracts of *Salvia hispanica* (11.12 g/l) and *Salvia hispanica* (6.17 g/l) for 24 h

	Mitotic index		Prophase		Metaphase		Anaphase		Telophase	
Sample	%	SD	%	SD	%	SD	%	SD	%	SD
Control	5.51	±0.76	34.31	±14.48	20.86	±6.42	11.89	±8.15	32.94	±9.65
Stevia	< 1.00		–		–		–		–	
Chia	< 1.00		–		–		–		–	

Stevia – water extract from *Stevia rebaudiana;* Chia – water extract from *Salvia hispanica*; Control – deionized water; n – number. Data are expressed as means ±SD (standard deviation).

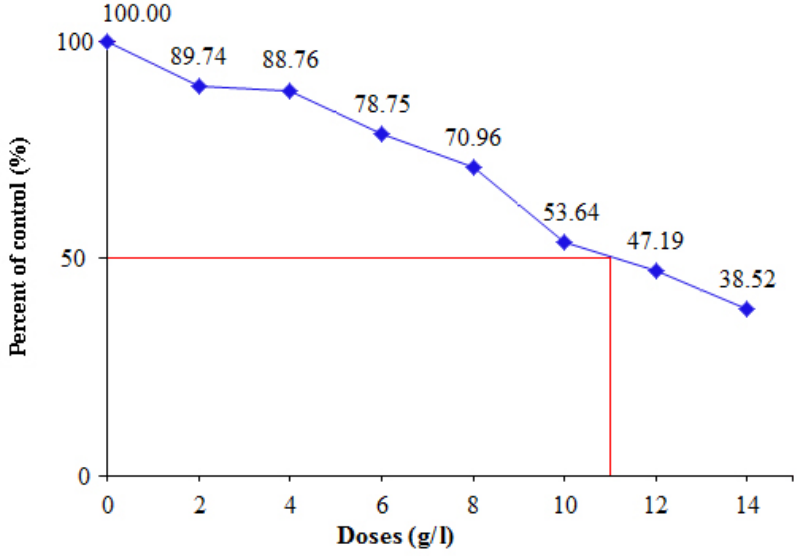

Fig. 1. Determination of the Effective Concentration causing 50% growth inhibition (EC50) value of water extracts of *Stevia rebaudiana* on root growth of *T. aestivum* L.

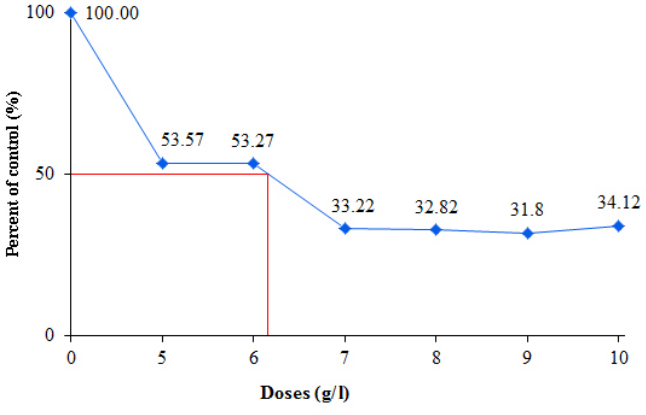

Fig. 2. Determination of the Effective Concentration causing 50% growth inhibition (EC50) value of water extracts of *Salvia hispanica* on root growth of *T. aestivum* L.

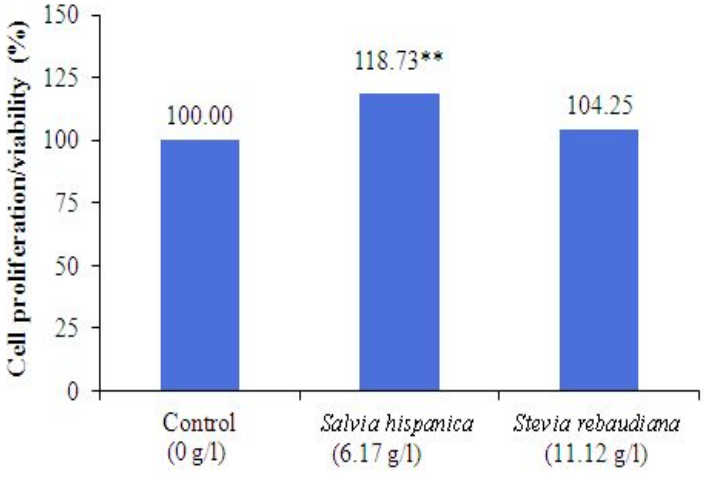

Fig. 3. Effect of *Stevia rebaudiana and Salvia hispanica* on proliferation/viability of human hepatoma cell line SK-HEP-1. **P ≤ 0.01.

In vitro cytotoxicity assay

Estimated EC50 values of plant extracts were used to evaluate effects on cell proliferation/viability (Fig. 3). After treatment cell viability was similar to those of control, respectively about 104% (stevia) and 119% (chia).

Discussion

Although stevia and chia are used mainly as functional foods, these plants contain various bioactive compounds. From this point of view, a matter of interest are studies on plant extracts as a source of allelochemicals and anticancer agents. In present study allelopathic and *in vitro* anticancer activity of water extracts of micropropagated stevia and chia grown in Bulgaria was evaluated. It is known that micropropagation represents an approach to develop plants with constant traits. On the other hand, because of variation in secondary metabolites, studies on plants from different geographical regions are important.

Effects of allelochemicals on sensitive plants can be easily tested in laboratory conditions: responses such as changes in seed germination, plant height and root length are estimated (Sampietro et al., 2009). *T*he results of present study revealed that water soluble allelochemicals in plants tested have no significant influence on seed germination. Moreover, a nonlinear effect was observed. The inconsistent effects of extracts tested on germination are in accordance with data about other plants (Mutlu & Atici, 2009). It is known that plant interaction is a *complex phenomenon* and even positive and negative effects could be observed at different concentrations of secondary metabolites (Baeshen, 2014).

Seedling growth was affected to great degree in comparison with germination. The established growth reduction could be attributed to inhibitory effects of allelopathic substances in water extracts. Chia exerted much stronger growth inhibition as compared with stevia. In our study, root growth was more sensitive than shoot growth. Stronger inhibitory effect on root length as compared to shoot length was reported in other studies (Kakati & Baruah, 2013). A possible explanation is that the permeability of allelochemicals to root is greater than to shoot (Nishida et al., 2005).

Allelochemicals can affect the plant growth by different manner: one of the principal mechanisms is the alteration in the mitotic index (Mohamed & El-Ashry, 2012; Reigosa et al., 1999). Based on root growth inhibition data EC50 values were estimated. EC50 determination is widely used as a first step in cytogenetic studies on medicinal plants extracts (Sarkar et al., 2012; Namkeleja et al., 2013). In present study both plants at concentration EC50 inhibited cell division. The decline of the mitotic index after treatment indicates the occurrence of a cytotoxic effect (Leme & Marin-Morales, 2009).

The results of present study confirmed observations that many plants used in traditional medicine show allelopathic effect (Kakati & Baruah, 2013). Plants with proven allelopathic activity are an important potential source for alternative pest management strategies (Macias et al., 2003). A matter of interest is to identify specific allelochemicals in chia, which revealed significant inhibitory effect.

Contemporary medicine uses different plant-derived compounds to treat cancer (Kakati & Baruah, 2013). Cancer diseases still remind a great concern to modern society and secondary metabolites in medicinal plants are widely studied as po-

tential anticancer agents. Cytotoxic effect on cancer cells *in vitro* can be detected using cell viability assay (Chen et al., 2015). In present study, stevia and chia water extracts had no influence on cancer cell line tested. One possible explanation could be the type of extraction, since in other study acetone and ethyl acetate extracts of *Stevia rebaudiana* were found to be cytotoxic to HEP2 cells, but water extract showed no pronounced antitumour activity (Jayaraman et al, 2008). So, our results confirm only the lack of anticancer activity of water extracts of plants tested, but not exclude future research on their possible medicinal application.

Conclusion

The results of present study revealed that water extracts from leaves of chia had much stronger allelopathic effect on the growth and development of *T. aestivum* L. as compared with stevia. The possibility of utilization of chia water extracts in agriculture needs to be studied further.

Water extracts from stevia and chia aerial parts had no influence on cell proliferation/viability of SK-HEP-1cell line.

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REFERENCES

Ahmed, M., Ting, I.P. & Scora, R.W. (1994). Leaf oil composition of *Salvia hispanica* L. from three geographical areas. *J. Essential Oil Res., 6,* 223 – 228.

Ali, N.M., Yeap, S.K., Ho, W.Y., Beh, B.K., Tan, S.W. & Tan, S.G. (2012). The promising future of chia, *Salvia hispanica* L. *J. Biomed. & Biotechnology*, *2012,* art. no. 171956.

Amato, M., Caruso, M.C., Guzzo, F., Galgano, F., Commisso, M., Bochicchio, R., Labella, R. & Favati, F. (2015). Nutritional quality of seeds and leaf metabolites of Chia (*Salvia hispanica* L.) from Southern Italy. *Eur. Food Res. & Technology*, *241*, 615 – 625.

Baeshen, A.A. (2014). Morphological and elements constituent effects of allelopathic activity of some medicinal plants extracts on *Zea mays*. *Int. J. Curr. Res. & Acad. Rev*., *2*(4)*,* 135 – 145.

Chen, J.-C., Chan, Y.-C. & Hwang, J.-H. (2015). Effects of tetrandrine and caffeine on cell viability and expression of mammalian target of rapamycin, phosphatase and tensin homolog, histone deacetylase 1, and histone acetyltransferase in glioma cells. *Tzu Chi Med. J., 27,* 74 – 78.

Devkota, A., Sharma, S., Ghimire, S.R. & Jha, P.K. (2013). Evaluation of allelopathic potential and phytochemical screening of some medicinal plant species of Nepal. *Int. J. Pharm. & Biol. Archive, 4,* 439 – 445.

Fiskesjö, G. (1985). The *Allium* test as standart in rnviromental monitoring. *Hereditas, 102*, 99 – 112.

Fujii, Y., Parvez, S.S., Parvez, M.M., Ohmae, Y. & Iida, O. (2003). Screening of 239 medicinal plant species for allelopathic activity using sandwich method. *Weed Biology & Management, 3,* 233 – 241.

Gautam, N., Mantha, A.K. & Mittal, S. (2014). Essential oils and their constituents as anticancer agents: a mechanistic view. *BioMed Res. Intern*., *2014*, art. no. 154105.

Gilani, S.A., Fujii, Y., Shinwari, Z.K., Adnan, M., Kikuchi, A. & Watanabe, K.N. (2010). Phytotoxic studies of medicinal plant species of Pakistan. *Pakistan J. Botany, 42,* 987 – 996.

Gupta, E., Purwar, S., Sundaram, S. & Rai, G.K. (2013). Nutritional and therapeutic values of *Stevia rebaudiana*: a review. *J. Med. Plants Res.,7,* 3343 – 3353.

Jayaraman S., Manoharan, M. S. & Illanchezian, S. (2008). *In-vitro* antimicrobial and antitumor activities of *Stevia Rebaudiana* (Asteraceae) leaf wxtracts. *Tropical J. Pharm. Res., 7,* 1143 – 1149.

Kakati, B. & Baruah, A. (2013). Allelopathic effect of aqueous extract of some medicinal plants on seed germination and seedling length of mung bean (*Vigna radiata* (L.) Wilczek. *Indian J. Plant Sci., 2,* 8 – 11.

Leme, D.M. & Marin-Morales, M.A. (2009). *Allium cepa* test in environmental monitoring: a review on its application. *Mutation Res.*, *682*, 71 – 81.

Macias, F.A., Marin, D., Oliveros-Bastidas, A., Varela, R.M., Simonet, A.M., Carrera, C. & Molinillo, J.M.G. (2003). Allelopathy as a new strategy for sustainable ecosystems development. *Biol. Sci. Space, 17,* 18 – 23.

Mohamed, F.I. & El-Ashry, Z.M. (2012). Cytogenetic effect of allelochemicals *Brassica nigra* L. extracts on *Pisum sativum* L. *World Appl. Sci. J., 20,* 344 – 353.

Montoro, P., Molfetta, I., Maldini, M., Ceccarini, L., Piacente, S., Pizza, C. & Macchia, M. (2013). Determination of six steviol glycosides of *Stevia rebaudiana* (Bertoni) from different geographical origin by LC–ESI–MS/MS. *Food Chem., 141,* 745 – 753.

Mutlu, S. & Atici, O. (2009). Allelopathic effect of *Nepeta meyeri* Benth. extracts on seed germination and seedling growth of some crop plants. *Acta Physiol Plant., 31,* 89 – 93.

Namkeleja, H.S., Tarimo, M.T. & Ndakidemi, P.A. (2013). Allelopathic effect of aqueous extract of *Argemone mexicana* L on germination and growth of *Brachiaria dictyoneura* L and *Clitoria ternatea* L. *Amer. J. Plant Sci., 4,* 2138 – 2147.

Nishida, N., Tamotsu, S., Nagata, N., Saito, C. & Sakai, A. (2005). Allelopathic effects of volatile monoterpenoids produced by *Salvia leucophylla*: inhibition of cell proliferation and DNA synthesis in the root apical meristem of *Brassica campestris* seedlings. *J. Chem. Ecol., 31,* 1187 – 1203.

Rank, J. (2003) The method of allium anaphase-telophase chromosome aberration assay. *Ekologija Vilnius, 1,* 38 – 42.

Reddy, L., Odhav, B. & Bhoola, K.D. (2003). Natural product for cancer prevention: a global perspective. *Pharmacology & Therapeutics, 99,* 1 – 13.

Reigosa, M.J., Sanchez-Moreiras, A. & Gonzales, L. (1999). Ecophysiological approach in allelopathy. *Crit. Rev. Plant Sci., 18,* 577 – 608.

Sampietro, D.A., Quiroga, E.N., Soberón, J.R., Sgariglia, M.A. & Vattuone, M.A. (2009). Bioassays with whole plants and plant prgans (pp. 377 – 398). In: Sampietro, D.A., Catalan, C.A.N. & Vattuone, M.A. (Eds.). *Isolation, identification and characterization of allelochemicals/natural products*. Boca RatonL CRS Press.

Sarkar, E., Chatterjee, S.N. & Chakraborty, P. (2012). Allelopathic effect of *Cassia tora* on seed germination and growth of mustard. *Turkish J. Botany, 36,* 488 – 494.

Taware A.S., Harke, S.N., Mukadam, D.S., Chavan, A.M. & Taware, S.D (2010). Effect of different extracts of callus and plantlets of *Stevia rebaudiana* (Bertoni) on seed germination of some agricultural crops. *African J. Biotechnology, 9,* 6675 – 6683.

Zayova, E., Nikolova, M., Dimitrova, L. & Petrova, M. (2016). Comparative study of in vitro, ex vitro and in vivo propagated *Salvia hispanica* (chia) plants: morphometric analysis and antioxidant activity. *AgroLife Sci. J., 5,* 166 – 173.

Zayova, E., Stancheva, I., Geneva, M., Petrova, M. & Dimitrova, L. (2013). Antioxidant activity of in vitro propagated *Stevia rebaudiana* Bertoni plants of different origins. *Turkish J. Biology, 37,* 106 – 113.

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