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RESEARCH AND CHARACTERIZATION OF DETERMINANTS CONTROLLING THE ACCUMULATION OF CERTAIN METALS IN THE LEAVES OF *DYSPHANIA AMBROSIOIDES*

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ABSTRACT

In this research, we are interested in the study of the leaves of the plant Dysphasia ambrosioides *and its extracts: on the one hand, by IR spectroscopic analysis, thermogravimetry (TGA), and determination of metals by atomic absorption spectrophotometer (SAA); and on the other, carrying out phytochemical screening of extracts of leaves of* D. ambrosioides.

Key words: heavy metals, *Dysphasia ambrosioides*, pollution, alkaloids, tannins, glycosids, flavonoids

RICERCA E CARATTERIZZAZIONE DEI DETERMINANTI CHE CONTROLLANO L'ACCUMULO DI ALCUNI METALLI NELLE FOGLIE DI *DYSPHANIA AMBROSIOIDES*

SINTESI

In questa ricerca gli autori si sono interessati allo studio delle foglie della pianta Dysphasia ambrosioides *e dei suoi estratti: da un lato, mediante analisi spettroscopica IR, termogravimetria (TGA) e determinazione dei metalli mediante spettrofotometro ad assorbimento atomico (SAA); e dall'altro, effettuando lo screening fitochimico di estratti di foglie di* D. ambrosioides.

Parole chiave: metalli pesanti, *Dysphasia ambrosioides*, inquinamento, alcaloidi, tannini, glicosidi, flavonoidi

INTRODUCTION

Today, a significant percentage of the drugs authorized by government agencies are naturally occurring molecules, or compounds derived therefrom (about 50%). As a consequence, there is a significant potential for discovering new molecules of therapeutic interest in plants. Among these plants we chose to study the *Dysphasia ambrosioides* (L) Mosyakin & Clemants. It is a wild species of tropical America naturalized in the Old World, an upright herb, annual or perennial, with a more or less pubescent branching stem. It is commonly employed as an antimicrobial, antifungal (Paul *et al.*,1993; Boutkhil *et al.*, 2009; Boutkhil *et al.*, 2011; Cicera *et al.*, 2018), anti-rheumatic, analgesic (Okuyama *et al.*, 1993), sedative, antipyretic (Gadano *et al.*, 2006), also used for the treatment of respiratory, urogenital disorders, and vascular, nervous, and metabolic disorders such as diabetes and high cholesterol (Cruz *et al.*, 2007), due to its cytotoxic (Ruth *et al.*, 2015), antioxidant, anti-inflammatory and anti-Leishmanial activities (Monzotea *et al.*, 2014; Luz *et al.*, 2017; Reyes-Becerril *et al.*, 2019).

Among pollutants generated by industrial activities, heavy metals (i.e., Cu, Pb, Cr, etc.) pose several concerns. These elements readily bio-accumulate and have a recognised eco-toxicity. Moreover, they are involved in several pathologies (in the central nervous system, liver, kidneys; and can also cause cancers and embryonic malformations) (Abrahams *et al.*, 2002).

Today, a lot of research investigates the impact of heavy metals on the rate of germination and plant growth. For example, Mihoub *et al.*, (2005) showed that during the germination of pea seeds (*Pisum Sativum* (L.)), the cotyledons in stressed grains gradually accumulate Cd and Cu, and retain high contents of Fe, Mg, and Zn. Some plants have little or no tolerance and die in contact with heavy metals. Others have defence reactions, and slow absorption by secreting acids which will increase the pH and consequently reduce the mobility of trace elements. Others are metal tolerant, and even accumulate them, concentrating them. These plants are said to be "hyper-accumulative" and metallophilic. The trace elements are absorbed by the roots and most often stay there. The translocation in the aerial parts (stems, leaves) varies depending on the metal and indicates an increase in the concentration of metals in the soil. Lead remains in the roots, while Cd passes more easily through the aerial parts. Studies have shown that certain plants, called metallophytes, are capable of developing normally on sites highly contaminated with various metals and some of these plants, qualified as hyper-accumulators (Brooks, 1998), are capable of massively storing metals in their aerial parts. There is also phyto-extraction, based on the use of hyper-accumulative plants, which absorb metals from the soil and accumulate them in aerial organs (McGrath, 1998). This method is effective for a wide variety of heavy metals (Pb, Cd, Ni, Zn…).

Phenolic compounds are a widely used class of secondary metabolites and are located in the vacuoles of plant cells, in the intercellular space as well as on the surface of plants. They form a large group of compounds which include simple phenols, such as phenolic acids, flavonoids (flavones, flavonols and anthocyanins) and polymerized phenols, such as tannins and lignins. They participate in defence reactions against pathogens, and in allelopathy protect cell structures from the unwanted effects of excess photochemical energy and ultraviolet radiation, especially UV-B rays. Phenolic compounds found in flowers and fruits have the property to colour these organs (Winkel-Shirley, 2001).

In this research, a preliminary investigation in order to study the leaves of the *D. ambrosioides* plant by determining its chemical composition was conducted by IR spectroscopy, thermogravimetric analysis (TGA), and determination of metals by atomic absorption spectrophotometer (SAA). A phytochemical screening for other compounds present in the aqueous extract was performed as well.

MATERIAL AND METHODS

Collection of samples

The leaves of *Dysphasia ambrosioides* were collected during spring, 2019, at Ain Orma park (33°53′36″N 5°32′50″W), which is located between the cities of Meknes and Khemissat, in the region of Fes-Meknes (Morocco).

After collection, the leaves of the plant were washed separately, dried at room temperature in a dry and ventilated space, and protected from light to avoid loss of active substances. After drying, the various organs were finely ground and powdered using an electric mill. The powder obtained was stored in closed jars and kept in absence of light.

Analytical techniques

Infrared spectroscopy was used to identify the chemical functions of organic molecules. Briefly, the infrared radiation is an electromagnetic radiation with a wavelength greater than that of the visible light but shorter than that of the microwave light. The infrared domain studied was between 4000 cm^{-1} and 400 cm^{-1}, which corresponds to the vibration energy domain of the bonds. The apparatus used in this analysis was Fourier transform infrared spectroscopy (IR-TR) type JASCO 4100.

Thermogravimetric analysis is a thermal analysis technique which consists of measuring the mass of a sample when it is subjected to temperature variations (or time) in an inert environment (Nitrogen, Argon, or Helium for high-temperature tests), or oxidant (dioxygen). The thermogravimetric analysis device (TGA) employed was the Shimadzu thermal analysis type. The curves recorded for

temperature ranged from 0 °C to 700 °C. The heating rate was equal to 10 °C/min.

For mineralization and dosing, 6 g of sample were put in a porcelain dish and calcined at 600 ºC in a muffle furnace (t=6 hours). The ash obtained was mineralized with 75% HNO_3 in a beaker and then brought to dryness until the mineralization discoloured (t=4 hours). The residue was filtered on Whatman-type filter paper.

The determination of heavy metals was carried out using a flame atomic absorption spectrometer (Shimadzu-type model AA-7000). The device was controlled by WIZARD software. A hollow cathode lamp (Hamamatsu Photonics K.K.) was used as the radiation source and a deuterium lamp for the correction of non-specific absorptions. The carrier gas used for the flame was a mixture of air-acetylene. The standard solutions were prepared by diluting the stock solutions with a concentration of 1000 mg/L. The calibration range was prepared according to the element to be assayed.

Several procedures were used to determine the different chemical groups contained in a plant organ. These are tests based on solubility tests, colouring, and precipitation reactions, as well as exams under ultraviolet light.

The quantitative study of the raw extract by means of spectrophotometric assays aimed at determining the total content of total polyphenols, total flavonoids, and condensed tannins. Three calibration curves were drawn for this objective and carried out for each type of assay. The results in gallic acid, quercetin and catechin equivalent are expressed in mg/g of dry matter.

Fifty grams of the powder was added to 500 mL of absolute ethanol, the mixture stirred for 24 hours at 4 °C, then let stand for a few hours. The mixture was then filtered through glass wool and then through sintered glass (funnel N° 03), the filtrate stored at 4 °C until use.

The determination of the total polyphenols was carried out by the Folin-Ciocalteu method described by Wende *et al.*, (2007) with some modifications. This colorimetric method is based on the reduction of the phosphotungsten-phosphomolybdenum complex of Folin reagent by the phenolic groups of the samples, yielding products of blue colouring in alkaline media. Briefly, 0.1 mL of the extract of was added to 2.5 mL of distilled water and 0.5 mL of Folin reagent. After 5 min, 1.0 mL of sodium carbonate (20%) was added to the reaction mixture and the whole incubated for 1 hour at room temperature. The absorbance was read at 765 nm using a UV spectrophotometer. The results are expressed in milligram equivalent of gallic acid/g of dry extract with reference to the calibration curve of gallic acid.

The determination of flavonoids was carried out according to the method of aluminium trichloride ($AlCl_3$) (Bahorun *et al.*, 1996); 1 mL of each extract (prepared in methanol) with suitable dilutions was added to 1 mL of the $AlCl_3$ solution (2% in methanol). After 10 minutes of incubation and reaction, the absorbance was read at 430 nm using a UV spectrophotometer. The results are expressed in mg equivalent of quercetin/g of dry extract with reference to the standard curve for quercetin.

The dosage of condensed tannins was carried out for the extract according to the method of Richard *et al.*, (1978) and Heimler *et al.*, (2006). At 400 µL of each sample or standard (prepared in methanol and in distilled water for Aq. E.) with suitable dilutions, 3 mL of the vanillin solution (4% in methanol) and 1.5 mL of concentrated HCl were added. After 15 min, the absorption was read at 500 nm. The concentration of tannins is deduced from the calibration range established with catechin and expressed in milligrams of catechin equivalent per gram of dry extract (mg EC/mg ES).

RESULTS AND DISCUSSION

The water content in samples is 4.48 %, which is a low value. Several factors could influence the water and dry matter content of the plant, such as the nature of the fibres, the age of the plant, the condition of the soil, and the shelf life of the plant after harvest. The infrared spectroscopic analyses of the calcined samples at temperatures of 110 °C, 325 °C, 450 °C and 600 °C are illustrated in Figure 1.

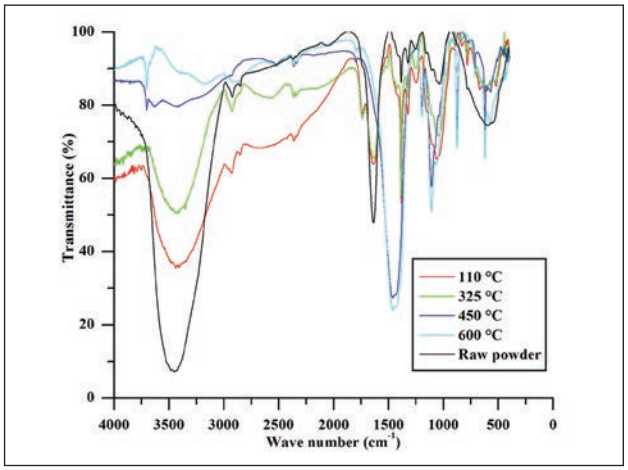

Fig. 1: IR spectrum (KBr) of leaves of *Dysphania ambrosioides* ***at different temperatures.***
Sl. 1: IR spekter (KBr) listov vrste *Dysphania ambrosioides* ***pri različnih temperaturah.***

The IR spectrum shows the presence of a broad and intense band around 3500 cm^{-1} attributable to the valence vibration band of the alcohol function $\nu_{(O-H)}$, and another band which appears around 2900 cm^{-1} attributable to the valence vibration band $\nu_{(C-H)}$. Similarly, we note the presence of a thin band around 1700 cm^{-1} relating to the valence vibration band of $\nu_{(C=O)}$. All of these bands assume that the powder contains organic molecules having alcohol and ketone. Fliou et al., 2019 analysed the

Daphne gnidium L. plant by infrared spectroscopy. The results obtained are similar to those found in this work.

At temperatures of 110 °C and 325 °C, we notice the persistence of the valence vibration bands: $\nu_{(OH)}$, $\nu_{(C-H)}$, and $\nu_{(C=O)}$ and a decrease in their intensity. At 450 °C, there is the disappearance of two bands relating to the vibration bands of the alcohol and ketone functions, and the appearance of a new band around 1480 cm^{-1} relating to the valence vibration band Ca ($CaCO_3$). This could be explained by the beginning of the disappearance of organic matter. At 600 °C, we notice the disappearance of organic matter, and the appearance of the bands relative to other mineral elements such as kaolinite, smectite, calcite and silicon oxide (Fig. 2) (Hachi *et al.*, 2002).

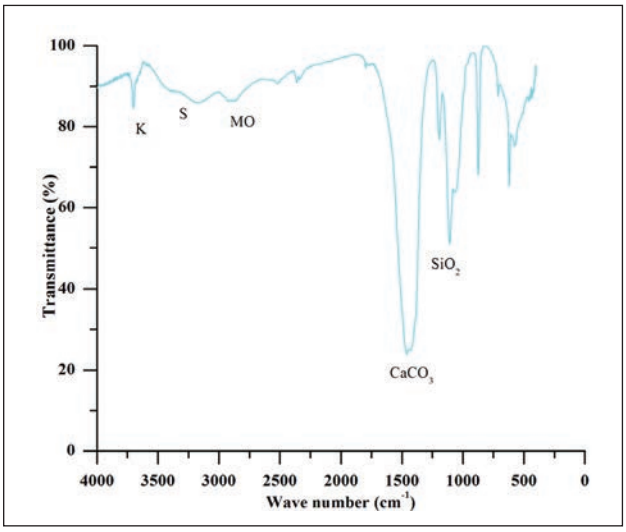

Fig. 2: IR spectrum of powdered leaves of** D. ambrosioides **at 600 °C. (K: Kaolinite [$Al_2Si_2O_5(OH)_4$], MO: Organic matter, S: Smectite, Calcite [$CaCO_3$]: Silicon oxide [SiO_2]).
Sl. 2: IR spekter listov v prahu vrste** D. ambrosioides **pri 600 °C. (K: Kaolinit [$Al_2Si_2O_5(OH)_4$], MO: Organska snov, S: Smectit, Kalcit [$CaCO_3$]: Silicijev dioksid [SiO_2]).

To follow the loss of sample mass during the rise of temperature, we used thermogravimetric analysis (TGA) and differential thermal analysis (DTA). The temperatures related to degradation rates were evaluated. The thermogram obtained is shown in Figure 3.

The thermal degradation of the sample can be identified by the decrease in its weight. The difference in mass is due to the endothermic and exothermic combustion reactions that occur.

The transformation process is characterized by thermal degradation presented by 3 stages: the first corresponds to a mass loss of 0.747 mg or 11.87 %. This loss, which is observed at a temperature of 110 °C, is attributed to

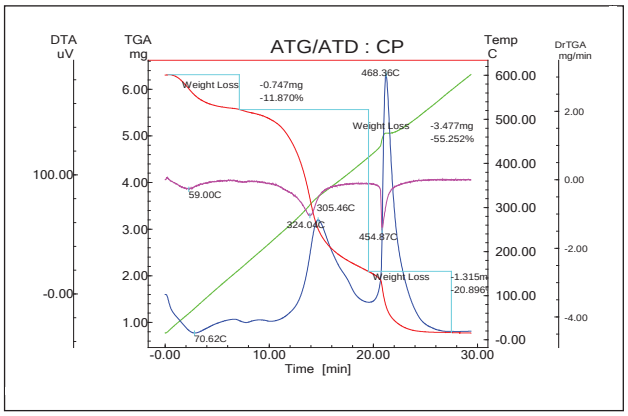

***Fig. 3: ATG/ATD curve of the raw powder of the leaves of** D. ambrosioides.*
***Sl. 3: ATG/ATD krivulja prahu listov vrste** D. ambrosioides.*

the evaporation of the water contained in the plant. The second step is observed at 325 °C, corresponding to the start of the thermal degradation of organic matter with a mass loss of 3.477 mg or 55.25%. Finally, the third stage, at 454.87 °C, records a mass loss of 1.315 mg or 20.90% and is related to the total destruction of organic matter (Tab. 1).

Tab. 1: Mass loss of leaves of** D. ambrosioides **as an effect of temperature.
Tab. 1: Izguba mase listov vrste** D. ambrosioides **pri različnih temperaturah.

Plant	Step	Temperature	A loss of mass (%)
Powder leaves of *Dysphania ambrosioides*	1	110 °C	11.87
	2	325 °C	67.12
	3	600 °C	88.02

The ATD diagram of the plant shows peaks indicating the different degradation reactions. An endothermic peak at 70.62 °C is attributed to the evaporation of absorbed water and two exothermic peaks, at 324.04 and 468.36 °C, are attributed to the degradation of organic matter. Indeed, the results of the calcinations confirm those of differential thermal analysis (DTA) by the loss of half of the organic matter at a temperature of 300 °C, and that this loss is considerable at 600 °C. The contents of heavy metal are presented in Table 2.

The results revealed a high retention of Na and Ca, with a content of 22.117 and 37.2633 mg/kg, respectively. These concentrations are below the authorized limit. Other elements such as Fe, Cu, Zn and Li are present at low contents, while Cd, Pb, K are almost non-existent. These results show that all the heavy metal contents are

***Tab. 2: Heavy metal content in leaves of** D. ambrosioides.*
***Tab. 2: Vsebnost kovin v listih vrste** D. ambrosioides.*

Metalelement	Content (mg/kg)	Content normal in plants by OMS (mg/kg)	Normal concentration (mg/Kg) (Kabata-Pendias, 1986)	Heavy metal content in the human body (mg/kg) (according to Schroeder, 1967)
Iron (Fe)	1.5175	-	-	60
Copper (Cu)	0.1256	150	-	1
Zinc (Zn)	1.1637	-	27 - 150	33
Cadmium (Cd)	0.003	0.3	0.05 - 0.2	-
Lead (Pb)	0.0145	10	5 - 10	-
Sodium (Na)	22.127	-	-	800
Lithium (Li)	0.1154	-	-	-
Potassium (K)	0.008	-	-	-
Calcium (Ca)	37.2633	-	-	19000

Tab. 3: Results of phytochemical screening of the extract of** D. ambrosioides. **(-): Absence, (+): Presence.
Tab. 3: Tab. 3: Rezultati fitokemičnega pregleda izvlečkov vrste** D. ambrosioides. **(-): Odsotnost, (+): Prisotnost.

			Aqueous extract of the leaves of D. ambrosioides
Alkaloids			+
Tannins	Catechic tannins		+
	Gallic tannins		-
Anthracenederivatives	Free anthracene		-
	Anthracenecombined	O-heteroside	-
		Heterosidegenin	-
		C-heteroside	-
flavonoids	Anthocyanins		-
	Flavones		-
	Flavanones		-
	Flavonols		-
	Leucoanthocyanins		+
	Catechol		-
Saponosides			-
Sterols and tri-terpenes			+++
Mucilage			+
Oses and holosides			+++
Prothocyanidols			-
Iridoids			-

lower than the standards proposed by the WHO, Kabata-Pendias, (1986) and Schroeder, (1967). This suggests that *D. ambrosioides* is not toxic with these trace elements. The results of total polyphenols, total flavonoids, and condensed tannins contents in the aqueous extract are summarized in Table 3.

Photochemical screening revealed the richness in this plant of secondary metabolites, such as alkaloids, catechic tannins, flavonoids (Leucoanthocyans), sterols and tri-terpenes, mucilages, oses and holosides. These results were found by Oliveira *et al.* (2017), who demonstrated that these secondary metabolites found in *D. ambrosioides* have positive effects in the fight against cattle ticks.

The results also show the absence of certain families, such as gallic tannins, anthracene derivatives, anthocyanins, flavones, flavonones, flavonols, catechols, saponosides, prothocyanidols and iridoids. The latter are considered to be powerful allelopathic agents, that is to say that they produce secondary metabolites which can alter the growth and/or the development of other systems (Rodrigues *et al.*, 2009; Lôbo *et al.*, 2008).

The concentration of total polyphenols is based on the regression equation (r^2=0.992) of the calibration range established with gallic acid (Fig. 4). It is expressed in milligrams of gallic acid equivalents per gram of the dry extract (mg EAG/g ES).

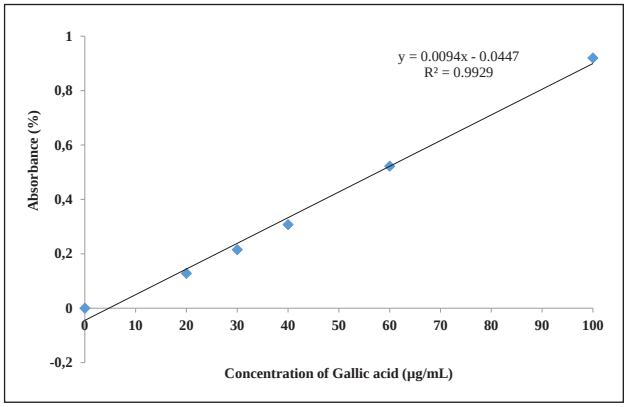

Fig. 4: Gallic acid calibration curve for the determination of total phenols.
Sl. 4: Umeritvena krivulja galne kisline za določevanje celokupnih fenolov

These results suggest that the ethanoic extract is rich in total phenolic compounds, with a content of 42.57 mg EAG/g ES. Previous works (Nowak *et al.*, 2016) on *Chenopodium* (L.) showed that the highest levels of polyphenols were observed in *Chenopodium album* (3.36 mg/g DW), seeds of *Chenopodium urbicum* (3.87 mg / g DW) and *C. urbicum* roots (1.52 mg/g DW). According to Dini *et al.*, (2010) the seeds of bitter *Chenopodium quinoa* contain

86.4 mg of AGE/10 g DW and of sweet *C. quinoa* 77.2 mg of AGE/10 g DW. *Chenopodium pallidicaule* has a higher total polyphenol content of 413 mg GAE/100 g DW (Dasgupta *et al.*, 2007).

The concentration of flavonoids was deduced from the calibration ranges established with quercetin (Fig. 5). It is expressed in milligrams of quercetin equivalent per gram of the dry extract (mg EQ/g ES). According to the calibration curve, the total content of flavonoids extracted from the extract of *D. ambrosioides* leaves with ethanol is of the order of 20.19 (mg EQ/g ES).

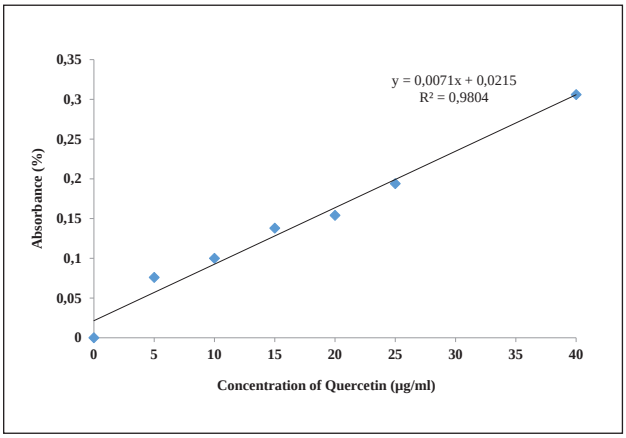

Fig. 5: Quercetin calibration curve for the assay of flavonoids.
Sl. 5: : Umeritvena krivulja za kvercetin za analizo flavonoidov.

The concentration of flavonoids was determined using the spectrophotometric method in the presence of aluminium chloride. The results obtained showed that the concentration of flavonoids in the extract is 20.19 mg EQ/g of ES. This value is lower than the value found by Tanzeel *et al.*, (2018), either a content of 57±1.41 µgQE/mg of extract. Sajjad *et al.*, (2016) have explained this variation in phenolic and flavonoid compounds in different parts of the plant by the polarity of the solvent and with antioxidant and medicinal properties. The calibration curve was constructed using catechin as a reference standard (Fig. 6).

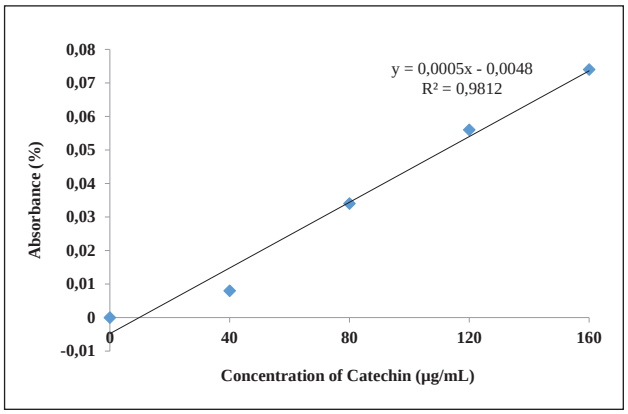

Fig. 6: Catechin calibration curve for the determination of condensed tannins.
Sl. 6: Umeritvena krivulja katehina za določevanje kondenziranih taninov.

According to the calibration curve, the content of condensed tannins in the extract of leaves of *D. ambrosioides* is of the order of 38.78 (mg EQ/g ES). The results showed that the content of condensed tannins in the ethanoic extract of the leaves of *D. ambrosioides* is 38.78 mg EQ/g ES. Upon comparison of these results with those of Ksouri *et al.* (2009) on *Tamarix gallica*, whose leaves recorded a total activity of 14.66 mg EAG/g DM, we note that the content of condensed tannins in the ethanoic extract of the leaves of *D. ambrosioides* can be considered to have strong antioxidant activity because they are very good scavengers for free radicals and also inhibit the formation of superoxide radicals.

CONCLUSIONS

Infrared spectroscopic analysis and differential thermal analysis have shown that the plant *D. ambrosioides* undergoes a loss of organic matter as the temperature increases. The remaining mineral matter was analysed by atomic absorption spectroscopy. The results showed that the plant contained certain metallic elements in small quantities, such as Na, Ca, Fe, Cu, Zn and Li, while the content of Cd and Pb was almost non-existent. Phytochemical screening of the extracts showed a significant presence of total polyphenols, total flavonoids and condensed tannins.

RAZISKAVA O DEJAVNIKIH, KI VPLIVAJO NA KOPIČENJE NEKATERIH KOVIN V LISTIH VRSTE *DYSPHANIA AMBROSIOIDES*

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POVZETEK

V pričujoči raziskavi so avtorji raziskovali liste rastline Dysphasia ambrosioides *in njihove izvlečke z uporabo IR spektroskopske analize, termogravimetrije (TGA), določali kovine z atomsko absorpcijsko spektrofotometrijo (SAA) ter opravili fitokemični pregled izvlečkov listov vrste* D. ambrosioides.

Ključne besede: težke kovine, *Dysphasia ambrosioides*, onesnaženje, alkaloidi, tanini, glikozidi, flavonoidi

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