DOI: 10.5281/zenodo.3249211
CZU 631.811.98:635.64

BIOLOGICALLY ACTIVE SUBSTANCES FROM *LINARIA GENISTIFOLIA* (L.) MILL AND THEIR POTENTIAL USAGE IN THE ECOLOGICAL AGRICULTURE

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Received: April, 12, 2019
Accepted: June, 05, 2019

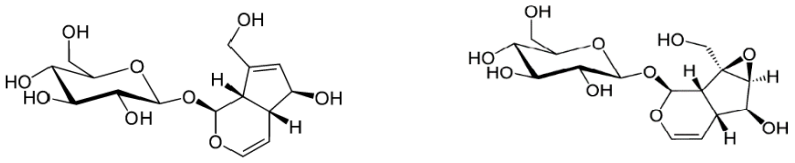
Abstract. The article starts with literature review on secondary metabolites, namely the iridoids contained in *Linaria* species (*Scrophulariaceae)*. It describes the biological activity of the iridoid glycosides; the importance and the benefits of the secondary metabolites and their eventual application in organic plant cultivation. Is further elucidated the profile of the biologically active substances predominant in the purified extract of *Linaria genistifolia* (L.) Mill., named by us genistifoliosides. Are investigated the biological activity and the stimulating effect of genistifolioside aqueous solution in concentration 0.01% on the tomato seed germination, plants' productivity (which is 33.9% higher) and the biochemical characteristics of the tomato fruit. It was elucidated, for the first time, the inhibitory effect of genistifolioside on the development of *Alternaria alternata* pathogenic fungi, affecting the vegetable crops; the 0.1% aqueous solution of genistifoliosides has the diameter of the fungi zone of inhibition 5.8 times bigger in comparison to the control sample and 4 times bigger in comparison to the certificated biopreparation. The purified extract of genistifoliosides exhibits properties of plant growth regulator as well as fungicidal properties and can be used in ecological agriculture.

Keywords: *Alternaria alternata, bioactivity, fungicidal, genistifoliosides, growth regulators, iridoids, tomatoes.*

Introduction

According to the conducted research, Linaria species (*Scrophulariaceae)* contain a wide variety of biologically active substances, such as iridoids, alkaloids, flavonoids, diterpenoids and others. A great part of the substances contained in different species of *Linaria* was isolated in individual state, identified and the structure of the new compounds was determined [1-12]. Several studies have shown an increased content of iridoid glycosides in *Linaria* (*L. dalmatica* (L.) Mill. *L. genistifolia* (var. *genistifolia* and var. *euxina*) (L.) Mill. *L. simplex* (Wild.), *L. pelisseriana, L. vulgaris* (L.) Mill. *and L. peloponnesiaca* Bois & Heldr, L. *aucheri, L. japonica* MIQ) species, [3-12]. Iridoids represent a large group of cyclopenta[c]pyran monoterpenoids, which provide a biogenetical and chemotaxonomic link between terpenes

and alkaloids and are found in the form of glycosides in plant families such as the *Apocynaceae, Scrophulariaceae, Verbenaceae, Lamiaceae, Loganiaceae* and *Rubiaceae,* etc. The most widespread iridoid glycosides in the vegetable world are derivatives of aucubin and catalpol Figure 1:



a) b)

Figure 1. a) Aucubin; b) Catapol.

The conducted research has confirmed that the *Linaria genistifolia* (L.) Mill. species, widespread in the wild flora of Moldova, is a rich source of biologically active substances, such as iridoid glycosides [9]. The substances act as secondary metabolites that can protect plants against insects, fungi, bacteria and viruses. Some secondary metabolites, such as alkaloids and iridoids synthesized by plants, have a protective role for plants, but are toxic to mammals. In small quantities, alkaloids can be used in pharmaceuticals, while in large ones, they are used as insecticides and poison, [13]. *In vitro* and *in vivo* studies have revealed that iridoids have neuroprotective, anti-inflammatory, immune-modulatory, hepatoprotective, cardioprotective, anticancer, anti-oxidant, antimicrobial, hypoglycaemic, hypolipidaemic, choleretic, antispasmodic and purgative properties [14-17].

It is well-known the importance of the secondary metabolites of plants as phytonutrients (phenolic acids, flavonoids, phytoestrogens, iridoids, etc.), which are contained in the functional foods of vegetal origin and are beneficial for the human health, [18]. Secondary metabolites with antioxidant, fungicides and immune-modulatory properties may be also used as plant growth regulators [19-26]. Using natural growth regulators in the organic plant cultivation is an agronomic practice of great interest, economically efficient and non-polluting, which does not impact the environment and maintains the fundamental agricultural resources. Vegetables, cereals, fruits, berries, etc. grown ecologically, without pesticides, but treated with low concentrations of biologically active substances of vegetal origin, are valuable functional foods, indispensable to human nutrition.

This study elucidates the biological activity of the purified extract of genistifoliosides (*GE*) obtained from *Linaria genistifolia* (L.) Mill. collected on the territory of Moldova, and describes the profile of the iridoid glycosides, which predominates in the above mentioned extract. In order to identify the main components, the fraction of iridoid glycosides (*IGF*) was obtained from the genistifoliosides extract by chromatographic methods. Research on the biological activity has shown that *IGF* exhibits strong fungicidal properties, but has a reduced activity as a growth regulator for vegetable plants; concurrently, *GE* exhibits both fungicidal and plant growth regulator properties [9, 24-26]. The conducted research and the bibliographic analysis suggest that the iridoids do not act in isolation, but may exert their properties when acting in synergy with other molecules e.g. polyphenols (acteoside, et.al.), which often co-occurs with iridoids [14]. Phyto synergy studies on iridoids (and other compounds) may be a rewarding research opportunity to better understand and unravel the complex interactions from which results a much stronger effect than that of an individual

compound. Hence, the biological activity of *GE*, which can be easily obtained through economical methods, was investigated.

Materials and methods

The plants of *Linaria genistifolia* (L.) Mill. were collected in flower from the scientific research field of Institute of Genetics, Physiology and Plant Protection, Kishinau, Moldova. The voucher specimen has been identified by the habilitated doctor in Biology Florea Vasile N. in the Laboratory of Natural Bioregulators.

Obtaining the purified extract of genistifoliosides (GE)

Fresh crushed aerial parts of *Linaria genistifolia* plant *(*500*g*) were extracted with 3 *L* of 50% aqueous ethanol. The ethanolic extract was concentrated through vacuum evaporation to an aqueous residue. The aqueous solution has been processed with chloroform (200 *ml*, 3 times), to remove lipids and chlorophyll and basified with NH_4OH. The aqueous fraction was dried in vacuum in the form of an azeotropic mixture with butan-1-ol, the residue was treated with acetone and dried, obtaining so a purified extract of genistifoliosides, as a beige powder of 7.8% yield. A major content of iridiod glycosides and a reduced content of polyphenol compounds and sugars were detected in the *GE* by a thin layer chromatography on Silufol plates, with the chloroform:methanol:water mobile phase (65:35:10, *v/v/v*) and in the presence of authentic samples. Under *UV*-250*nm* ultraviolet light, the spots of the polyphenolic compounds became fluorescent. The chromatogram was developed by spraying with Ehrlich reagent (2.0*g* of *p*–dimethylaminobenzaldehyde (*DMAB*) in 50 *ml* of 95% ethanol and 50 *ml* of concentrated hydrochloric acid). The spots of the iridoid glycosides became reddish-brown and the spots of the polyphenols turned blue. Were detected 7 spots that belong to iridoid glycosides and 2 spots that belong to polyphenols.

Obtaining the iridoid glycosides fraction (IGF)

5.0*g* of *GE* was applied on the Sephadex *LH-20* column. The column was eluted with mixture of $MeOH\text{-}H_2O$ (1:10 *v/v*); the collected eluates were combined according to the chromatographic mobility (*TLC* on the Silufol) and evaporated through vacuum distillation to dryness. A fraction of 3.6*g* of iridoid glycosides was obtained, for which was registered the *FT-IR* spectra (the Bruker Vertex 70 spectrophotometer in the 400 – 4000cm^{-1} range). The *IGF* was further chromatographed on silica gel columns until obtaining 4 main components; the structure of the iridoid glycosides was determined through ^{1}H and ^{13}C *NMR* spectroscopy and comparison with bibliographic data [9].

Testing the biological activity of GE

In order to select the optimal solution concentration and exposure time, the growth regulating activity of *GE* on plants was tested under laboratory conditions. Tomato seeds were treated with 0.0001%, 0.001%, 0.005% and 0.01% aqueous solutions, with the exposure time ranging from 15 minutes to 24 hours. As a control version the water soaked seeds were employed. For each case, 4 repetitions of 100 seeds each were made. The plant response was assessed on the basis of its germination, the length of embryonic root and seedling [27]. Before being sown, the tomato seeds were wetted by spraying with the most effective *GE* solution (0.01%), deriving from the 300*ml* of solution per 10*kg* of seeds. After 20 minutes (the optimal exposure time), the seeds were dried to free-flowing and sown in open field. The plants on the experimental fields were wetted by drip irrigation. The influence of *GE* on the

tomatoes' productivity and biochemical indicators [28] was determined at the Institute of Scientific Research In Agriculture.

To assess the fungicidal properties of the *GE*, under laboratory conditions, was applied the paper disc method [29]. The agar nutrient potato-glucose medium was melted and inoculated with *Alternaria alternata*. Inoculated medium was poured in sterile Petri dishes, 20*ml* each. After solidification, filter paper discs, soaked with 0.1%, 0.01%, 0.001% *GE* aqueous solutions and 0.01% Ecostim solution (certificated biopreparation) [26], were placed on the surface of the medium. The water soaked discs served as a control. The experiment was carried out in 3 replicas. The Petri dishes were kept for 7 days in the thermostat at 25^0C. The diameter of the fungi inhibition zones was measured with the ruler. The average value of the size, the absolute error and the average absolute error were calculated.

Results and discussions

From the *L. genistifolia* (L.) Mill. plant, collected on the territory of Moldova during its blossom, was obtained the purified extract of genistifoliosides (*GE*), the iridoid glycosides being detected in it as major products and polyphenols as minor products by thin-layer chromatography on the Silufol plates. The iridoid glycosides were separated by placing the *GE* on the Sephadex column and eluating the fraction of iridoid glycosides (*IGF*), for which the *FT-IR* spectra was registered, Figure 2. As per the absorption bands, we conclude that the mixture consists of glicosylated compounds (absorbtion bands $\nu_{max}\ [cm^{-1}]$ 2900-2850, 1150-918 belong to sugars). The glycosylated iridoids were further isolated by silica gel chromatography column. By physic-chemical methods, ^1H and ^{13}C *NMR* spectroscopy and by comparison with bibliographic data, was determined the structure of the 4 iridoid glycosides with a major content of: 5-*O*-allosylantirrinoside, antirrinoside, linarioside and 6-*β*-hidroxiantirride [9].

According to the bibliographic data, *L. genistifolia* plant also contains other iridoid glycosides: 5-*O*-glucosylantirrinoside, antirride, genistifolin, *E*- and *Z*- *p*-coumaroylantirrinoside; genistifolin being considered the taxonomic marker of *L. genistifolia* [4].

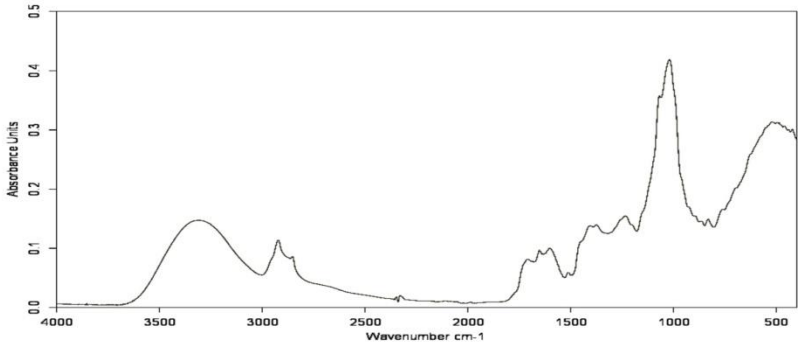


Figure 2. FT-IR spectra of iridoid glycosides fraction from *Linaria genistifolia*.

The recent study of the biological activity indicates that *IGF* exhibits fungicidal activity against *Fusarium oxysporum* and *Helminthosporium avenae* pathogenic fungi root rot [9]; however, the treatment of the tomato seeds before sowing with an *IGF* solution resulted into an inhibitory effect on the germination.

A plant growth stimulating and prominent antifungal activities were determined after applying the *GE* solutions [24-26, 30]. This could be explained by the synergistic action of all

the extract's components (iridoid glycosides and polyphenols), also noted by other researches [14].

Laboratory tests with aqueous solutions of *GE,* of different concentrations (0.0001%, 0.001%, 0.005% and 0.01%) and with tomato seeds' soaking time varying from 15 minutes to 24 hours, were conducted before open field usage. It was determined that treating the seeds with *GE* increases germination, while the length of the embryonic root and seedling is higher in comparison to the control group (water). Following the results of the laboratory tests, were selected the optimal exposure time and the optimal concentration of the *GE* solution (0.01%). In mechanical sowing, pre-sowing soaking of seeds for a long time is not recommended because of their swelling. Since no significant differences in germination rates from soaking time were found during laboratory testing, we recommended soaking the seeds for 20 minutes in field tests.

Treating the tomato seeds before sowing in open field with a 0.01% of genistifoliosides solution obtained from *L. genistifolia,* influenced the plant germination and productivity. Indicators of field germination of tomatoes on the 20^{th} day after sowing with the use of treated seeds exceeded the control variant by more than 2 times, which ensured the appearance of simultaneous and aligned shoots. The use of the *GE* had an impact on the date of entry into the fruiting phase of tomatoes: plants began to bear fruits 2-4 days before the control. Yield was higher than in the control variant by 10.3$t \cdot ha^{-1}$, which is 33.9%,"Table 1". Additional 10.3$t \cdot ha^{-1}$ of tomato fruits were obtained at the experimental production sites.

Table 1

The effect of *GE* from *Linaria genistifolia* on productivity of tomatoes.

Variant	**Yield** ($t \cdot ha^{-1}$)	**± compared to control** ($t \cdot ha^{-1}$)
Control, H_2O	30.4	
GE	40.7	10.3
$HPC_{0.5}$	6.9	

Studies of the chemical composition of tomato fruit allowed to determine that the use of secondary metabolites *GE* had a stimulating effect on the biochemical characteristics of the final product "Table 2". Thus, pre-sowing treatment of tomato seeds with a solution of *GE* provided a 12.3% increase in the content of vitamin C in fruits and a 10.3% decrease of acidity, compared to control samples.

Table 2

Influence of *GE* from *Linaria genistifolia* on the quality of tomato fruit.

Variant	**Dry matter** (%)	*	**Total sugar** (%)	*	**Acidity** (%)	*	**Vitamin C** ($mg \cdot 100g^{-1}$)	*
Control, H_2O	5.4		3.0		0.39		21.1	
GE	4.8	-11.1	2.7	-10.0	0.35	-10.3	23.7	12.3
$HCP_{0,5}$	0.5		0.4		0.1		4.8	

Note: * % to the control

By testing the fungicidal action of the *GE* extract, obtained from *L. genistifolia* plant, it has been proven that genistifoliosides inhibit the development of pathogenic fungi *Alternaria alternata,* a fungus which has been recorded to cause leaf spot and other diseases to over 380

host species of plant. This pathogen infects tomato plants and is often referred to as *Alternaria* stem canker of tomato.

The recorded results have shown that the highest fungicidal activity has an aqueous solution of *GE* with a concentration of 0.1%. The diameter of the inhibition zones of *Alternaria alternata* fungi is 5.8 times bigger in comparison to the control sample and 4 times bigger in comparison to the certificated biopreparation solution, "Table 3".

Table 3.

The influence of *GE* on *Alternaria alternata* fungi development.

Variant	**Mass fraction** (%)	**Fungi inhibition diameter zone** (*mm*)	*	**
Control, H_2O		4.3±0.4		
Ecostim	0.01	6.3±0.9	<0.5	
GE	0.1	25.0±0.7	<4.8	< 3.0
GE	0.01	20.7±1.1	<3.8	< 2.3
GE	0.001	12.3±0.4	<1.9	<1.0

Note: * - % to the control,

** - % to Ecostim (the certificated biopreparation solution).

Conclusions

The profile of the iridoid glycosides contained in the purified extract of genistifoliosides, obtained from Linaria genistifolia (L.) Mill. plant, collected on the territory of Moldova, was described. In order to identify the main components, the fraction of iridoid glycosides was obtained by chromatographic methods. Further was investigated the biological activity of the genistifoliosides. It was determined that treating the tomato seeds before sowing with an 0.01% aqueous solution of genistifoliosides, stimulates the seed germination, increases the plant productivity by 33.9% and influences tomato's biochemical indicators. For the first time, it was elucidated the inhibitory effect of the genistifoliosides on the development of the *Alternaria alternata* pathogenic fungi that affects the vegetable crops. It was determined that the 0.1% aqueous solution of genistifoliosides has the diameter of the fungi zone of inhibition 5.8 times bigger in comparison to the control sample and 4 times bigger in comparison to the certificated biopreparation. The research carried out proved that the purified extract of genistifoliosides exhibits both plant growth regulator properties and fungicidal properties and can be applied in the ecological agriculture.

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