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COMPARATIVE ANALYSIS OF *Artemisia annua* EXTRACTS OBTAINED BY MODERN AND CLASSIC EXTRACTION TECHNIQUES

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Abstract

The aim of our study was to assess the artemisinin and phenolic compounds content of extracts obtained from Artemisia annua by classic solvent extraction comparatively with the extract obtained by microwave. For the classic technique, the solvents used were water, methanol, ethanol, and acetone. For microwave extraction, the solvent was ethanol 90% (v/v). The phenolic compounds were identified by HPTLC and artemisinin was also quantified by HPLC. In all samples rutin, hyperoside, chlorogenic and cafeic acids were identified. The highest quantity of artemisinin was found in extracts obtained by microwave extraction. The highest DPPH radical scavenging was obtained for aqueous extract, whereas methanolic extract exhibited almost a constant activity in different concentrations. The results obtained for total antioxidant capacity by the phosphomolibdenum method were similar to the DPPH method.

Key words*: Artemisia annua, artemisinin, microwave extraction.*

INTRODUCTION

Artemisia annua in a perennial plant from *Asteraceae* family, indigenous from temperate Asia. It is used in Chinese traditional medicine for a long time for anti-malarial, immune-suppressive, anti-inflammatory and anti-cancer properties (Alesaeidi & Miraj, 2016). Now it's naturalized in countries from temperate climates, including Romania. The chemical composition includes sesquiterpenoids, flavonoids, coumarins, proteins, and steroids. The main chemical compound of the plant is artemisinin (sesquiterpenoid) (Ikram & Simonsen, 2017). Based on scientific research, for the treatment of uncomplicated malaria due to *Plasmodium falciparum*, the World Health Organization (WHO) recommends artemisinin-based combination therapy (ACT) Also, the WHO statement shows that further studies are necessary, in the field of fundamental and clinical research (WHO, 2012). In 2015 the estimated number of people infected with malaria parasites in sub-Saharan Africa was 114 million (WHO, 2016).

Based on research studies WHO does not recommend the utilisation of *Artemisia annua* in different forms (as tea or compacted plant material), because of the low content of artemisinin in the plant (WHO, 2012).

Recent studies have shown that flavones and artemisinin have synergic anti-malarial activity (Czechowski, 2019).

Because of the previous scientific results, the extraction techniques of artemisinin are very important. The efficiency of extraction depends on the chosen technique, respectively on the raw material, solvent - solid ratio, the temperature of extraction and duration of the extraction (Zhang et al., 2018).

Safety risks such as solvent toxicity and the presence of solvent residues in extracts, together with low yield, have stimulated the development of other extraction technologies, clean or environmentally friendly technologies, which can minimize or eliminate the use of organic solvents.

These techniques are also known as cold extraction techniques, in which the stability of the extracted compounds is not affected and the energy required for extraction is reduced (Tiwari, 2015).

Our goal in this study is to comparatively evaluate the chemical composition in artemisinin and phenolic compounds and antioxidant capacity of extracts of *Artemisia*

annua obtained by classic solvent extraction and microwave extraction techniques.

Microwaves are electromagnetic fields in the range of 300 MHz to 300 GHz, with two perpendicular oscillating fields, the frequencies of the electric field and the frequencies of the magnetic field. The solvent enters the solid matrix by diffusion, and the solute is dissolved to reach a concentration limited only by the characteristics of the solid (Angiolillo et al., 2015).

Microwaves are a non-contact heat source that can generate more efficient heating, accelerate energy transfer and reduce the thermal gradient. Several classes of compounds such as essential oils, antioxidants, pigments, flavors and other organic compounds can be effectively separated using this method (Li et al., 2013).

According to Leadbeater (2014), the use of microwave equipment is a flourishing technology, because it is possible to generate higher temperatures easily, safely and in a reproducible manner; reaction time can be reduced; the yield can be increased; and the purity can be improved, compared to conventional heating methods. This technique can be performed either with or without the addition of any solvent (Oroian & Escriche, 2015).

Li et al. (2013) provide an overview of the techniques that are available for extracting bioactive compounds using microwaves, without solvents. They have shown that this can be an alternative to other techniques, with the benefits of time reduction, energy consumption, solvent use, and no CO_2 emissions.

Grigoras et al. (2012) conducted a comparative study of conventional methods, maceration and green extraction using pressurized liquid, ultrasound, and microwave. The microwave assisted methodology provided the highest concentration of the bioactive compounds in the extract obtained.

MATERIALS AND METHODS

Raw material - Artemisia annua was purchased from Dorel Plant SRL. A voucher specimen of *Artemisia annua* is deposited in INCDCF-ICCF Herbarium.

Sample preparation: Classic extraction - the samples were prepared by extraction with ethanol (T1), water (T2), methanol (T3), and acetone (T4) - vegetal material/solvent rate - 1/10 m/v, for 1 h at the boiling temperature of the solvent. The process was followed by filtration of the extracts, solvent evaporation at reduced pressure until the precipitates appeared. Each precipitate were dissolved in 50 ml 50% ethylic alcohol (v/v) and frozen until analysis.

Microwave-Assisted Extraction: raw material was extracted under the influence of microwave energy using 90% ethanol (v/v) with the variation of extraction parameters as follows:

Codification of extracts	Raw material (G	Pressure (W)	Temperature (°C)	Time (min)
M1	30	180	30	8
M2	30	330	60	8
M3	30	330	40	6
M4	30	330	40	12

The extract thus obtained was evaporated in vacuum and redissolved in 15 ml 50% ethylic alcohol (v/v) and frozen until analysis.

HPTLC Analysis - Fingerprint chromatography of the samples for the identification of the chemical compounds was made according to the method described by Wagner and Bladt, 1996. The samples (3-7 µl) and references substances of phenolic compounds and artemisinin (1-5 µl) (10^{-3}M T5 - artemisinin, T6 - hyperoside, T7 - chlorogenic acid, T8 - chologenic acid, T9 - caffeic acid, T10 - rutin Sigma-Aldrich) were spotted on 20 x 10 Silica gel 60F254 TLC plate in 10 mm band length. The spots were made with a Hamilton- Bonaduz, Schweiz syringe, and CAMAG LINOMAT 5 instrument. For artemisinin identification the mobile phase was n-hexane: ethyl acetate 75:25 (v/v) (system A) and for polyphenolic compounds was ethyl acetate-acetic acid-formic acid-water 100:11:11:27 (v/v/v/v) (system B). For pre-saturation, the mobile phase was put in the TLC twin-chamber for 30 min at ~20°C, before the analysis. After development (up to 90 mm), plates were dried and visualized, for B system in Natural Product followed by PEG4000 reagent and for A in sulphuric anisaldehyde. The fingerprints were evaluated at UV with WinCats and VideoScan software for A system and in visible light for B the system.

HPLC Analysis - Chromatographic separation was achieved on a LaChrom CLASIC MERCK-HITACHI system, with DAD detector; a Kromasil 100-5C18 150*4.6 mm column at 30

± 1°C, using gradient elution. Quantification of artemisinin was performed using a mobile phase consisting of two solutions - phosphate buffer solution, pH = 3.0 as A solution and acetonitrile as B solution at an initial flow rate of 1 ml/min; with an injection of 20 ìl.

*Free radical scavenging w*as made for different concentrations (1%, 0.1%, 0.01% in methanol) of the extracts and measured against 1, 1 diphenyl-2-picryl hydrazyl radical (Sigma-Aldrich) (Sanchez-Moreno et al. 1998). Briefly, 2950 ml of the DPPH methanolic solution (0.0025 g/l) were mixed with 50 µl aliquots of the extract. For blank solution, the sample was replaced with methanol. After standing for 30 minutes of the room temperature for the solution was measured UV absorbance at 517 nm. The DPPH% scavenging effect was calculated using the following equation:

% inhibition = $\{(D_0 - D_1)/D_0\}$ x 100.

Where D_0 is the absorption of blank solution and D_1 is the absorption of the extract.

Total antioxidant capacity (TAC) assay - was made for ethanolic solution of the extracts (concentration 1%, 0.1%, 0.01%) according to Prieto et al., 1999, phosphomolybdenum method. To 2.7 ml of phosphomolybdenum solution (0.6 M sulphuric acid, 28 mM sodium molybdate, and 4 mM ammonium phosphate) was added 0.3 ml of extract solution. The absorbance of the samples, incubated for 90 minutes at 95^0, was measured after cooling them to room temperature at 695 nm with UV-VIS spectrophotometer. The blank solution was ethanol. The antioxidant capacity was expressed as ascorbic acid equivalent (AA) to 1 mg of the active substance. The calibration curve is linear for ascorbic acid in the range of 0.001 to 1 mg/ml, n = 6, $r^2 = 0.999$.

RESULTS AND DISCUSSIONS

The HPTLC fingerprint profile is utilized as an identification method of compounds, compounds classes, of plant species respectively.

The fingerprint of *Artemisia annua* extracts - A system - (Figure 1) is characterized by the presence of artemisinin (T5) compound in all four samples as a pink spot at Rf~0.4.

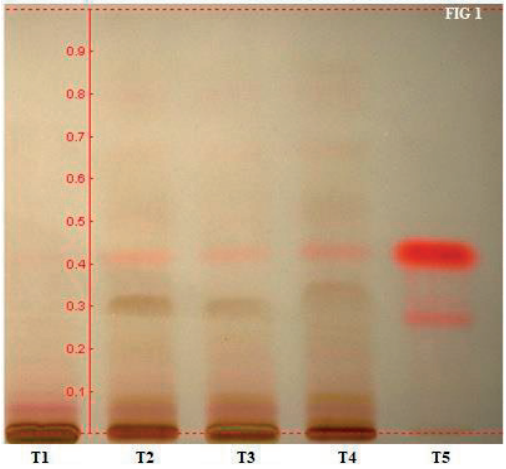

Figure 1. HPTLC fingerprint of *Artemisia annua* extracts - identification of artemisinin (reference substance)

In Figure 2, *the polyphenolic fingerprints of Artemisia annua extracts - B system - are* characterized *by the presents of all four reference substances:*

Reference substance	*Rf*	*Description*
T6 - hyperoside	Rf~0.68	orange fluorescent spot
T7 - chlorogenic acid	Rf~0.53	blue fluorescent spot
T8 - caffeic acid	Rf~0.95	blue fluorescent spot
T9 - rutin	Rf~0.45	orange fluorescent spot

In all four samples was also identified two green spots with Rf between 0.2 and 0.3 and an orange sport at Rf~0.18, mostly due to flavonoid glycosides and two blue florescent spots at Rf~0.8 and Rf~0.9, as caffeoyl and dicaffeoyl quinic acids (chlorogenic acids derivates).

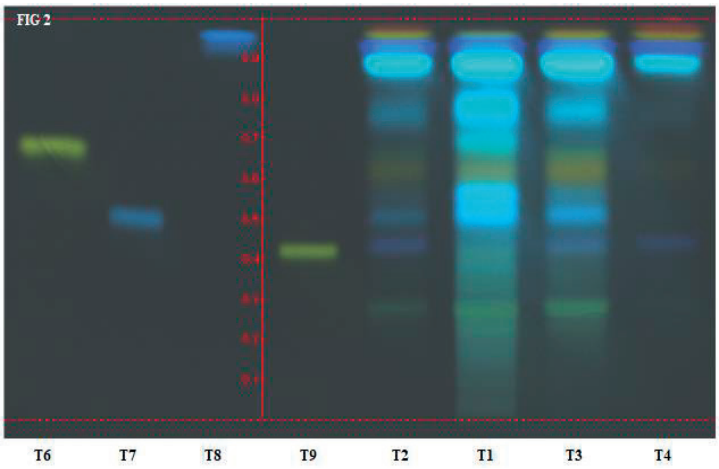

Figure 2. HPTLC fingerprint of *Artemisia annua* extracts - identification of phenolic compound

HPLC was performed for the quantification of artemisinin in all samples. The results are presented in Table 1.

Table 1. Artemisinin content in extracts (mg/100 ml)

Extract	Artemisinin (mg/100 ml)
T1	34.12
T2	13.39
T3	12.65
T4	22.23
M1	27.39
M2	60.37
M3	21.45
M4	92.45

Antioxidant activity was performed by DPPH (1,1 diphenyl-2-picryl hydrazyl) radical scavenging method for all the extracts obtained. The DPPH assay method is based on the reduction of the stable free radical DPPH (Shekhar & Anju, 2014). Changing the solution color from purple to yellow reflects the ability to scavenge DPPH free radical. (Brighente et al., 2007; Ionita, 2005).

Total antioxidant capacity assay (TAC) is based on the reduction of Mo (VI) to Mo (V) creating a green colored phosphomolybdenum at V complex, acidic pH, in the presence of extracts that contain antioxidants compounds. (Wan et al., 2011).

The next two figures present the antioxidant activity of the extracts obtained by the two methods DPPH - Figure 3 and TAC- Figure 4.

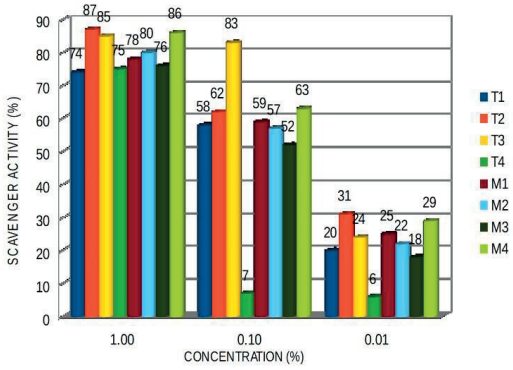

Figure 3. Scavenger activity of the DPPH

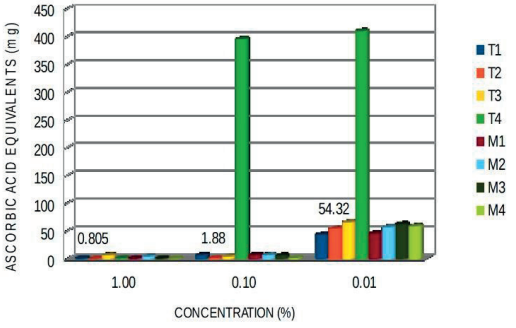

Figure 4. Total antioxidant capacity

The results obtained for both methods show that the antioxidant activity the T1 - water extract and M4 ethanol microwave extract have the highest effect, T1 being slightly superior.

The antioxidant activity is mainly attributed to polyphenolic compounds by research results (Cai et al., 2004; Kırca & Arslan, 2008; Surveswaran et al., 2007). There are also recent studies about water extract obtained from *Artemisia annua* that contain artemisinin and have DPPH radical scavenging activities if 91%, respectively higher than extracts obtained in other solvents (Kim et al., 2015). A review made by Alesaeidi & Miraj in 2016, shows that artemisinin and its flavonoids (a class of polyphenols) have a synergistic effect not only for anti-malarial activity, but also for immunosuppressive, anti-inflammatory, and anti-cancer properties of the plant activity.

CONCLUSIONS

The present study indicates that the water extract obtained by the solvent classical extraction technique and the last one of the extracts obtained by microwave technique are the most valuable for antioxidant properties, and also that the modern technique has superior results for artemisinin extraction.

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