SOME EFFECTS INDUCED BY THE HYDROALCOHOLIC EXTRACT OF *TRIFOLII RUBRI FLOS* (RED CLOVER FLOWERS) ON *TRITICUM AESTIVUM* L. PLANTS

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Abstract. Red clover (*Trifolium pratense*) hydroalcoholic extract was prepared by extraction of powdered dried flowers with ethanol 70% v/v (1:13.33), by reflux for two hours. This was diluted with distilled water to give the final concentrations of 0.5, 1 and 5% (v/v) (TPEx1, TPEx2 and TPEx3). These concentrations were tested for their effects on seed germination and seedlings growth of wheat (*Triticum aestivum*) in a laboratory experiment. Distilled water was used as a control (C). After the 10 days of experiment, we evaluated seed germination and seedling growth (root and shoot length, fresh and dry biomass) of wheat.

INTRODUCTION

Plants have been used to prevent and treat various human diseases throughout history (Akinboro and Bakare, 2007). Epidemiological studies suggest that a reduced risk of cancer is associated with increased consumption of vegetables and fruits. The beneficial effects observed in these studies have been attributed to the presence of numerous polyphenol compounds with antioxidant and free radical scavenging - properties. In addition, among the active polyphenol compounds, flavonoids have been shown to have antioxidative and anticarcinogenic properties (Okiemy Akeli *et al*., 2010). Despite the current availability of a multiplicity of anticancer agents, there is a continuous search for new compounds that may be more effective and safe (Kumar and Singhal, 2009). As a consequence it is extremely important the employment of genotoxicity tests to identify their possible mutagenic potential (Celik and Aslnatürk, 2010; Saulo *et al*., 2009). The most common vegetal species used as plant models for the evaluation of cytogenetic and mutagenic effects of plant extracts and drugs are represented by *Vigna radiata* (Kumar and Singhal, 2009), *Vicia faba*, *Tradescantia paludosa*, *Pisum sativum*, *Hordeum vulgare*, *Triticum aestivum, Secale cereale, Crepis capillaries* and *Allium cepa* (Akinboro and Bakare, 2007).

Literature presents some studies conducted to elucidate the potential effects of different vegetal extracts on *Triticum* species. In comparison with numerous species of genus *Brassica*, Mason - Sedum *et al.* reported that water extracts from residues of the genus *Brassica* significantly reduced root and coleoptile length of wheat with little effect on germination. In bioassay experiments, Bialy *et al*. showed that inhibition of seed germination occurred upon treatment with chemicals derived from species of the genus *Sinapis* (Abu- Romman *et al*., 2010). Mesquite (*Prosposis juliflora*) aqueous leaf extract was tested for its effects on seed germination and radicle length of *Triticum aestivum* var- Lok. It caused a pronounced inhibitory effect on seed germination and root length, proportionally to the concentration of the extract (Siddiqui *et al*., 2009). The effects of *Euphorbia hierosolymitana*- aqueous leachate on germination, seedling growth, and total chlorophyll and protein contents of wheat were examined (Abu- Romman *et al.,* 2010). A cultivar of barley (*Hordeum vulgare*) was found to be phytotoxic to durum wheat *(Triticum durum*) and bread wheat (*T. aestivum*). For both wheat species, radicle growth was more depressed than coleoptile growth, though stimulation of seedling growth was observed for durum wheat (Ashrafi *et al*., 2008; Ashrafi *et al.,* 2009). The phytobiological testing of some vegetal extracts of *Mentha piperita* and *Trigonella foenum- graecum* on wheat (*Triticum aestivum*, *Dropia* cultivar) did not show significant clastogenic and cytotoxic effects (Gille *et al*., 2006). Treatments with two flavonoid compounds, rutin and quercetin, respectively, slightly reduced the germination percent of wheat in case of quercetin, and rutin strongly inhibited the seeds germination at maximum concentration (0.1%). Also, they influenced wheat seedlings growth in a different way, depending on their chemical structure and concentration (Cretu *et al.*, 2006a; Cretu *et al*., 2009).

Some hydroalcoholic extracts of *Medicago herba*, *Glycine semen* and *Trifolii rubri flos* were tested for their phytobiological effects on wheat (*Triticum aestivum*, *Dropia* cultivar). The results indicated also positive and negative effects on growth and development of wheat seedlings (Cretu *et al*., 2006b). Some products with flavonoid compounds, including vegetal extracts of *Medicago herba*, *Glycine semen* and *Trifolii rubri flos* and other vegetal powders, induced some inhibitory effects of seeds germination and seedlings growth, without reference of vegetal species combination (Cretu *et al*., 2006c).

Trifolium pratense L. (red clover) is rich in isoflavonoids. The major active estrogenic isoflavonoids are biochanin A, daidzein, formononetin and genistein. It also contain carbohydrates: arabinose, glucose, glucuronic acid, rhamnose, xylose (following hydrolysis of saponins glycosides), polysaccharide (a galactoglucomannan); flavonoids:

isorhamnetin, kaempferol, quercetin, and their glycosides; saponins; coumaric acid; phaseolic acid; salicylic acid; vitamins and minerals. Traditionally red clover has been used for chronic skin disease, whooping cough and especially for eczema and psoriasis (Barnes *et al*., 2007).

In our study, we investigated the effects of a red clover 70% hydroalcoholic extract on wheat (*Triticum aestivum*, *Dropia* cultivar) seeds germination, root and shoot length, and fresh and dry biomass.

MATERIALS AND METHODS

Extract preparation. The fluid hydroalcoholic extract of *Trifolium pratense* (red clover) flowers (TPEx) was obtained from powdered dried material by reflux with 70% ethanol (1:13,33), for two hours. The hydroalcoholic extract was filtered through a textile filter, and used as a stock extract. This was diluted with distilled water to give the final concentrations of 0.5, 1 and 5% (v/v) (TPEx1, TPEx2 and TPEx3).

Extract analysis. The stock extract was qualitative and quantitatively analyzed. The qualitative analysis consisted in phytochemical screening and spectroanalytical profile by HPTLC and UV/VIS absorption spectroscopy.

Phytochemical screening was done by specific chemical reactions for the identification of phytochemicals presence in the stock extract (Ciulei *et al*., 1994).

Spectroanalytical profile was done in order to detect the presence of hyperoside (from flavone O- glycosides class) and biochanin A (from isoflavonoids class) in red clover extract (TPEx).

Detection of hyperoside - Equipment: CAMAG LINOMAT IV, CAMAG TLC 3 Scanner, WINCATS Planar Chromatography Manager. Chromatographic conditions: Stationary phase - HPTLC plates G60F254 10 x 10 cm, 0.2 mm thickness (Merck); Wavelength - 366 nm after derivatization; Mobile phase - ethyl acetate: methanol: water: formic acid = 25:1:1.5:3 v/v; Derivatization - 0.5% diphenylboryloxyethylamine (Natural Product, NP) in ethyl acetate, followed by 5% polyethylene glycol - 4000 (PEG) in dichloromethane; Reference - hyperoside.

Detection of biochanin A - Equipment: CAMAG LINOMAT IV, CAMAG TLC 3 Scanner, WINCATS Planar Chromatography Manager. Chromatographic conditions: Stationary phase - HPTLC plates G60F254 10 x 10 cm, 0.2 mm thickness (Merck); Wavelength - 254 nm; Mobile phase - chloroform: methanol: water: glacial acetic acid = 24:12:4:0.2 v/v; Reference - biochanin A.

UV/VIS absorption spectrum of red clover extract was done with a CARY 50 UV/VIS spectrophotometer, by reading the extract maximum absorption in the 200 - 400 nm range.

The total flavonoids and polyphenols of red clover stock extract (TPEx) were quantitatively evaluated. Total flavonoid content was determined by following colorimetric aluminium chloride method and calculated as rutin (g/100 ml). The absorbance of reaction mixture was measured at 430 nm with a CARY 50 UV/VIS spectrophotometer (Cretu *et al*., 2011). Total polyphenol content was determined by Folin-Ciocalteu method and expressed in terms of gallic acid equivalent, which is a common reference compound. The absorbance of reaction mixture was measured at 760 nm with a CARY 50 UV/VIS spectrophotometer (g/100 ml) (Cretu *et al*., 2011). Results were presented as mean ± SD (standard deviation).

All reagents were of purity grade.

Seeds treatment. One hundred seeds of *Triticum aestivum* L. (*Dropia* cultivar from Secuieni Agricultural Research and Development Station, Neamt) were treated with different concentrations (0.5%, 1% and 5%) of the red clover – 70% hydroalcoholic extract, for 12 hours. Distilled water was used as a control (C). Seeds were washed with distilled water and placed on an inert material, in hydroponic system (constant level of water) and maintained under 23 ± 1^0C and natural light (day/night alternance, with a 12 hours photoperiod), for 10 days. The experiment was performed in the laboratory of the Commercial Society for Medicinal Plant Research and Processing "PLANTAVOREL" S.A. Piatra Neamt, between 1.03- 10.03. 2010.

Seeds bioassay. After the 10 days of experiment, germination was determined by counting the number of germinated seeds and expressed as total percentage, the root and shoot lengths by measuring representative seedlings. Fresh and dry weights were calculated by seedlings separation into root and shoot parts, and fresh samples were dried at room temperature (Cho *et al*., 2007). The results of root and shoot length measurements were evaluated statistically by Student's test.

RESULTS AND DISCUSSION

Analysis of red clover extract

The result of the phytochemical screening (Cretu *et al*., 2011) revealed that tannins, reducing sugars, aminoacids, flavonoids, flavonoid glycosides, polyphenols, coumarins, sterolic

saponins were present in the hydroalcoholic extract of *Trifolium pratense* (flowers) (TPEx), (Table 1).

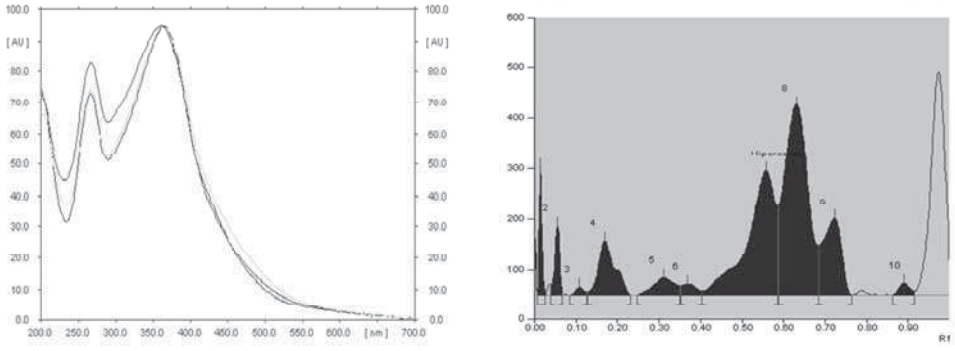
The HPTLC profiles identified the flavonoid compounds represented by hyperoside (Fig. 1a, b) and biochanin A (Fig. 2a, b) in the red clover 70% hydroalcoholic extract (TPEx).

The UV/VIS absorption spectrum evidenced maximum peaks at 206, 208, 213, 214.9, 219, 224, 229.1 and 263 nm (Fig. 3). According to literature, the peaks in the 210 - 310 nm range are due to the phenolic group, and those in the 255 - 280 nm range are specific to the flavonoids. Also, the 255 - 270 nm range are due to the aromatic structures and the chromopherous groups >C=O (Manole, 2008).

Table 1. Phytochemical screening of the 70% hydroalcoholic extract of *Trifolium pratense* flowers

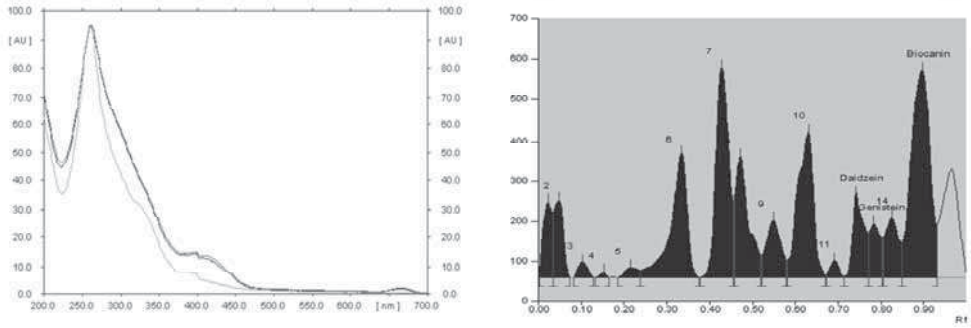
Phytochemical	Inherence
Hydroalcoholic extract	
Tannins	+ + + (dark - green)
Reducing sugars	+ + +
Alcaloids	-
Aminoacids	+
Flavonoids	+ + +
Polyphenols	+
Hydrolized hydroalcoholic extract	
Anthracyanosides	-
Coumarins	+ + +
Cardiotonic heterosides	-
Sterolic saponins	+ + (red ring)
Triterpens	-
Flavonoid glycosides	+
Proanthocyanidols	-
Anthocyanosides	-

“+”= present; “-“= absent.



a) **b)**

Fig. 1. Hyperoside detection: a) UV spectrum of hyperoside in red clover extract; b) Analogue curve of red clover extract at 366 nm after derivatization.



a) **b)**

Fig. 2. Biochanin A detection: a) UV spectrum of biochanin A in red clover extract; b) Analogue curve of red clover extract at 254 nm.

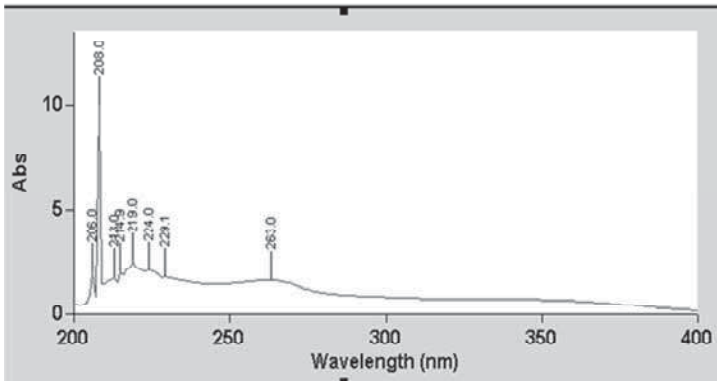


Fig. 3. UV/VIS spectrum of the 70% hydroalcoholic extract of *Trifolium pratense* flowers

Total content of flavonoid compounds (as rutin) and polyphenols (as gallic acid) in red clover stock extract is presented in Table 2.

Qualitative (phytochemical screening, HPTLC profile and UV/VIS absorption spectrum) and quantitative (total flavonoid and polyphenol content) analyses revealed the complex composition of the 70% hydroalcoholic extract of *Trifolium pratense* flowers (TPEx).

Table 2. Total flavonoid content in red clover 70% hydroalcoholic extract

Phytochemical content	Red clover extract (TPEx)
Total flavonoids as rutin (g/100 ml)	0.0873 ± 0.0007*
Total polyphenols as gallic acid (g/100 ml)	0.1305 ± 0.0024*

*= mean of three determinations ± SD (standard deviation)

Seed bioassay

The effect of red clover extract at different concentrations on the germination of wheat is shown in Table 3. The control treatment produced a germination rate of 96%. Compared to control (C), the maximum seed percentage was shown at TPEx1 treatment. Germination reduction was about 1% after treatment with TPEx2 extract. Regarding this results, we can observe that the tested hydroalcoholic extract of *Trifolium pratense* flowers did not significantly influence seed germination at all concentrations, compared to control. Also, stimulation or inhibition of seed germination was not proportional to the concentration of the extract.

The study of table 4 revealed that red clover extract stimulated root length, compared to control, with a maximum stimulation of 16% at TPEx2 treatment. Minimum and maximum concentrations determined a similar stimulation, of 9% and 8%, respectively. Regarding shoot length, we can observe a slightly inhibitory effect at TPEx1 and TPEx2 treatments (with 3% and 4%, respectively), where shoot length dropped to 96.11 mm and 94.76 mm, respectively, compared to control.

Table 3. Effect of red clover extract on germination (G %) of *Triticum aestivum* seeds

Variants	G %
Control (C)	96
TPEx1 (0.5%)	98
TPEx2 (1%)	95
TPEx3 (5%)	96

Table 4. Effect of red clover extract on seedling growth of *Triticum aestivum*

Variants	Root length (mm)				Shoot length (mm)			
	x ± sx		s%	C=100	x ± sx		s%	C=100
Control (C)	121.35	2.87	19.64	100	98.81	2.35	19.79	100
TPEx1 (0.5%)	132.90	2.54	16.00	109	96.11	1.99	17.29	97
TPEx2 (1%)	141.16	2.48	16.01	116	94.76	1.56	14.95	96
TPEx3 (5%)	131.08	2.52	17.85	108	98.71	1.94	18.25	100

In the present study, response indices revealed that the inhibition of growth parameters of seedlings was more pronounced than seed germination. The inhibitory effect of the tested concentrations - TPEx1 and TPEx2 - on shoot length of wheat may be related to the presence of allelochemicals including tannins, flavonoids and phenolic acids. Due to this, shoot length appeared more sensitive to allelochemicals than root length. This effect is slightly contradictory to some literature results, according to which, the alleopathic plant extracts generally have more pronounced effects on radicle, rather than hypocotyl growth. This may be attributed to the fact that radicles are the first to come in contact with allelochemicals (Ashrafi *et al*., 2008). Furthermore, these might be due to synergistic effect rather than single one. Phenolic acids have been shown to be toxic to germination and plant growth processes (Siddiqui *et al.,* 2009). Other studies have reported that the response to allelochemicals may be dependent on concentration (Ashrafi *et al*., 2009).

Red clover extract determined stimulation of root fresh biomass at all concentrations compared to control, ranging between 29 and 44% (Table 5). This is correlated with increasing extract concentration, at TPEx1 and TPEX2 treatments. Root dry biomass also increased as the

increasing of extract concentration, in case of TPEx1 and TPEx2 treatments. The response of wheat shoots was similar to that of roots; stimulation ranging is between 20 and 54% for fresh biomass, and 2 and 22% for dry biomass (Table 5). Also, we can observe a linearity between increasing of extract concentration and fresh and dry biomass stimulation.

Table 5. Effect of red clover extract on total fresh and dry biomass of *Triticum aestivum* seedling

Variants	Root				Shoot			
	Fresh biomass (g)		Dry biomass (g)		Fresh biomass (g)		Dry biomass (g)	
	T	C=100	T	C=100	T	C=100	T	C=100
Control (C)	4.1	100	0.511	100	3.5	100	0.636	100
TPEx1 (0.5%)	5.3	129	0.520	102	4.2	120	0.649	102
TPEx2 (1%)	6.2	151	0.613	120	4.9	140	0.762	120
TPEx3 (5%)	5.9	144	0.559	109	5.4	154	0.774	122

T = total fresh and dry biomass (g)

Results regarding fresh and dry biomass of roots are correlated with those obtained for the previous parameter - root length, respectively. In both cases, it took place stimulation, compared to control. Although fresh and dry biomass of shoot was stimulated, its length was inhibited at TPEx1 and TPEx2 treatments. In this case, the accumulation of fresh and dry biomass in treated wheat may reflect a thickening of shoots compared to control, an amplification of this process, rather than shoot cells elongation.

But, these results regarding total fresh and dry biomass did not offer us enough data as a result of the variable number of seedlings in each treatment variant. Thus, we calculated the individual fresh and dry biomass of wheat roots and shoots (Table 6).

Table 6. Effect of red clover extract on the individual fresh and dry biomass of *Triticum aestivum* seedling

Variants	Root				Shoot			
	Fresh biomass (mg)		Dry biomass (mg)		Fresh biomass (mg)		Dry biomass (mg)	
	M	C=100	M	C=100	M	C=100	M	C=100
Control (C)	59.42	100	7.41	100	50.72	100	9.22	100
TPEx1 (0.5%)	75.71	127	7.43	100	60.00	118	9.27	101
TPEx2 (1%)	74.70	126	7.39	99	59.04	116	9.18	99
TPEx3 (5%)	68.60	115	6.50	88	62.79	124	9.00	98

M = mean of individual fresh and dry biomass (mg)

Data presented in Table 6 revealed that the individual fresh biomass of root and shoot was stimulated, ranging between 15 and 27%, and 16 and 24%, respectively. Our results showed that stimulation was not linear with increasing of extract concentration. Thus, stimulation of the individual fresh biomass of root decreased as the extract concentration increased. In case of shoot individual fresh biomass, maximum stimulation was observed at TPEx3 treatment, compared to control. Individual dry biomass of root was inhibited at TPEx2 and TPEx3

treatments, significantly in the second case, with 12%, compared to control (Table 6). Also, the individual dry biomass of shoot was slightly inhibited at TPEx2 and TPEx3 treatments, (with 1% and 2%, respectively).

CONCLUSIONS

In our study, we have investigated the effects of a red clover - 70% hydroalcoholic extract at different concentrations on wheat plants. We have evaluated seed germination, root and shoot length, and fresh and dry biomass. Our work was carried out in a laboratory experiment.
Our treatments did not significantly influence seed germination at all extract concentrations. Also, stimulation or inhibition of germination capacity was not proportional to the concentration of the tested extract. These treatments stimulated root length and slightly inhibited shoot length. They also determined the increase of total fresh and dry - root and shoot biomass, at all concentrations and proportional to the increasing of extract concentration in case of wheat shoot. The last two concentrations decreased the individual dry biomass of root and shoot, significantly for root.

REFERENCES

Abu- Romman S., Shatnawi M., Shibli Rida, 2010: *Allelopathic Effects of Spurge (Euphorbia hierosolymitana) on wheat (Triticum durum).* Am-Euras. J. Agric. & Environ. Sci., 7 (3): 298 - 302.
Akinboro A., Bakare A. A., 2007: *Cytotoxic and genotoxic effects of aqueous extracts of five medicinal plants on Allium cepa Linn.* Journal of Ethnopharmacology, 112: 470 - 475.
Ashrafi Z. Y., Sadeghi S., Mashhadi H. R., Alizade H. M., 2008: *Study of allelopathical effects of barley on inhibition of germination and growth of seedling green foxtail.* Journal of SAT Agricultural Research, 6: 1 - 6.
Ashrafi Z. Y., Sadeghi S., Mashhadi H. R., 2009: *Inhibitive effects of barley (Hordeum vulgare) on germination and growth of seedling quack grass (Agropyrum repens).* Icelandic Agricultural Sciences, (22): 37 - 43.
Barnes Joanne, Anderson Linda A., Philipson J. D., 2007: *Herbal Medicines, Third Edition, Red clover*, Published by the Pharmaceutical Press, An imprint of RPS Publishing: 498.
Celik T. A., Aslnatürk Ő. S., 2010: *Evaluation of Cytotoxicity and Genotoxicity of Inula viscosa Leaf Extracts with Allium Test.* Journal of Biomedicine and Biotechnology, 1 - 8.
Cho Y.Y., Oh S.B., Oh M.M., Son J.E., 2007: *Estimation of individual leaf area, fresh weight and dry weight of hydroponically grown cucumbers (Cucumis sativus L.) using leaf length, width and SPAD value.* Scientia Horticulturae, vol. 111, Issue 4, 16 February: 330 - 334.
Ciulei I., Grigorescu Em., Stanescu Ursula, 1994: *Plante medicinale. Fitochimie si fitoterapie, vol.1.* Editura Medicala, Bucuresti: 229 – 234.
Cretu Ruxandra, Gille Elvira, Danila Doina, Floria Fl., Mihailescu Roxana, 2006a: *Phyto – biological testing of some flavonoid compounds of vegetal origin. Note 3. Phyto – biological testing of some flavonoid compounds- based products. Analele Stiintifice ale Universitatii “Alexandru Ioan Cuza” (Serie noua), Sectiunea Genetica si Biologie Moleculara,* VII: 165 - 170.
Cretu Ruxandra, Gille Elvira, Danila Doina, Floria Fl., Bara I. I., 2006b: *Phyto – biological testing of some flavonoid compounds of vegetal origin. Note1. Phyto – biological testing of rutoside and quercetol.* Analele *Stiintifice ale Universitatii “Alexandru Ioan Cuza” (Serie noua), Sectiunea Genetica si Biologie Moleculara*, VII: 171 - 174.
Cretu Ruxandra, Gille Elvira, Danila Doina, Floria Fl., Mihailescu Roxana, 2006c: *Phyto – biological testing of some flavonoid compounds of vegetal origin. Note2. Phyto – biological testing of vegetal extracts from Medicago herba, Glycine semen and Trifolii rubri flos.* Analele *Stiintifice ale Universitatii “Alexandru Ioan Cuza” (Serie noua), Sectiunea Genetica si Biologie Moleculara*, VII: 175 - 180.
Cretu Ruxandra, Gille Elvira, Danila, Doina, Floria Fl., 2009: *Effects induced by singular and combined treatments with gamma radiation and flavonoid compounds on Triticum aestivum L.* Analele Stiintifice ale Universitatii “Alexandru Ioan Cuza” (Serie noua), Sectiunea Genetica si Biologie Moleculara, X: 55 - 62.
Cretu Ruxandra, Mihailescu Roxana, Verdes Ramona, Mitroi Gabriela, Iacob Elena, Chiriac Maria, Mierlici Ionela, 2011: *The qualitative and quantitative characterization of Trifolii rubri flos and Violae tricoloris herba hydroalcoholic extracts.* Romanian Biological Sciences, Vol. IX, No. 1 - 4: 77 - 78.

Gille Elvira, Floria Fl., Mihailescu Roxana, Danila Doina, 2006: *The Phytobiological Testing of Some Extracts of Mentha piperita and Trigonella foenum- graecum used to obtain food supplements*. Romanian Biological Sciences, IV (3 - 4): 1 - 10.

Kumar V. L., Singhal A., 2009: *Germinating seeds of the mung bean, Vigna radiata (Fabaceae), as a model for the preliminary evaluation of cytotoxic effects of drugs.* Biocell, 33 (1): 19 - 24.

Manole Elena, 2008: *Vegetal extracts that are used for the protection of the polymers used as food packing material.* Journal of Agroalimentary Processes and Technologies, 14: 288 - 292.

Okiemy Akeli Marie-Genevieve, Ongoka P. R., Gatouillat Gr., Attibayeba Lavaud Catherine, Madoulet Claudie, 2010: *Cytotoxic effect induced by Morinda morindoides leaf extracts in human and murine leukemia cells.* African Journal of Biotechnology, 9 (39): 6560 - 6565.

Saulo Sousa M., Silva Pamela S., Campos J. M. S., Viccini L. F., 2009: *Cytotoxic and genotoxic effects of two medicinal species of Verbenaceae.* Caryologia, 62 (4): 326 - 333.

Siddiqui Sazada, Bhardwaj Shilpa, Khan S. S., Meghvanshi, M. K., 2009: *Allelopathic Effect of Different Concentration of Water Extract of Prosopsis Juliflora Leaf on Seed Germination and Radicle Length of Wheat (Triticum aestivum Var – Lok -1).* Am- Euras. J. Sci. Res., 4 (2): 81 - 84.

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