CHEMICAL COMPOSITION AND ANTIOXIDANT ACTIVITY OF *FICUS ELASTICA* ROXB. EX HORNEM AND *RAPHANUS SATIVUS* L. SELECTIVE DRY EXTRACTS WITH POTENTIAL ANTIDIABETIC ACTIVITY

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Abstract

The aim of our study consisted in obtaining, phytochemical and antioxidant activity evaluation of two selective dry extracts from the leaves of *Ficus elastica* Roxb. ex Hornem and *Raphanus sativus* L., with potential use in metabolic diseases. The results of the phytochemical assessment of the two hydroethanolic dry extracts (the employed hydroethanolic mixture were: 70% ethanol (v/v) for *Fici elastici folium* and 50% ethanol (v/v) for *Raphani sativi folium*) indicated the following: *Fici elastici extractum* (FEE) - 3.6140±0.4000 g% flavonoids expressed as hyperoside, 5.950 ± 0.3800 g% phenolcarboxylic acids expressed as chlorogenic acid, 13.3299 ± 0.1583 g% total phenolic compounds expressed as tannic acid; 2. *Raphani sativi extractum* (RSE) - 6.7300 ± 0.6600 g% flavonoid expressed as hyperoside, 3.1263 ± 0.1500 g% phenolcarboxylic acids expressed as chlorogenic acid, 21.4175 ± 0.9454 g% total phenolic compounds expressed as tannic acid. The HPLC analysis confirmed the presence of apigenin-7-glucoside in *Raphani sativi extractum*. Evaluation of the antiradicalar activity of the dry extracts was determined by scavenger activity of free radicals, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+) and by ferric reducing power method. The antioxidant activity was expressed as EC50 mg/mL. The following results were obtained: EC50_{DPPH} = 6.4166 ± 0.3329 mg/mL, EC50_{ABTS} = 0.0768 ± 0.0020 mg/mL, EC50_{ferric reducing power} = 0.4027 ± 0.0016 mg/mL (for FEE); EC50_{DPPH} = 7.5100 ± 0.1414 mg/mL, EC50_{ABTS} = 0.2611 ± 0.1893 mg/mL, EC50_{ferric reducing power} = 1.3935 ± 0.0111 mg/mL (for RSE).

Rezumat

Scopul lucrării a constat în obținerea, caracterizarea fitochimică și evaluarea activității antioxidante a două extracte uscate, selective, obținute din frunzele de *Ficus elastica* Roxb. ex Hornem și *Raphanus sativus* L., cu potențială activitate în tratamentul afecțiunilor metabolice. Extracte hidroalcoolice uscate (obținute în etanol 70% pentru *Fici elastici folium* și în etanol 50% pentru *Raphani sativi folium*) conțin: *Fici elastici extractum* (FEE) - 3,6140 ± 0,4000 g% flavone exprimate în hiperozidă, 5,950 ± 0,3800 g% acizi fenol-carboxilici exprimați în acid clorogenic, 13,3299 ± 0,1583 g% polifenoli totali exprimați în acid tanic; *Raphani sativi extractum* (RSE) - 6,7300 ± 0,6600 g% flavone exprimate în hiperozidă, 3,1263 ± 0,1500 g% acizi fenol-carboxilici exprimați în acid clorogenic, 21,4175 ± 0,9454 g% polifenoli totali exprimați în acid tanic. Analiza HPLC a evidențiat prezența apigenin-7-glucozidei în RSE. Activitatea antioxidantă a extractelor a fost determinată pe baza capacității de scavenger a radicalilor liberi DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS•+ (acidul 2,2'-azinobis-3-etilbenzotiazolin-6-sulfonic) și pe baza capacității de reducere a ferului. Capacitate antioxidantă a fost evaluată pe baza EC50 (μg/mL). S-au obținut următoarele rezultate: EC50_{DPPH} = 6,4166 ± 0,3329 mg/mL, EC50_{ABTS} = 0,0768 ± 0,0020 mg/mL, EC50_{reducerea ferului} = 0,4027 ± 0,0016 mg/mL (pentru FEE); EC50_{DPPH} = 7,5100 ± 0,1414 mg/mL, EC50_{ABTS} = 0,2611 ± 0,1893 mg/mL, EC50_{reducerea ferului} = 1,3935 ± 0,0111 mg/mL (pentru RSE).

Keywords: microscopic exam, polyphenols, apigenin-7-glucoside, antioxidant capacity

Introduction

The species of the *Ficus* genus (*F. racemosa*, *F. glomerata*, *F. indica*, *F. carica*) exhibit numerous therapeutic effects such as: anti-pyretic, gastro-protective, antiulcer and antidiabetic. The leaves of *Ficus elastica* are less studied and possess parasitocidal, antioxidant, antiallergic and diuretic properties [20, 40].

Raphanus sativus L. is a cruciferous vegetable that is widely consumed across the globe. Different parts of this species are used for medicinal purposes to treat digestive disorders such as: indigestion, digestive inflammation, diarrhoea and abdominal pain. The radish leaves are reputed to poses anti-cancer activity, anti-inflammatory and antidiabetic effects [2, 29]. Taking into consideration the scientific data, the aim of our work was obtaining, phytochemical characterization and evaluation of the antioxidant capacity of two selective dry extracts from the leaves of *Ficus elastica* Roxb. ex Hornem and *Raphanus sativus* L. with potential use in metabolic diseases (diabetes mellitus).

Materials and Methods

Vegetal samples

Ficus elastica Roxb. ex Hornem and *Raphanus sativus* L. leaves have been used as material. Herbal products were dried and stored in laboratory conditions. The research was carried out in several steps: 1) verification of herbal products identity based on macroscopic and microscopic characteristics; 2) obtaining and characterization of two selective dry extracts 3) determination of extracts antioxidant activity.

The herbal products identity was determined based on macroscopic (morpho-anatomical characteristics) and microscopic (performed on surface preparations and cross/longitudinal sections) assays (Labophot 2 Nikon/digital Leika DMS 1000 microscope, ob. 4x, 10x, 40x, 100x) [10].

Obtaining and characterization of selective dry extracts. 556 g *Fici elastici folium* and 977.32 g *Raphani sativi folium* were heated twice on a reflux condenser with 70% ethanol (for *Fici elastici folium*) and 50% ethanol (for *Raphani sativi folium*) for 60 min. using 1:10 herbal product/solvent ratio (for the first extraction) and 1:5 (for the second one). After cooling, the combined filtrates were concentrated using a rotary evaporator and then freeze-dried. The solvent choice and concentration were based on scientific literature data [19, 30, 39, 42, 43]. The extraction yield is expressed as the percentage of the total mass of the dry extract (M_{ext} without water content) with respect to the mass of the raw material (M_{pv}) loaded onto to the flask for solvent extraction: $Y\% = (M_{\text{ext}}/M_{\text{pv}}) \times 100$. The obtained dry extracts were encoded as follows: *FEE* - *Fici elastici extractum* and *RSE* - *Raphani sativi extractum*.

Spectrophotometric assays. The flavonoids content (FL) was determined based on the chelating reaction with aluminium chloride [14]. Phenolcarboxylic acids (PAC) were assessed based on formation of oxymes in the presence of sodium nitrite/hydrochloric acid and sodium hydroxide [15]. The total phenolic (TPC) content was evaluated based on the capacity to reduce molybdenic(VI) compounds [36]. For all spectrophotometric determinations a Jasco V-530 spectrophotometer (Jasco, Japan) was used. The following calibration curves were used to calculate the content of active compounds: hyperoside (linearity range: 7.32 - 34.16 $\mu\text{g/mL}$, $r = 0.9998$, $n = 12$), chlorogenic acid (linearity range: 0.0113 - 0.0527 mg/mL , $r = 0.9998$, $n = 6$) and tannic acid (linearity range: 2.0 - 12.0 $\mu\text{g/mL}$, $r = 0.9990$, $n = 10$).

HPLC analysis was carried out using a previously published external standard method with gradient elution and UV detection [15].

Evaluation of the antiradicalar activity of *FEE* and *RSE* was determined by means of the following methods: the scavenger activity towards 2,2-diphenyl-1-picrylhydrazyl (DPPH) [8,26], 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS $^{•+}$) [9,33] free radicals and ferric reducing power [28].

Preparation of samples for antioxidant activity evaluation

For *FEE*: 0.5000 g dry extract were dissolved in 25 mL or 50 mL of 70% ethanol (v/v) by ultrasonication. The stock solutions concentration were 1% (for DPPH assay) and 2% (for ABTS $^{•+}$ and ferric reducing power assays).

For *RSE*: 0.5000 g dry extract were dissolved in 25 mL of 50% ethanol (v/v) by ultrasonication. All antioxidant assays were performed using a 2% stock solution.

Scavenging activity of free radical DPPH (2,2-diphenyl-1-picrylhydrazyl) [8, 26]. Briefly, 0.3 mL of different concentration of analysed extracts (0.8 - 10 mg/mL for *FEE*; 2.5 - 15 mg/mL for *RSE*) were mixed with 2.7 mL of freshly prepared DPPH $^{\bullet}$ ethanolic solution (120 μL , $\text{Abs} = 0.68 \pm 0.01$). After 30 min of incubation, in the dark at room temperature, the absorbance was measured at 517 nm. Ethanol was used as blank. The concentration of analysed dry extracts that inhibited 50% of the DPPH free radical (EC_{50} , mg/mL) was determined graphically from the linear regression curve plotted between percent (%) of inhibition and dry extracts concentrations (mg/mL).

Radical scavenging of the extracts were calculated as follows and expressed in terms of EC_{50} (mg/mL extract that scavenge 50% of DPPH free radical).

$$\% \text{Inhibition} = \frac{\text{Abs}(t = 0 \text{ min}) - \text{Abs}(t = 30 \text{ min})}{\text{Abs}(t = 0 \text{ min})} * 100,$$

where Abs (t = 0 min) - absorbance of DPPH solution; Abs (t = 30 min) - absorbance of DPPH solution with sample, after 30 min.

ABTS^{•+} radical cation scavenging assay was performed according to Re R *et al.* method [9, 14, 33]. The ABTS^{•+} radical cation was generated by incubation of ABTS diammonium salt (7 mM) with potassium persulphate (2.45 mM) in the dark, at room temperature for 16 h. The absorbance of the ABTS^{•+} radical solution was equilibrated to a value of 0.700 ± 0.02 at $\lambda = 734$ nm after dilution with ethanol. Briefly, *FEE* and *RSE* stock solutions were diluted with 70% ethanol and 50% ethanol in order to obtain solutions with equivalent concentrations of 0.03 - 0.16 mg/mL (for *FEE*) and 0.2 - 1 mg/mL (for *RSE*) (test solutions). 0.5 mL of the tested solutions were treated with 3 mL of ABTS^{•+} free radical and the mixture was kept in the dark. The absorbance of the free radical cation was measured at $\lambda = 734$ nm before (A_{start}) and 6 min. after adding the extractive solutions (A_{end}). Ethanol was used as a blank. The ability to scavenge the ABTS^{•+} free radical was calculated according to the following formula: $\text{ABTS}^{\bullet+} \text{ radical scavenging activity (\%)} = (A_{\text{start}} - A_{\text{end}}) / A_{\text{start}} \times 100$. The concentration of analysed dry extracts that inhibited 50% of the ABTS^{•+} free radical (EC_{50} , mg/mL) was determined graphically from the linear regression curve plotted between percent (%) of inhibition and dry extracts concentrations (mg/mL).

Ferric reducing power. The reducing power assay was determined according to Oyaizu M method [28]. Briefly, *FEE* and *RSE* stock solutions were diluted with 70% ethanol and 50% ethanol in order to obtain solutions with equivalent concentrations of 0.03 - 1.6 mg/mL (for *FEE*) and 0.2 - 2 mg/mL (for *RSE*) (test solutions). 2.5 mL of the test solutions were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and

2.5 mL of 1% potassium ferricyanide. Samples were kept at 50°C in a water bath (Raypa, Spain) for 20 min. After, 2.5 mL of 10% trichloroacetic acid was added and the mixture was centrifuged at 2500 rpm for 5 min. (Universal 16 centrifuge). The upper layer (2.5 mL) was mixed with 2.5 mL water and 0.5 mL of a 0.1% ferric chloride solution. The absorbance was measured at $\lambda = 700$ nm, after 10 min., against a blank that contained all reagents except for tested solutions. A higher absorbance indicates stronger reducing power. The analysed dry extracts concentration providing 0.5 of absorbance (EC_{50} mg/mL) was determined graphically from the linear regression curve plotted between absorbance and test solutions concentrations (mg/mL).

Statistic analysis. For each dry extract, three samples were analysed and all assays were carried out in triplicate ($n = 3$). The results are expressed as mean $\pm$ standard deviation. Standard deviation was determined using Microsoft Office (Excell, 2010).

Results and Discussion

The macroscopic and microscopic examination confirmed the identity of the raw materials based on the correspondence with the characters described in the scientific literature [6, 25, 35, 38]. The analysis of both surface preparations and transverse/longitudinal sections for *Fici elastici folium* revealed an asymmetric heterogenous structure of the lamina with a bistratified palisade (Figure 1a); the adaxial epidermis shows a thick cuticle (characteristic to xerophytic plants) with moniliforme thickening of cell walls (Figure 1b); the hypodermis is characterized by large cells, some of them contain round or oblong shaped gray cystoliths (Figure 1c).

Figure 1.

Anatomical elements for *Fici elastici folium*: a) asymmetric heterogenous structure of the lamina, ob.40x; b) epidermic cells with moniliforme thickening of cell walls and round cystoliths, ob. 40x; c) cystoliths, ob.100x; d) non-articulated laticifers, ob.40x; e) anomocytic stomata, ob.10x; f) prismatic crystals, ob.40x

The midrib has a characteristic shape (a prominent arch on the abaxial face); subepidermally one can note a mechanic tissue (angular collenchyma) with inequal thick cell walls. Numerous non-articulated laticifers were observed in the parenchyma (Figure 1d). The vascular tissue of the leaves is represented by collateral bundles, which are disposed as an abaxial arch. The abaxial epidermis has numerous stomata submerged in the cuticle (with supra-stomatic chambers) (Figure 1e), due to plant's specific adaptation to environmental conditions. In the surface preparations we have observed numerous prismatic crystals along the veins (Figure 1f). The observed anatomical elements are in agreement with the scientific data [6, 7, 16,

35, 38], however the type and shape of the observed cystoliths represent an original aspect of our research. For *Raphani sativi folium*, we have observed a specific structure of the mesophyll, with four layers of palisade and a spongy parenchyma (Figure 2a). However, other authors, Miyake and co-workers have observed only one or two layers of palisade [25]. The midrib presents in the upper-third only one collateral bundle surrounded by colenchimatic pericycle; in the lower-third, it has five opened collateral bundles. Secondary veins show bundles without colenchimatic pericycle. The parenchyma cells have cellulosic walls. On the lower epidermis, rare unicellular trichomes were observed (Figures 2b and 2c).

Figure 2.

Anatomical elements for *Raphani sativi folium*: a) asymmetric heterogeneous structure of the lamina, ob.10x; b) unicellular trichomes (digital microphotography); c) unicellular trichomes ob.10x

The extraction yield was 13.84% for *FEE* and 26.05% for *RSE*. Hydroalcoholic dry extracts have the same organoleptic characteristics of the raw materials; *FEE* is green-brownish coloured, while *RSE* is brown-greenish coloured. Both extracts have a specific smell. The results of the quantitative determinations are found in Table I. *RSE* has a higher content of TPC and FL than *FEE*. PAC prevails in *FEE* when compared to *RSE*. Differences related to extracts obtaining procedures (different solvent, time of contact, raw material:solvent ratio) and raw material quality makes the comparison of our data with the ones from scientific literature difficult. The flavonoid content of *FEE* (36 mg/g hyperoside or equivalent to 23.48 mg quercetin/g extract) is lower than the results found by Preeti and co-workers (43 mg quercetin/g extract) [32]. In opposition, *RSE* has a higher content of flavonoids

compared to other authors results (67.30 mg hyperoside/g extract equivalent to 43.64 mg quercetin/g extract vs. 10.423 mg quercetin/g extract found by Goyeneche and co-workers) [17]. Data related to PCA total content weren't found in other reports, therefore a comparison was not possible. The TPC content for *RSE* falls within the limit found by other authors (21.4175 g% TPC expressed as tannic acid is equivalent to 2.1408 g% TPC expressed as gallic acid vs. 0.659 mg% TPC expressed as gallic acid, Goyeneche and co-workers, and 9.80g% TPC expressed as gallic acid, Eugenio and co-workers) [13, 17]. In the case of *FEE*, the TPC content is lower than other findings (13.32 g% TPC expressed as tannic acid equivalent to 2.27 g% TPC expressed as catechin vs. 5.88 g% TPC expressed as catechin) [41].

Table I

Results of the spectrophotometric determinations

Dry extract	g% FL	g% PCA	g% TPC
<i>FEE</i>	3.6140 ± 0.4000	5.9507 ± 0.3800	13.3299 ± 0.1583
<i>RSE</i>	6.7300 ± 0.6600	3.1263 ± 0.1500	21.4175 ± 0.9454

FEE - *Fici elastici extractum*, *RSE* - *Raphani sativi extractum*, PCA = phenolcarboxylic acids; FL = flavonoids; TPC = total phenolic content. Results are mean ± SD (standard deviation) (n = 3)

The HPLC analysis of *RSE* confirmed the presence of apigenin-7-glucoside, but not of rutin. Both compounds are mentioned by scientific data [1, 31]. Also, phenol-carboxylic derivatives like ferulic acid and caffeic

acid were not identified, despite other research data [31].

The chromatographic analysis of *FEE* indicated a main compound (unidentified due to lack of standard) with two absorption bands in the UV/visible region,

similar to flavans derivatives compounds: 284 nm (main band; Band I in the 250 - 285 nm range representing A ring absorption) and 320 nm (Band II in the 320 - 385 nm range representing the B ring absorption) [34].

We suppose that this compound belongs to the flavonoidic group, namely tannins due to several reasons: i) UV maxima at 284 nm falls within the UV features of condensed tannins (280 - 300 nm) and is very similar to other catechin derivatives from other *Ficus* sp. (270 nm - gallo catechingalat; 275 nm - epigallocatechin; 250 nm - catechin, 280 nm - epi-afzelechin) [23,27]; ii) a tannin compound from *Manihot esculenta* is characterised by UV maxima at 320 nm (and UV maxima at 280 nm) [23]; iii) a lack of other UV maxima describing other flavans derivatives, like 520 nm - for coloured

substances (anthocyanidins) or 360 nm - for flavonol group [3]; iv) tannins presence in *Fici elastici folium* [32].

Our results regarding *in vitro* antioxidant activity showed that *FEE* had the best antioxidant activity independent of the method used, since lower values of EC_{50} (mg/mL) were observed compared to *RSE* (Table II). The scavenger activity of *FEE* against DPPH free radical was established for 0.8 - 10 mg extract/mL, and the highest inhibition percent 65% was found at 10 mg/mL (Figure 3a). The antioxidant activity is lower compared to a methanolic dry extract ($IC_{50} = 6.4166 \pm 0.3329$ mg/mL vs. $27.17 \mu\text{g/mL}$ [32]. *RSE* scavenges DPPH free radical at a higher domain of concentration (2.5 - 15 mg/mL), the highest inhibition percent 85 % was found at 15 mg/mL (Figure 4a).

Table II

EC_{50} (mg/mL) values for the analysed dry extracts

Dry extract	ANTIOXIDANT ASSAY - EC_{50} (mg/mL)		
	DPPH	ABTS ⁺	Reducing power
<i>FEE</i>	6.4166 ± 0.3329	0.0768 ± 0.0020	0.4027 ± 0.0016
<i>RSE</i>	7.5100 ± 0.1414	0.2611 ± 0.1893	1.3935 ± 0.0111

FEE – *Fici elastici extractum*, *RSE* – *Raphani sativi extractum*. Results are mean $\pm$ SD (standard deviation) (n = 3)

TPC content is not well-correlated with the scavenger activity towards DPPH free radical, probably because other (unquantified, but mentioned) compounds like, scopoletin [31], polysaccharides [31] or sterols [32] may act as antioxidants against DPPH [4, 22, 44]. Another explanation is that other compounds like

ascorbic acid, amine (tyrosine), sulphur compounds (for *Raphani sativi folium*) or carboxylic acids (acetic and formic acids) may interfere with Folin-Ciocalteu reagent overestimating the TPC content of the extracts [5].

Figure 3.

Antioxidant capacity of *FEE*: a - DPPH assay, b - ABTS assay, c - ferric reducing power

As shown in Figure 3b, the scavenger activity towards $\text{ABTS}^{+\cdot}$ free radical for *FEE*, at the highest concentration (0.16 mg/mL) was 97.87%. Regarding ferric reducing power, the absorbance values varies between 0.1068 (at 0.032 mg/mL) and 1.7034 (at 1.6 mg/mL) (Figure 3c). The comparison of our results with other authors work was difficult, since most scientific studies refers to the antioxidant capacity of other *Fici* sp. *folium* (*Ficus glomerata* Roxb., *Ficus carica* Linn. or *Ficus benjamina* L.) [11, 12, 18]. However, we assume that phenolic compounds

(determined by us using both spectrophotometric and chromatographic methods) together with triterpenes, sterols or minerals (mentioned by scientific literature) might be responsible for the overall antioxidant capacity [37].

RSE also scavenged $\text{ABTS}^{+\cdot}$ free radical in a dose-dependent manner (Figure 4b). The dry extract displayed scavenging activity with values ranging from 43.12% (at 0.2 mg/mL) to 82.41% (at 1 mg/mL). Regarding its ferric reducing power, at the highest concentration the absorbance was 0.6766 (Figure 4c).

Figure 4.

Antioxidant capacity of *RSE*: a - DPPH assay, b - ABTS assay, c - ferric reducing power

According to other authors, *Raphani sativi folium* have also shown antioxidant activity, but the comparison with our results was hard due to different extraction solvents and methods [17, 21]. According to our results flavones (apigenin-7-glucoside) and phenolcarboxylic acids are responsible for the antioxidant activity. Moreover, the scientific literature foresees the presence of other phenols (caffeic acid, *p*-coumaric acid, caffeoylmalic acid, epicatechin, sinapic acid, tyrosol, kaempferitrin) [17], which are well known as metal chelators, reducing agents, hydrogen donors, singlet oxygen quenchers and superoxide radical scavengers. Additionally, they are also involved in activation of humans antioxidant defence system, since they enhance superoxide dismutase, catalase and glutathione peroxidase activities [24]. Over and besides, minerals (zinc, manganese, magnesium - which are reported for radish leaves) [17] might also be involved in the overall antioxidant activity.

Conclusions

The phytochemical study reveals a diverse chemical composition and high polyphenolic content for both extracts, especially in the case of *Raphani sativi*

extractum. The scavenger activity against free radicals was proved for both extracts, although *Fici elastici extractum* exhibited the highest antioxidant potential when compared to *Raphani sativi extractum*.

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