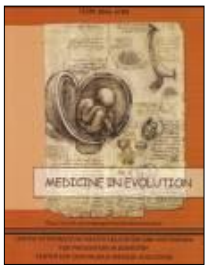
RESEARCHES REARDING OBTAINING SELECTIVE EXTRACTS WITH HYPOGLYCEMIANT PROPERTIES FROM VEGETAL INDIGENOUS PRODUCTS (CICHORII HERBA AND FRAXINI FOLIUM)NOTE II. CONTRIBUTIONS TO THE PHARMACOGNOSTICAL AND PHYTOBIOLOGICAL STUDY ON CICHORII HERBA



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ABSTRACT

*The **aim** of the present research is to verify the identity and the citotoxicity of Cichorii herba (through microscopic, chemical and phytobiologic exams), and to determine the extraction parameters for the further development of a pharmacological-active extract.*

Using microscopic exam, anatomic elements belong to Asteraceae family were identified. The chemical analysis established that flavonoids (rutin, hiperoside), hydroxycinanamic acid derivatives (caffeic acid, chlorogenic acid), tannin, reducing compounds, monosaccharides, polyholosides and non-alkaloid nitrogen compounds are present in the raw material.

The spectrophotometric analysis has revealed a higher content of flavones and phenolcarboxilic acids in the 70% ethanolic extract compared to the 50% methanolic extract.

The Triticum bioassay (Constantinescu method) revealed a mitoinhibitory effect only at high concentrations 5% (prophases, unorganised metaphase and tropokinetic telophase), frequent areas with inhibited kinesis, interphases with difficult to observe nuclei (slightly contracted aspect). The other main concentration effects on radix elongation not remarkably different from the control

***Keywords**: Cichorii herba, polyphenols, mitoinhibitory effect.*

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INTRODUCTION

Cichorii herba is used to treat digestive disorders, adjuvant in slimming belts, anorexia, and gout [2, 8]. *Cichorii herba* does not have a monograph in any of the pharmacopoeias (Romanian Pharmacopoeia 10-th edition, European Pharmacopoeia 7-th edition, United State Pharmacopoeia 31-th) and it is not mentioned in the literature for hypoglycemic activity.

The product contains: polyphenolic acids (cichoric acid chlorogenic acid), flavones (hiperoside), sesquiterpenic lactones (lactucin, lactucopicrin, 8-desoxy lactucin), guaianolid glycosides (cichoroisides B and C), hydroxycoumarins (umbelliferone) and polyynes [9]. Some of these constituents (hiperoside, caffeic acid, chlorogenic acid, isochlorogenic acid) are mentioned in the literature for their inhibitory effect on the aldose-reductase, on 11- β HSD, and AGE compounds (which are involved in the pathogenesis of diabetes and aging processes) [7]. Considering that the presence of flavones, coumarins and polyphenolic acids could be involved in regulating glucose metabolism and vascular disorders induced by hiperglicemia, we have in view to obtain selective extracts, standardized in flavones and phenolic acids.

The aim of this study is to verify the identity and the quality of the raw material (through microscopic, chemical and phytobiologic exams), and to establish the extraction parameters, in view to elaborate the technologic process for to manufacture a pharmacologic-active extract.

MATERIAL AND METHOD

The raw material was supplied by S.C. Fitoterapia S.A., Bucharest. For identity verification, the following tests were performed: macroscopic exam, microscopic exam (powder preparations clarified with 800g/L solution of chloral hydrate and stained with 10g/L solution of phloroglucin hydrochloride; Zeiss Imager D1 microscope, ob x 40), specific chemical reactions and chromatography - TLC for polyphenols [plate: TLC silica gel GF254 (Merck); mobile phase: anhydrous formic acid: glacial acetic acid: water: ethyl acetate = 11: 11: 26: 100 (v / v / v / v); reference substances: 0.1 mg/mL methanolic solutions of rutoside (Fluka), hiperoside (Roth), caffeic acid (Merck), chlorogenic acid (Roth); test solution (SA): 1g of vegetal powder was extracted with 10 mL metanol R under a reflux condenser for 30 min, filtered and concentrated to 1 mL; detection: successive spaying with methanolic solution 10g /L diphenylboryloxyethylamine = DPBA and 50g/L of polyetilen glycol 400, examine in ultraviolet light at 365 nm] [3, 4].

In order to verify the quality, the following active principles were assayed, using specific spectrophotometric methods: flavones (using spectrophotometric method based on the reaction with AlCl3/CH_3COONa, according to Romanian Pharmacopoeia 10th edition, *Cynarae folium* monograph), phenolcarboxilic acids (using spectrophotometric method based on the formation of oxymes in the presence of sodium nitrite and sodium molibdate).

The standard calibration curves were obtained using rutin and caffeic acid, respectively.

For the spectrophotometric assay a UV-VIS Cecil Series 2000 spectrophotometer was used [5, 6, 10].

The cytotoxicity was tested using the Constantinecu phytobiological method (the Triticum test), which involved analysing the influence of an aqueous solution on the radicular growth and on the cellular division in the roots of the germinated cariops.

As a biological reagent, embryonic roots of wheat karyopses (*Triticum aestivum*, race Boema obtained from SC Adaflor SRL Zebil, (Tulcea), harvested in 2010), germinated and treated in laboratory conditions were used. The 5% solution obtained from *Cichorii herba* from the reflux method at 30 min. was used for testing. From the primary solution (5%) several dilutions were made, thus being obtained solutions of 3.33%; 2.5%; 1.66%; 0.33% and 0.033% concentrations.

The solutions to be tested were brought in Petri dishes having a diameter of 15 cm, and then the wheat karyopses were introduced (the main rooth had to be 10 mm length). The dishes were covered with the lid and then the karyopses were left in contact with the solutions for 5 days. In parallel a control sample was prepared, in which the test solution was replaced by distilled water. Root elongation was evaluated at the same time for 5 days. For the microscopic study, the embryonic root of two karyopses from each Petri dish was sectioned at a distance of 5 mm from the tip and it was stained (by slight heating) with diluted orcein, a dye with great affinity for chromatin in an acid medium (the acid medium is necessary for the hydrolyse of the chromatic material from meristems, then the hydrolysed chromatic material is stained in red). The stained sections were then examined by immersion in cedar oil using Nikon Labophot 2 microscope (ob 40x and 100 x).

The statistical processing of the data (the influence of the extracts on the *Triticum* embryonic root growth) was performed using the Minitab commercial package (version 15.0). The normality assumption was checked through the Anderson-Darling test and the homoschedasticity assumption through the Levene's test (at a significant level of 0.05). As the normality assumption was violated, the non-parametric Kruskal-Wallis test was used to compare multiple samples, and Mann-Whitney test was applied for the *post-hoc* subgroup analysis (comparing the groups treated with extracts at various concentration levels with the control group).

Differences were reported as statistically significant using a 0.05 threshold level [1]. 50% methanol and 70% ethanol were used to determine the extraction parameters using as the reflux method for 30 min.

RESULTS

The morphologic and anatomic characteristics of the raw material correspond to those mentioned in scientific literature for *Cichorii herba*. Using specific reactions, the following active principles were identified: flavones, tannin, reducing compounds, phenollcarboxilic acids, monosaccharides, polyholosides and non-alkaloid nitrogen compounds.

Using TLC technique, hiperoside (Rf = 0.56), caffeic acid (Rf = 0.90) and chlorogenic acid (Rf = 0.40) were identified - fig. 1 A and B (A = UV light at 366 nm, B = DBPA + UV light at 366 nm).

Rutin (Rf = 0.30) were not identified. The spectrophotometric analysis has revealed a higher quantity of flavones (g%, expressed as rutoside) in

the 70% ethanolic extract (0.160-0.194%) compared to that in the 50% methanolic extract (0.109-0.173%) and a lower quantity of phenolcarboxilic acids (g%, expressed as caffeic acid) in the 50% methanolic extract (2.0895 – 2.7724%) compared to that in the 70% ethanolic extract (2.2178- 2.9510%). So, we consider 70% ethanol is the proper solvent for to obtain the selective pharmacologic-active extract.

The *Triticum* bioassay (Constantinescu method) revealed a mitoinhibitory effect only at high concentrations 5%.

The other main concentration effects on radix elongation not remarkably different from the control.

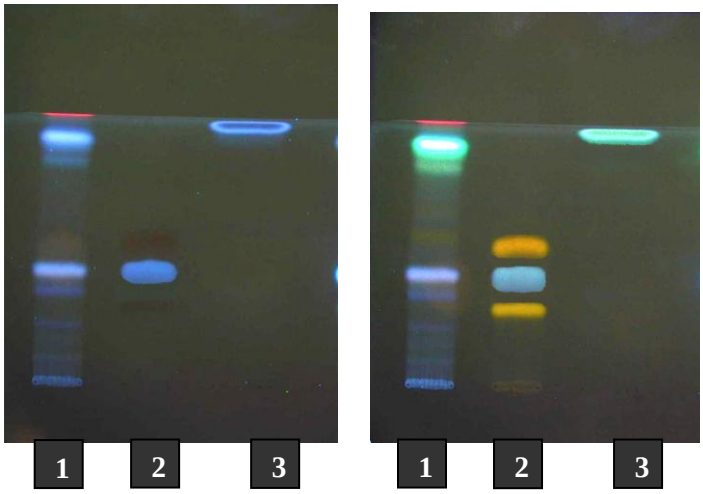

Fig. 1. TLC chromatogram for flavones and polyhhenolic acids from C*ichorii herba*
A (u.v.), B (DBPA + u.v.). 1- SA; 2- mixture of reference substances (from top downwards: hiperoside, chlorogenic acid, rutin); 3- caffeic acid

Table 1 Kruskal-Wallis test results for the *Cichorii herba* extracts (comparison of the root length measured for various extract concentration levels and the control group).

Concentration	**N**	**Median**	**Average rank**	**Z**
M	11	2,00	36,7	-0,58
0,03%	11	1,90	34,8	-0,87
0,33%	12	2,20	55,8	2,48
1,66%	12	2,10	45,0	0,73
2,50%	11	2,10	48,0	1,15
3,33%	12	2,10	48,6	1,31
5,00%	11	1,40	12,0	-4,39
Global	80		40,5	
H = 25.83 DF = 6 P = 0.000				
H = 26.09 DF = 6 P = 0.000 (adjusted for *ties*)				

The statistical tests (Kruskal-Wallis and Mann-Whitney, respectively, the latter being used for post-hoc comparison with the control group) confirmed the observations suggested by the graphical representation of the data

(fig.2) for each extract, the Kruskal-Wallis test revealed statistically signify-cant differences ($p<0.001$) within the samples compared, as may be seen from table I. The results of the post-hoc analyses (between the values seen for each concentration level and the control group, respectively) showed statistically significant differences ($p<0.05$) for the 5.00 concentration only.

These differences and their statistical significance were maintained throughout the 5 days of the experiment.

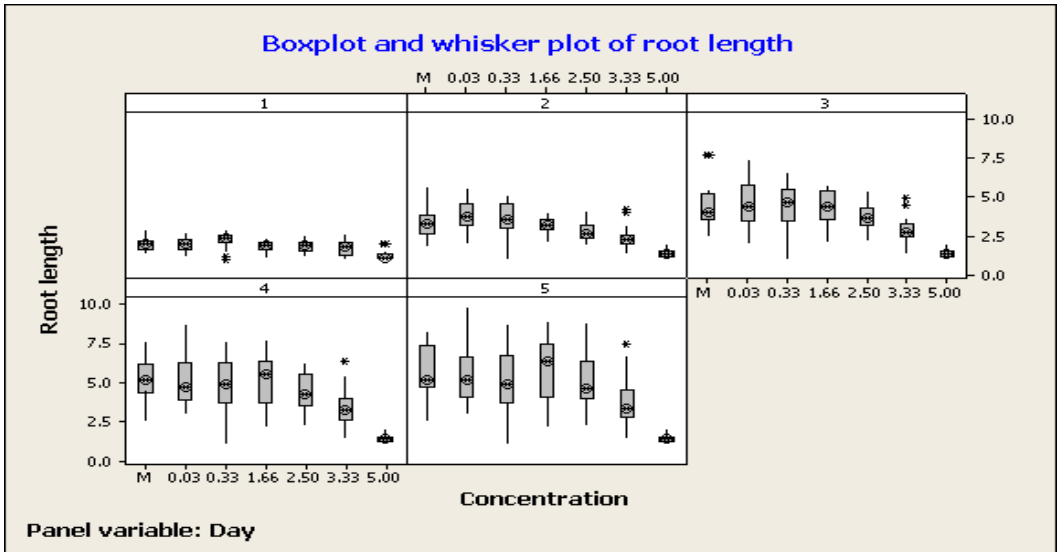

Fig.2. Box and whisker plot showing the influence of the Cichorii extract on embryonic wheat roots, depending on the measurement day (each day in a separate panel) and concentration. M – control group; the other numbers indicate the extract concentration. Interquartile box, median and outliers are shown.

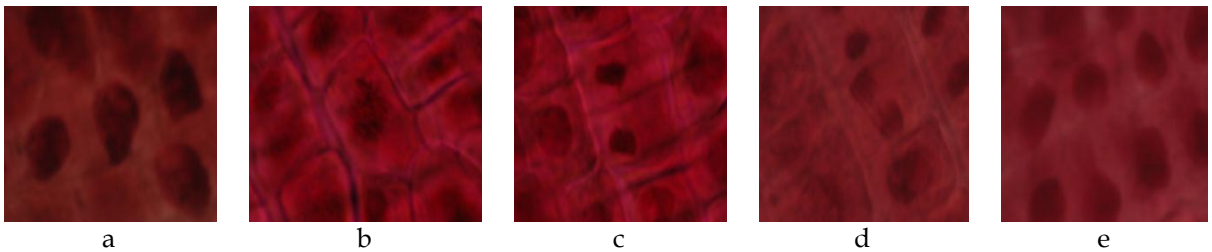

Fig. 3. The karyokynetic's film change : a- interphases with nucleii of various forms ; b- metaphasees in tropokinesis; c- telophases in tropokinesis; d- anaphases in tropokinesis; e- areas with inhibited kinesis

DISCUSSIONS

Cichorii herba meets current quality rules, do not show cytotoxic properties, a statistically significant effect mitoinhibitor and rare type cromatoclazic changes against the control was observed only at very high concentrations (5%).

The best solvent for obtaining a pharmacological-active extract is ethanol 70%.

Acknowledgement

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