Phytotoxic and Antioxidant Activities of Leaf Extracts of *Ailanthus altissima* Swingle

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Leaf extracts of Ailanthus altissima (Mill.) Swingle (Simaroubaceae, smooth sumac) were evaluated for their phytotoxic and antioxidant activity. Tests on germination and growth of Lactuca sativa L. (Asteraceae, lettuce) seeds in the presence of extracts and of a synthetic herbicide by comparison were made. Antioxidant activity of extracts was determined by DPPH free radical scavenging and ABTS methods. Total phenols content and flavonoids amount were quantified spectrophotometrically. Extracts showed a very good inhibitory activity on germination seeds and growth of lettuce seedlings. Several compounds with potential phytotoxic activity were identified by GC-MS analysis, while the antioxidant activity could be accounted for by the high content of flavonoids found in extracts.

Keywords: Ailanthus altissima (Mill.) Swingle, phytotoxic activity, antioxidant activity

Ailanthus altissima (Mill.) Swingle (*Simaroubaceae*), known as the tree of heaven or ailanthus originating from China, grows spontaneously or it is cultivated as an ornamental tree. It was introduced to Europe in the 18th century. In traditional Chinese medicine the roots, bark and leaves of this tree are still used today for their medicinal properties astringent, hair growth stimulator, for treatment of dysentery, intestinal haemorrhage, cardiac palpitation, asthma, epilepsy [1]. Ailanthus has been reported for other uses such as: host plant to feed silkworms [1], good source of wood for papermaking [2,3]; root and bark extracts have been mentioned as having allelopathic properties on species like garden cress seeds, redroot pigweed, velvetleaf, yellow bristlegrass, barnyard grass, pea and maize [4,5]. Some studies have shown that ailanthone, compound which is considered responsible for alelopathic properties is also a good antimalarial agent [6]. In our previous work was shown that ailanthus leaf extract has antifungal effects which can be increased by heat shock, visible light and calcium [7].

Analyses of chemical composition of various extracts of ailanthus reported the presence of aliphatic C_6 compounds (alcohols, aldehydes, acids, esters), sesquiterpenes (*beta*-caryophyllene, *alpha*-humulene, *gamma* and *delta* cadinene), oxigenated monoterpenes (linalool, geraniol, *alpha*-terpineol) [8], polyphenols (hydroxycinnamic acids, flavonoids) [9], quassinoids [10, 11], alkaloids [12].

In this work the biological activity of ethanol extracts from the leaf of the ailanthus tree species growing spontaneously in Romania was evaluated. Phytotoxic activity of extracts was evaluated by tests on germination and growth of *Lactuca sativa* L. (lettuce, *Asteraceae*) seeds and was compared with that of a common herbicide Clinic 360 SL. Antioxidant activity was determined using DPPH free radical scavenging method and TEAC assay. Extracts were analyzed for their chemical content by GC-MS and total phenols content and flavonoids amount were determined spectrophotometrically.

Experimental part

Materials and methods

Ethanol (S.C. P.A.M. Corporation S.R.L., Romania), 1,1-diphenyl-2-picrylhydrazyl (DPPH·) (Sigma, Germany), Folin-Ciocalteu reagent, aluminum chloride hexahydrate, potassium acetate (Scharlau, Spain), sodium carbonate (Merck, Germany), gallic acid (Riedel-deHaën, China), quercetin hydrate (Wako, Japan), hydrogen peroxide 37%, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), potassium persulfate, potassium phosphate mono- and dibasic (Sigma-Aldrich, USA), *N*-(phosphonomethyl)glycine - Clinic 360 SL (360g/L) – herbicide, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), butylated hydroxytoluene (BHT) (Alfa Aesar, Germany).

Extracts preparation

Extracts were obtained by reflux and ultrasound methods. For both methods, 5 g of dry grounded leaves were extracted for 1 hour with ethanol: water = 70% (v/v) (vegetal material: extraction solvent ratio = 1:20, w/v). Thus, each hydro-alcoholic solution has 5% concentration. The extracts were centrifuged to discard the solid debris and the supernatants were filtered.

The vegetal species *Ailanthus altissima* (Miller) Swingle was identified by a biologist at the Faculty of Biology – Botanical Garden, University of Bucharest, Romania and a voucher specimen (number - BUC 400644) has been deposited in the herbarium.

Phytotoxicity assay

Sample preparation

Alcoholic extracts obtained were rotary evaporated at low pressure. The residues obtained were re-dissolved in distilled water (up to 100 mL) and then filtered. to get the stock solutions (considered as having 5% concentration). Two more extract concentrations (2.5 and 1.25% respectively) were obtained by diluting with distilled water the 5% corresponding stock solutions of the extracts. Before applying on lettuce seeds (from Mefim AGRO SRL, Romania) - 20 seeds per each Petri dish for germination tests and 10 seedlings for growth tests - extracts were

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sterilized by filtering through mixed cellulose ester membrane filter (0.45μm, ADVANTEC, Toyo Roshi Kaisha, Ltd., Japan).

Three concentrations of herbicide used for comparison (0.75 mg/mL, 0.38 mg/mL and 0.19 mg/mL respectively) have been used in the experiment. The highest concentration (0.75 mg/mL) was made by dilution of herbicide with distilled water, according to the indications from the product tag. From this solution, the other two concentrations were obtained by corresponding dilutions.

Phytotoxicity test

Tests on seed germination and on seedling growth and calculations were realized according to our previous work [13-17]. Two replicates were prepared for each sample.

Statistical analysis

Statistical analysis was done using Origin 6.0 program. Results were subjected to one way analysis of variances and the mean values were separated at $p < 0.05$ by applying 2-sample *t*-test.

GC-MS analysis of ethanol extracts

The GC–MS analysis of extracts in ethanol 70% (v/v) of ailanthus was done according to Popa et all. [7, 18]. An Agilent 6890 N gas chromatograph interfaced with an Agilent mass selective detector 5975B (Agilent Technologies, USA). Oven temperature program: 100–320^{0}C, at 10^{o}C/min; the injector parameters were: initial temperature 250 ºC, pressure 6.89 psi, split ratio 2:1, carrier gas helium. For the separation of the sample components a HP-5MS 5% phenylmethyl siloxane capillary column was used with the following characteristics: max. temperature 325°C, nominal length 30.0 m, nominal diameter 250.0 μm, nominal film thickness 0.25 μm, initial flow 1.0 mL/min, the mode of functioning was constant flow. Interface temperature was 280^{0}C; standard electronic impact (EI), MS source temperature: 230^{0}C; MS quadrupole temperature: 150^{0}C; mass scan range: 29 – 850 amu at 70 eV, total analysis time 39 min. One microliter of sample was injected into the system.

To identify the constituents from extracts, the mass spectra obtained for each compound was compared with those in the MS library (Nist, Wiley) and the reverse fit factors have been given.

Antioxidant activity determination of ethanol extracts

DPPH free radical scavenging activity was determined spectrophotometrically and according to Lungu et all., Lee et all. [19, 20]. 500 μL solution of standard/sample mixed with 500 μL DPPH· solution (0.135 mM in 70% (v/v) ethanol) were kept in dark for 30 min. Absorbance of these solutions were determined at λ = 514 nm. For calibration curve, solutions of Trolox in ethanol of concentrations between 2.5 – 25 μM were prepared. Inhibition percentage was calculated according to (1):

$$\%I = [(\text{absorbance of control} - \text{absorbance of sample}) / \text{absorbance of control}] \times 100 \quad (1)$$

Calibration curve inhibition percentage as a function of Trolox concentration (micromoles/L) was drawn. The equation of obtained curve was: %I = 0.9781 x concentration of standard + 50.73, correlation coefficient $r^2/n = 0.9900/6$ (n = number of determinations).

2,2-Azino-Bis(3-ethylbenzthiazoline-6-Sulphonic acid) (ABTS) radical scavenging activity was done according to Re et all. [21] 7mM ABTS water solution was mixed with 2.45 mM potassium persulfate solution at volumetric ratio 1:1. The mixture was kept in the dark at room temperature for 16 h. Before analysis, $ABTS^{\cdot+}$ obtained solution was diluted with 5 mM phosphate buffer (PBS) *p*H 7.4 to an absorbance of 0.70 ± 0.02 at 734 nm. In a 1 cm cuvette 990 μL $ABTS^{\cdot+}$ solution and 10 μL standard/extract were homogenised and the absorbance read after 1 minute. For calibration curve, solutions of Trolox in PBS of concentrations between 0.5 and 2.5 mM were prepared. The equation of linear domain of curve the inhibition percentage *vs.* Trolox concentration was: y = 17.31x – 4.66 (where y = percentage of inhibition, x = concentration of standard) and $r^2/n = 0.9847/5$.

Antioxidant activity values of extracts were expressed as milimoles/L Trolox equivalents for both methods.

Total phenols and flavonoids content determinations of ethanol extracts

Total phenols content of extracts obtained in ethanol 70% (v/v) was determined according to Folin-Ciocalteu method [22]. A mixture of 250 μL of each standard solutions (gallic acid in ethanol 70% v/v) or extracts, 2.5 mL aqueous solution of Folin-Ciocalteu (10% v/v) and 2 mL sodium bicarbonate solution (1 mol/L) were allowed to stand at room temperature for 15 min and then absorbance was measured at wavelength of 760 nm against blank (ethanol solution). Calibration curves absorbance *vs.* concentration of standard solutions was drawn. The equation of curve obtained was: y = 0.003x – 0.048, (where y = absorbance, x = concentration of standard), $r^2/n = 0.9970/8$ and linearity domain was 20 – 120 mg/L. The results were expressed as mg gallic acid equivalents (GAE) /100g dried plant material (dw).

Total flavonoids content of ethanol extracts was determined by aluminum chloride method [19, 22, 23]. 1 mL standard/extract mixed with 0.2 mL of 10% w/v aluminum chloride, 0.2 mL of 1 M potassium acetate and 5.6 mL of distilled water was kept at room temperature for 30 min and the absorbance of the reaction mixture was measured spectrophotometrically at 428 nm. Concentrations between 20-80 mg/L of rutin standard solutions were use for calibration curve, equations of obtained curve was y = 0.001x – 0.005, $r^2/n = 0.9930/7$ and linearity domain 10 - 200 mg/L. Flavonoids content was expressed as mg rutin equivalents (RE)/100g dw.

All determinations were carried out in triplicate.

Results and discussions

Phytotoxic effect of ailanthus extracts on germination of lettuce seeds

Data regarding the phytotoxic effect of extracts on lettuce seeds germination is presented in table 1.

The results obtained showed that all extracts totally suppressed germination of lettuce seeds, excepting 1.25% extract obtained by ultrasounds extraction where the

Extract concentration (%, w/v)	RI	
	Reflux	Ultrasounds
5.00	-1[a]	-1[a]
2.50	-1[a]	-1[a]
1.25	-1[a]	-0.85[a]

RI: responsive index = (number of lettuce seed test germinated in day 5/ number of control seeds germinated in day 5) - 1. If -1< RI > 0 extracts inhibit germination and if 0 < RI > 1extracts have stimulator effect; a - p < 0.001 (t-test)

Table 1
RESPONSE INDEX OF AILANTHUS ETHANOLIC EXTRACTS AT DIFFERENT CONCENTRATIONS

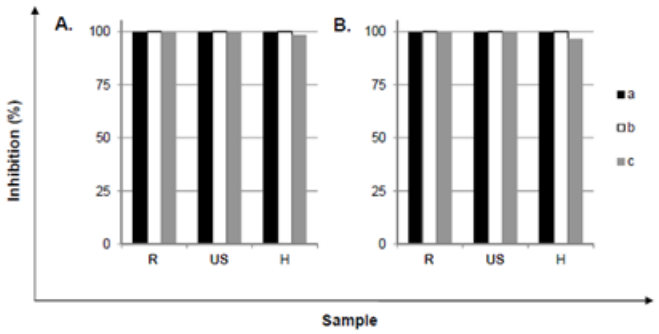

Fig. 1. Effect of ailanthus extracts on lettuce seedlings growth. A. root and B. shoot
R: reflux extract; US: ultrasound extract; H: herbicide; I(%): inhibition percent = $(L_{control}-L_{treatment})/L_{control}$ x 100, where $L_{control}$ = radicle length of lettuce control seedlings, $L_{treatment}$ = radicle length of the seedlings treated with extracts; a = 5%; b = 2.5%; c = 1.25% concentrations of extracts and respectively a = 0.75 mg/mL; b = 0.38 mg/mL; c = 0.19 mg/mL concentrations in case of herbicide

Table 2
CHEMICAL COMPOSITION OF EXTRACTS BY GC-MS

RT [min]	Compound	R (%)	Reverse fit factor	RT [min]	Compound	US (%)	Reverse fit factor
2.87	Cytosine	0.69	586	3.04	2,2,dimethyl-1,3-dioxolane-4-methanol	2.6	939
3.33	1,2,3-Propanetriol	1.49	899	3.35	1,2,3-propanetriol	0.91	905
5.15	N-Amyl acetate	2.69	770	5.29	1,3-dioxolane-4-methanol-2,2,dimethyl-acetate	1.02	929
6.31	Octanoic acid	2.36	924	6.43	octanoic acid	15.87	935
7.20	Triethylene-glycol-methyl ether	0.56	839	7.23	triethylene-glycol-methyl ether	0.6	859
8.36	Butanoic acid, 2,2-dimethyl	3.62	566	8.39	butanoic acid, 2,2-dimethyl	25.76	543
11.13	Guanosine	2.12	800	8.89	butanoic acid propyl ester	0.83	619
11.82	d-Galactose	0.58	550	11.02	guanosine	1.36	799
13.52	Butanoic acid, 2,3-dihydroxypropyl ester	4.01	904	13.53	butanoic acid, 2,3-dihydroxypropyl ester	9.2	902
13.97	d-Galactose, 6-deoxy	1.95	601	14.80	hexanoic acid, 2-ethyl, ethyl ester	16.44	462
14.80	Hexanoic acid, 2-ethyl, ethyl ester	24.26	470	15.98	mirystic acid	1.2	859
15.99	Mirystic acid	3.56	872	16.62	methyl 3,4,ethyliden-alpha-D-galactopyranoside	0.75	535
16.70	Octanoic acid propyl ester	1.10	506	17.26	butanoic acid 1,2,3,propanetriylester	1.69	924
17.14	Neophytadiene	5.52	919	18.75	palmitic acid	3.8	879
18.76	palmitic acid	13.28	880	19.76	stearic acid	0.12	617
19.50	stearic acid	1.35	718	20.71	phytol	7.28	906
19.73	oleic acid	0.59	844	23.69	glycerol tricaprylate	4.66	686
20.69	phytol	3.40	907	29.38	dinonanoin monocaprylin	0.58	401
20.98	6,9-pentadecadien,1-ol	6.89	800				
23.68	glycerol tricaprylate	5.25	697				
25.15	palmitin, 2-mono	0.91	731				
28.31	2,6,10,15,19,23-hexamethyl-2.6.10,14,18,22-tetracosahexaene	1.59	854				
29.36	dinonanoin monocaprylin	0.74	385				
31.07	cholest-, 5-on, 3-ol	1.35	451				
32.26	stigmasterol	1.49	428				
32.73	*gamma*-sitosterol	3.82	378				
	Total %	**95.17**				**94.67**	

RT – retention time (minutes); R – reflux extract, US – ultrasounds extract, Reverse fit factor – is a match factor calculated by the computer search system and indicates the closeness of fit between the obtained spectra and the reference spectra from the library. A reverse fit factor value of 1000 corresponds to a perfect match. Thus a reverse fit factor greater or equal to 800 represents reasonable matches with library spectra.

inhibition of germination was not total but quite significant. The herbicide used for comparison has no effect on germination because it is not effective as pre-emergence herbicide.

Phytotoxic effect of extracts on the growth of lettuce seedlings

The effect of ethanol extracts on root and shoot of lettuce seedling is presented in figure 1.

As can be observed in figure 1A, root growth inhibition was 100 % for all experiments. In figure 1B we can see that the inhibitory effect of leaf extracts on shoot seedlings is 100%.

When seedlings were treated with herbicide the root and shoot seedling growth inhibition percent was also 100%, except the case when 0.19 mg/L herbicide solution was applied the inhibition percent was slightly lower but still significant (fig. 1A,B). These results showed that ailanthus leaf extracts have good potential as herbicide [24].

GC-MS qualitative analysis of ailanthus extracts

The results of GC-MS qualitative analysis of ailanthus ethanol extracts are presented in table 2.

Table 3
ANTIOXIDANT ACTIVITY AND POLYPHENOLS CONTENT OF AILANTHUS ETHANOL EXTRACTS

Extract	Antioxidant activity		Polyphenols content	
	DPPH method (TE, mM)	ABTS method (TE, mM)	Total phenols (g GAE/100 g dw)	Flavonoids (g RE/100 g dw)
A-R	0.155 ± 0.013	8.93 ± 0.44	0.716 ± 0.080	5.34 ± 0.19
A-US	0.159 ± 0.0010	9.11 ± 0.21	0.553 ± 0.0040	5.41 ± 0.14

A: ailanthus; R: reflux; US: ultrasounds; TE: Trolox equivalents; GAE: gallic acid equivalents; RE: rutin equivalents

A total of 27 compounds were identified, among which saturated fatty acids, sterols, vitamin E, neophytadiene, phytol and others in smaller percentage.

The ailanthone (a diterpene) was not detected by GC-MS in analyzed leaf extract. The literature data reported ailanthone as the main component responsible for herbicidal activity in ailanthus [25] and is found in higher amounts in bark of this specie. Even if this compound was not detected still the extracts showed very good phytotoxic activity suggesting that there are other compounds from chemical composition of analyzed leaf extracts have phytotoxic activity. For example, some of fatty acids identified in extracts (decanoic acid, undecanoic acid) may have an important contribution to this type of activity, fact reported by some authors [26, 27]. Literature also mentions polyphenols (cinnamic acid, chlorogenic acid, rutin) [28, 29] with phytotoxic activity. Chlorogenic acid and rutin were also identified qualitatively by LC-MS (data not shown). Further studies can be done to establish the contribution of these compounds at phytotoxic activity of ailanthus extracts and a possible mechanism.

Antioxidant activity determination

Antioxidant activity values of ethanol extracts determined by DPPH and ABTS methods and expressed as mM Trolox equivalents (TE) are presented in table 3.

Values of antioxidant activity for the two extracts are quite similar: aprox. 0.16 mM TE of extract when DPPH method was used and respectively around 9 mM TE in case of ABTS method applied (table 3). The extraction method does not seem to influence the chemical content and antioxidant activity of extracts.

Total phenols and flavonoids content determination

TP and flavonoids contents are shown in table 3. As can be observed there are no significant differences between the values of TP of the two extracts, the same happens in case of flavonoids content of extracts.

Flavonoids are found in considerable higher amounts than TP content.

Conclusions

The ailanthus leaf extracts showed strong inhibitory effect on germination of lettuce seeds for concentrations starting from 2.5%, for both reflux and ultrasounds extracts. The inhibitory effect on growth and development of root and shoots seedlings was maximum even for the smallest concentrations of extracts used (1.25%). This effect is similar to the one of used synthetic herbicide at a concentration of 0.38 mg/L. This suggested that ailanthus extracts have a high phytotoxic activity. Compounds with potential phytotoxic activity determined by chromatography in extracts (fatty acids, polyphenols), confirm this idea.

Extracts also showed good antioxidant activity, results that are in agreement with the phenols content determined spectrophotometrically, especially flavonoids.

Acknowledgements: The authors are grateful to Prof. Dr. Paulina Anastasiu, director of the Botanical Garden Dimitrie Brandza" University of Bucharest (Romania) for identifying the ailanthus species.

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Manuscript received: 7.10.2015