Chemical Characterization of *Artemisia Annua L.* Extract
Assessment of Antioxidant Activity *in vitro* and *in vivo* Toxicity Studies

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The purpose of the study was to demonstrate in vitro antioxidant activity and in vivo toxicity of the Artemisia Annua L. extract. The plant was harvested from Bihor area (Cri'ul Repede and Negru river valleys), Romania. Preparation of the plant product and of the lyophilizated extract was carried out in accordance with the Romanian Pharmacopeia Xth Edition. Lyophilized extract was evaluated in terms of polyphenol content using HPLC method. Antioxidant activity was highlighted using the DPPH, ABTS and FRAP methods. Hepatic, renal and haematological toxicity studies have been performed on laboratory mice. For this purpose blood and organs were collected. Biochemical and haematological parameters were determined on the blood samples and histopathological examination was performed on organs. In vitro antioxidant effect of Artemisia Annua L extract and its lack of in vivo toxicity were demonstrated. It is desirable to obtain a new phytoproduct harvested from spontaneous flora of Romania with antioxidant / antitumoral properties and which is devoid of toxicity.

Keywords: extract, antioxidant, toxicity, phytoproduct

There has been an increase in the reevaluation of traditional medicinal plants worldwide, with extensive research on various plant species and their therapeutic properties being carried out. Traditional medicinal plant remedies have been highlighted as alternative medicines that are less likely to cause adverse side effects, unlike synthetically generated by chemical substances [1-6].

Artemisia annua L, which is also known as *sweet wormwood* and *Qinghao*, *Asteracea* family, is an annual herb, native to temperate Asia but naturalized throughout the world. It has fern-like leaves, bright yellow flowers and a camphor-like scent [7].

Artemisia annua L has been used for at least 1600 years in traditional Chinese herbal medicine to treat symptoms associated with malaria. Herba *Artemisia annua* was cited in the Classified Materia Medica as a food supplement related to longevity, a characteristic probably associated with its antiparasitic and antioxidant properties [8,9]. Has been used also, for many centuries in traditional Asian medicine for the treatment and prevention of fever and chills [1]. In previous studies, *Artemisia species* has been reported to exhibit anticancer, antioxidant, antidiabetic, antihepatitis, antihypertensive, antibacterial, antifungal, anti-inflammatory and antiviral effects [10-13].

A variety of compounds have been extracted from *Artemisia annua L* such as sesquiterpenoids, flavonoids, coumarins, lipids, phenolics, purines, steroids, triterpenoids, aliphatics and artemisinin [14,15].

Free radicals in the form or reactive oxygen and nitrogen species are an integral part of normal physiology, and free radical reactions occur throughout the human body. Overproduction of these reactive species can occur due to oxidative stress caused by imbalances in the body's antioxidant defence system and free radical formation. These reactive species can interact with biomolecules, causing injury and death. So, reactive oxygen species (ROS) such as the superoxide anion, hydroxyl radical and hydrogen peroxide are known as inducers of arteriosclerosis, cancer, diabetes and aging [16-18].

Antioxidants can remove ROS, therefore, many methods for evaluating their activity have been reported [19-22]. Among these methods, the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays have been widely used [23-25].

Phenolic compounds exert multiple biological effects, including antioxidant and free radical-scavenging abilities. Their anti-radical property is directed toward hydroxyl radical and superoxide anion, highly reactive species, especially the last one being one of the main ROS involved in the DNA damage processes [26-29].

The first objective of this study was to demonstrate *in vitro* antioxidant activity of *Artemisia annua L* herba harvasted from Bihor area, Romania. The second objective of the study was to demonstrate the lack of toxicity of *Artemisia annua L* extract. In the future, it is desirable to obtain a new active phytoproduct without toxicity.

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Experimental part

Materials and methods

Plant matrial and extraction

Artemisia annua L. (*Asteraceae*) was harvested from Bihor area (Cri'ul Repede and Negru river valleys), Romania. The plant harvest was performed according to the Good Agricultural and Collection Practices rules, from an unpolluted area. The vegetal product (herba) was dried at 40°C in an oven for total moisture removal and then was crushed according to the Romanian Pharmacopoeias 10th edition (passing through the sieve number I) [30].

Artemisia annua L. extract was prepared by maceration with alcohol, accordingly to the Romanian Pharmacopoeias 10th edition (the ratio vegetal product/ alcohol 30° = 20%), at room temperature (20°C), for 24 h, with periodic mixing, pressed and filtered [30]. The hydroalcoholic extracts were centrifuged and supernatants were evaporated to dryness under vacuum in a rotavapor. The dry extract was dispersed in 10 mL distilled water and frozen at -25°C. Finally, they were brought into the lyophilizer using Freeze dryer Alpha 1-2 Christ (Martin Christ, Osterode am Harz, Germany). The lyophilized extracts were weighed and transferred to a dry sealed container. A brown powder with homogeneous, spongy appearance was obtained.

Identification and quantification of polyphenols in Artemisia Annua L. extract

Identification of polyphenols from *Artemisia Annua L* extract was performed using a system ACME 9000 HPLC equipped with an UV detector. It was used a column Phenomenex OOF-4439-Y0, gemini c18 150 x 3, 110A. The elution was an isocratic one, using a mobile phase with the following composition - water: methanol: acetic acid (700:300:2) (v/v). The following parameters were used: liquid flow 0.4 mL/min, injection volume 20µL, column temperature 25°C. The detection was performed at 300 and 360 nm wavelengths. The polyphenols were identified by comparing the retention time of the compounds from the extract with that of the standard quercetin solution.

The standard quercetin solution was prepared by dissolving 0.01 g quercetin in a mixture of methanol:water (50:50) and then brought into a 5 mL rated balloon (concentration of the solution: 2 mg/mL).

1.6201 g of *Artemisia Annua L* extract was dissolved in a mixture of methanol:water (50:50) and brought into a 10 mL rated balloon. The solution showed a slight opalescence and for this reason it was filtered and diluted 1:10 with the same solvent mixture.

The standard and the extract solutions were analyzed three times to determine the retention times and the peak area.

The determination of *in vitro* antioxidant capacity of the lyophilized extract

Measurement of DPPH radical scavenging activity

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) radical scavenging activity was assessed according to Kikuzaki et al [31]. In this assay, 50 µL extract of fraction solutions with different concentrations were added with 1.0 mL of 0.4 mM methanolic - DPPH and brought up with methanol to 5.0 mL. The mixture was shaken vigorously using a vortex and left to stand for 15 min, at room temperature, in dark. The scavenging effect on the DPPH radical was read using spectrophotometer (Genesys-10) at 517 nm. The radical scavenging activity was expressed as radical scavenging percentage using the following equation: % Inhibition = $[(A_B-A_A)/A_B] \times 100$; where, A_B = absorption of blank sample (t = 0 min.), A_A = absorption of test extract solution (t = 15 min.). The DPPH solution without sample solution was used as control.

ABTS method

ABTS method (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) named also *TEAC method* (Trolox Equivalents Antioxidant Capacity) is a spectrophotometric method which measures the ability of compounds to scavenge the $ABTS^+$ cation radical in relation to Trolox. Shortly, the $ABTS^+$ cation radical was produced by reacting the $ABTS^+$ solution (7mM) with potassium persulfate (2,45 mM) solution, keeping the mixture in dark at room temperature for 16 h. ABTS stock solution was diluted in order to obtain an absorbance of 0.7±0.02 at 734 nm. After addition of 25µL extract to 2.5 mL of diluted $ABTS^+$, the solution was mixed very well (using vortex) for 30 seconds and the interaction between the antioxidants and the $ABTS^{\bullet+}$ was monitored spectrophotometricaly at 734 nm, exactly at 1 minute. The calibration curve was linear for the range of Trolox concentrations between 0.125 and 2 mmol/L. The ABTS value was obtained using the following equation based on the calibration curve: $y = 1629 x + 98.94$ ($R^2 = 0.998$), where x = absorbance and y= µmol Trolox equivalent [32,33].

FRAP method

FRAP method (Ferric Reducing Antioxidant Power) is a simple spectrophotometric method that assesses the antioxidant power of the studied samples, being based on the reduction of ferric tripyridyltriazine complex [Fe(III)-TPTZ] by a reductant, at an acid *p*H. The stock solutions included: 300 mM acetate buffer; 270 mg $FeCl_3 \cdot 6 H_2O$ dissolved in 50 mL distillated water; 150 mg TPTZ and 150 µL HCl, dissolved in 50 mL distillated water. The working FRAP solution was freshly prepared by mixing 50 mL acetate buffer, 5 mL $FeCl_3 \cdot 6 H_2O$ solution and 5 mL TPTZ solution. Trolox was used as a standard solution, the calibration curve was made for concentrations between 0 and 300 µM, having a correlation coefficient $R^2 = 0.9956$ and the regression equation ($y = 0.0017x + 0.0848$), where x = absorbance registered at 595 nm and y = µmol Trolox equivalent [34-36].

The reagents used for the evaluation of "in vitro" antioxidant activity were purchased from Sigma-Aldrich (St. Louis, MO, USA).

In vivo toxicity study of the Artemisia Annua L. extract

Animal care and experimental procedures

A subacute toxicity test was performed on 10 adult Swiss mice, 34.5 ± 2.5g body weight, 5 male and 5 females. The mice were supplied by Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania. Mice were housed in standard polypropylene cages, at optimum density and in standard laboratory conditions (temperature 25 ± 1 °C, relative humidity 55 ± 5%, and 12 h light/dark cycle). The mice had free access to standard granular diet and water. All the procedures performed on laboratory animals, completed with the European Directive 2010/63/EU, and the national low 43/2014 for Protection of Animals Used for Scientific Purposes. This project was legally approved by the Comity for Bioethics of UMF (accord no.369/28.IX.2017) and the Veterinary Sanitary Direction and Food Safety (aut. no. 90/10.X.2017). All animals received daily *Artemisia annua L* extract in a dose of 1000 mg d.s./ kg b.w., for 14 days long. The body weight and general clinical status was recorded every day. At the end

the blood was drown from the orbitary sinus under deep narcosis, then the animals were euthanized by narcotic overdose. We performed gross examination immediately after, and fragments of kidney and liver were removed, fixed in buffered formalin and embedded in paraffin wax. Later, the hematoxiline eosine stain was done for histopathology. The blood was used to perform the biochemical and haematological examinations [37].

Biochemical determinations

Liver toxicity study (transaminases, albumin, total protein determinations) and renal toxicity study (creatinine, urea determinations) were performed using a screen point semiautomatic analyzer, STAT - FAX 1904 Plus, Global Medical Instrumentation, Inc. 6511 Bunker Lake Blvd. Ramsey Minnesota, 55303 USA and special determination kits.

Hematological determinations

Hematologic toxicity testing (erythrogram, leukogram, thrombogram) was performed using an Abacus Junior Vet, Diatron, 3 Diff Messtechnik, Budapest, Hungary. In this purpose, we determined the following haematological parameters: red blood cells (RBC), haemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red blood cells distribution width (RDW); white blood cells (WBC), lymphocytes (LYM), middle cells (MID), granulocytes (GRA); total platelet count (PLT), platelet hematocrit (PCT), mean platelet volume (MPV) and platelet distribution width (PDWs).

Histopathological analysis

Histopathological analyses of hepatic and renal tissues were performed using an Optical Microscope Olympus l CX41.

Statistical analysis

All data are reported as the mean ± Standard Deviation (S.D.). Statistical values were obtained using GraphPad Prism version 5.0 for Windows, GraphPad Software, San Diego California USA.

Results and discussions

Identification and quantification of polyphenols in Artemisia Annua L. extract

We evaluated the content of polyphenols for the extract of *Artemisia Annua L* and we expressed them in quercetin. HPLC method was used for the identification and quantification of quercetin. The results obtained by the HPLC method are shown in table 1 and figures 1 and 2.

The determination of in vitro antioxidant capacity of the lyophilized extract

In vitro antioxidant properties of *Artemisia Annua L.* were determined by three methods, DPPH, ABTS and FRAP. The results are shown in table 2. Scavenging effect of the plant extract, determined by DPPH method, was measured as percentage of inhibition (%) of DPPH radical, and the antioxidant capacity of extracts from ABTS and FRAP methods was measured as Trolox equivalents (TE).

Compound	Area (mAU)	Retention time (min)	Wavelength (nm)	Assay (mg/100g extract)
Quercetin	519.6537	2.0167	360	4.1

Table 1
IDENTIFICATION AND QUANTIFICATION OF QUERCETIN IN *Artemisia Annua L* EXTRACT USING HPLC METHOD

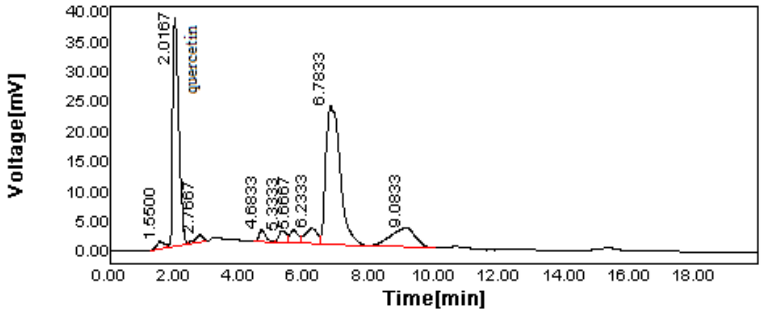

Fig.1. HPLC chromatogram of *Artemisia annua L* extract

Extract	DPPH (%)	ABTS (µmol TE/g)	FRAP (µmol TE/g)
Artemisia annua L.	9.03±1.11	109.26± 5.98	98.35± 0.00

*- TE = trolox equivalent

Table 2
THE ANTIOXIDANT CAPACITY OF *Artemisia Annua L.* EXTRACT

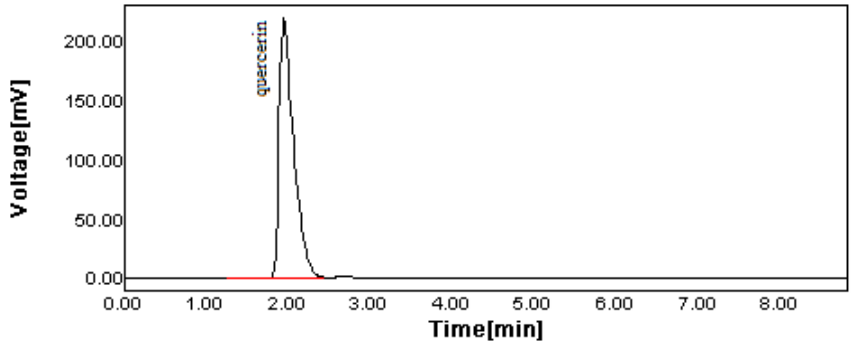

Fig.2. HPLC chromatogram of standard quercetin solution

Table 3

THE INFLUENCE OF *Artemisia Annua L* EXTRACT ON BIOCHEMICAL PARAMETERS

Mice group	Urea (mg/dL)	Creatinine (mg/dL)	ALAT (U/L)	ASAT (U/L)	Total protein (g/dL)	Albumin (g/dL)
Male	15.97±2.35	0.350±0.130	32.56±1.34	125.78±45.98	5.54±0.22	3.45±0.32
Female	19.90±6.52	0.360±0.182	46.89±2.54	185.76±56.72	6.06±0.18	3.67±0.78

Normal values: Urea 12-28 mg/dL, Creatinine 0.3-1 mg/dL, ALAT 26-77U/L, ASAT 54-269 U/L, Total protein 3.5-7.2g/dL, Albumin 2.5-4.8g/dL (Mean±S.E.M.) (n=5) [38]

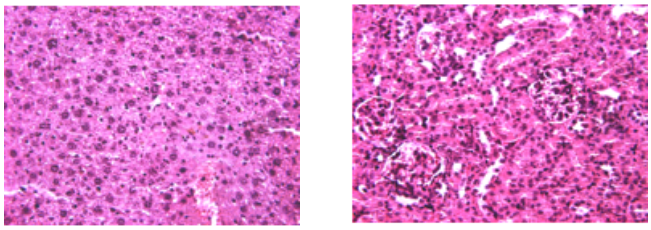

Fig. 3. Histopathological analyses of hepatic and renal tissues.

Table 4

THE INFLUENCE OF *Artemisia Annua L* EXTRACT ON ERYTHROGRAM

Mice group	RBC (10^{12}/L)	HGB (g/dL)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dL)	RDWs (fl)
Male	8.58±0.81	13.08±1.31	41.08±3.01	47.87±1.34	15.24±0.19	31.84±0.68	33.59±1.24
Female	8.89±1.19	14.12±1.59	44.36±3.96	49.89±3.62	15.88±0.88	31.83±0.73	35.36±1.58

Normal values: RBC 7-12.5 10^{12}/L, HGB 10.2-16.6 g/dL, HCT 39-49 % (Mean±S.E.M.) (n=5) [38]

Mice group	WBC (10^9/L)	LYM (10^9/L)	MID (10^9/L)	GRA (10^9/L)
Male	9.02±1.93	6.49±1.14	0.23±0.04	2.30±0.91
Female	9.65±1.77	6.80±0.46	0.29±0.09	2.56±1.21

Normal value: WBC 6-15 10^9/L (Mean±S.E.M.) (n=5) [38]

Table 5

THE INFLUENCE OF *Artemisia Annua L* EXTRACT ON LEUKOGRAM

Mice group	PLT (10^6/L)	PCT (%)	MPV (fl)	PDWs (fl)
Male	905.60±149.11	0.59±0.09	6.56±0.23	7.04±0.23

Table 6

THE INFLUENCE OF *Artemisia Annua L* EXTRACT ON THROMBOGRAM

The results presented in table 2 demonstrate the *in vitro* antioxidant activity of the *Artemisia Annua L.* lyophilized extract.

In vivo toxicity study of the Artemisia Annua L. extract

Table 3 shows that biochemistry parameters fall within normal limits [38].

The histological study of the *liver* showed a normal structure, the hepatocytes look normal, are separated by sinus capillaries, bile ducts and supportive tissue. Remack's cellular cords are well-acclaimed, intralobular support tissue is represented by connective tissue, collagen fibers and reticulin in a reduced amount (fig. 3).

The histological study of the *kidney* revealed a normal aspect of renal parenchyma, in the kidney cortex the renal glomerulus is well defined. Counted tubes present intact cells with spherical core located centrally. The conjunctivo-vascular tissue in the cortical stroma is poorly represented (fig. 3).

So, biochemistry determinations reflected normal kidney and liver function, aspect confirmed by histopathology studies.

Tables 4-6 show also that haematological parameters fall within normal limits [38].

There were no significant changes in body mass, all the groups in the study having a slight upward trend.

The biochemical and haematological parameters did not change, keeping them within normal limits in all the animals under study. Animals did not show clinical changes during the experiment, all animals survived to the end. These findings suggest that the *Artemisia Annua L.* extract is safe.

Conclusions

In the present study we highlighted *in vitro* antioxidant activity of *Artemisia Annua L* extract and we demonstrated the lack of toxicity for *Artemisia annua L* extract *in vivo*. In our point of view, these findings justify further studies. So, we purpose to prove *in vivo* antioxidant activity and to demonstrate de antitumor efficacy of *Artemisia Annua L* extract *in vitro* and *in vivo.* This extract of *Artemisia Anuua L* might be the basis of a new pharmaceutical remedy using a plant harvested from the spontaneous flora of Romania.

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