NEW SOURCES OF CONDENSED TANNINS - INVESTIGATION OF BRANCHES OF SAME SCHRUBS SPECIES THROUGH HPTLC ANALYSIS

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Abstract. *R. canina L., H. rhamnoides L. and P. spinosa L. species are used traditionally as medicinal plants due to phytotherapeutical and nutritional potential of fruits. The aim of this study is to identify and quantify the catechin and epicatechin in branches of these species, using qualitative (HPTLC) and quantitative (HPTLC densitometry) methods. HPTLC fingerprinting of crude methanolic extracts showed specific peaks, with different Rf values, corresponding to catechin (at Rf 0.46) evidenced in branches of all three species and epicatechin (at Rf 0.43) highlighted only in samples of P. spinosa. The quantitative evaluation by HPTLC densitometry indicated the amount of catechin in branches of R. canina (0.17% g/g in dried plant material), H. rhamnoides (0.10% g/g in dried plant material) and P. spinosa (0.25% g/g in dried plant material) and the amount of epicatechin in branches of P. spinosa (0.22% g/g in dried plant material). These vegetal species can be considered a new source of catechins.*
Keywords: dog rose, sea buckthorn, blackthorn, condensed tannins, densitometry

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1. Introduction

Condensed tannins are a group of polyphenolic compounds belonging to flavonoid class (flavan 3-ols) present in high concentration in a variety of medicinal plants. They are recognized to posses antibacterial [1,2], antifungal [3], antioxidant [4-7], antihypercholesterolemic [8,9], antimutagenic[10], antiviral [11] and anticarcinogenic activities [6,12], their effect consisting in improving human health by preventing various diseases. Catechins have received considerable attention due to their various biological activities, in particular by their effects on arteriosclerosis [13] and their scavenger ability of reactive oxygen species [14,15].

The condensed tannins occur almost universally in ferns and gymnosperms and are widespread among the angiosperms, especially in trees and shrubs. Regarding the localization of condensed tannins, they are found distributed throughout the organs of the plant; are accumulated in larger amounts in bark, branches, leaves, flowers, where they are found free or combined with proteins, alkaloids, mucilages [16-21].

Rosa canina L. (Rosaceae) , *Hippophae rhamnoides* L. (Elaeagnaceae) and *Prunus spinosa* L. (Rosaceae) species are used traditionally as medicinal plants due

to phytotherapeutical and nutritional potential of fruits. The branches belonging to these species can also represent new sources of condensed tannins.

Rosa canina (dog rose) is a shrub native to Europe, western Asia and north-eastern Africa. The nutritive and therapeutical value of the mature dog rose fruits (*Cynosbati fructus*) is conferred by their content in: sugars, organic acids, pectins, flavonoids, tannins, carotenoids, macro- and microelements etc. [22-25]. Seeds contain oil, minerals and fatty acids (mainly linoleic, oleic, linolenic, palmitic, stearic and arachidonic acids) [26]. The nutritive, therapeutical and ecological value of dog rose turns it into a species of great perspective, from which also the branches can be used as a new source of condensed tannins, with antioxidant potential.

Hippophae rhamnoides, commonly known as sea buckthorn, is a shrub in which all parts of it are considered an important source of large number of bioactive substances. The branches are less investigated, some of the existing studies showing that acetonic sea buchthorn extracts inhibits the growth of cancer cells due the presence of catechin [27]. In other types of ethanolic and methanolic extracts hydrolysable tannins (gallic acid), flavonoids (rutin) and condensed tannins (epicatechin) were evidenced [28].

Prunus spinosa (blackthorn or sloe) is a perennial plant growing as a shrub on slopes of wild uncultivated areas. The infusion obtained from branches is used in the tratment of hypertension [29] and the macerated fruits in gastrointestinal disturbances. *P. spinosa* contains significant quantities of phenolic antioxidants [30] mainly including phenolic acids (neochlorogenic and caffeic derivatives) and type proanthocyanidin A [31]. The concentration of bioactive compounds in extracts of branches, leaves and fruits from *P. spinosa* depends on morpho-anatomical element (branches, leaves and fruits) and on solvent used for extraction. Ethanolic extract obtained from the branches has higher antioxidant capacity compared to that resulted from leaves and fruits [31]. Branches could be an important natural source for extracts which can be used to prevent oxidative stress that leads to many diseases due to their content in condensed tannins.

High performance thin layer chromatoghraphy is an ideal tool for separation and visualization of the phytocompounds from herbal materials and extracts. The image of HPTLC coupled with a digital scanning profile is more attractive to herbal analysts for constructing herbal chromatographic fingerprint by means of HPTLC and provide adequate information and parameters for comprehensive identification, assesment and comparison of major active constituents fingerprint serving as a basis for demostrating their biological activities.

This study aimed the identification and quantification of catechin, as a valuable biomarker in branches (*Rosa canina, Hippophae rhamnoides* and *Prunus spinosa*) and epicatechin in branches of *Prunus spinosa*, by qualitative (HPTLC) and quantitative (HPTLC densitometry) methods. Although different morphologic elements of these vegetal species contain significant compounds with medicinal

properties, there are not enough published data regarding the phytochemical constituents and also the HPTLC profiling of extracts from branches of dog rose, sea buckthorn and blackthorn. HPTLC is an important linear method, used in drug, extracts and other herbal products analysis. A major advantage of HPTLC is its ability to analyze several samples simultaneously using a small quantity of mobile phase. The combination of the densitometric method by HPTLC with UV-VIS spectrophotometry provides a comprehensive assessment of the content of condensed tannins, which can be used for identification and evaluation of these compounds in branches of *R. canina*, *H. rhamnoides* and *P. spinosa*.

2. Materials and methods

2.1. Plant material

Branches of *R. canina* , *H. rhamnoides* and *P. spinosa* were collected from a plateau area with temperate continental climate (Siret Valley, Bacău county, Romania), in September 2016. Plant material was dried in a well-ventilated room, in a single layer, protected from direct solar light. The dried branches were grounded using a laboratory mill Microton MB550, to obtain particles with medium size of 0.8 mm diameter. The samples were stored in a clean desiccator until were extracted and used for HPTLC investigation.

2.2. Chemicals and reagents

The following reference standards were used: (+)-catechin (min. 99% HPLC from Fluka, Sigma-Aldrich, Saint-Louis, MO, USA) and (-)-epicatechin (ROTICHROM TLC from Carl Roth, Mannheim, Germany). All the reagents were of analytical grade or pure: toluene, 99.7% (Sigma-Aldrich), ethyl acetate, min. 99.5% (Sigma-Aldrich), formic acid p.a. (Merck Germany), methanol p.a., min. 99.8 % (Chempur Poland), ferric chloride, min. 98.5%, extra pure, anhydrous (Carl Roth). HPTLC plates G60 F254, 200x100mm (Merck, Darmstadt, Germany) were used as stationary phase for HPTLC identification and HPTLC densitometric assay.

2.3. Preparation of extracts and reference solution

An amount of 5g of powdered dried material from each vegetal species were weighed accurately and dispersed separately in 100 mL of methanol. A batch extraction was performed at room temperature (20-23^{0}C) for 24 hours. After the extraction time has elapsed, each extract was filtered through a textile filter and used for qualitative and quantitative analyzes: HPTLC, UV/VIS spectrophotometry and densitometry. Reference solutions of (+)-catechin and (-)-epicatechin were prepared with a concentration of 0.2 mg/mL in methanol for each and stored at 4°C until use.

2.4. HPTLC identification and densitometric assay

HPTLC chromatographic study was done according to literature [32-35] using the equipment CAMAG LINOMAT IV, TLC 3 Scanner and as software WINCATS Planar Chromatography Manager.

2.5. Chromatographic procedure

Plant material extracts: (17 µL – RCMe; 15 µL – HRMe; 15 µL – PSMe) and aliquots (6 µL – catechin; 6 µL – epicatechin) of reference solutions were applied separately as bands (start position – 33 mm from left side and 10 mm from the bottom, distance between bands – 6 mm, bandlength – 14 mm, delivery speed – 6 s/µL) to silica gel 60 F254 – precoated HPTLC plates, 20 x 10 cm, using Camag Linomat IV automatic sample applicator.

For all samples, the plates were developed in a saturated vertical-developing chamber at room temperature (20-22°C) for 30 minute, using as mobile phase a mixture of toluen: ethyl acetate: formic acid (12:12:2). The development distance was 7 cm. After the developing, the plates were air dried at room temperature. A visualising agent was selected based upon the class of phytoconstituents found in the preliminary phytochemical screening tests. The visualising reagent helps in visualisation as well as confirmation of the identify of the phytoconstituents. A 5% ferric chloride solution was used for spraying the plate, for visualising of condensed tannins in the extracts, followed by heating it at a temperature of 100°C for 10 minute. The processing of the chromatogram was carried out using CAMAG Reprostar 3 with digital video camera, on derivatized plate in visible light (Fig. 1). The *Rf* values of phytochemicals in plant material extracts of dog rose, buckthorn and blackthorn are presented in Table 1.

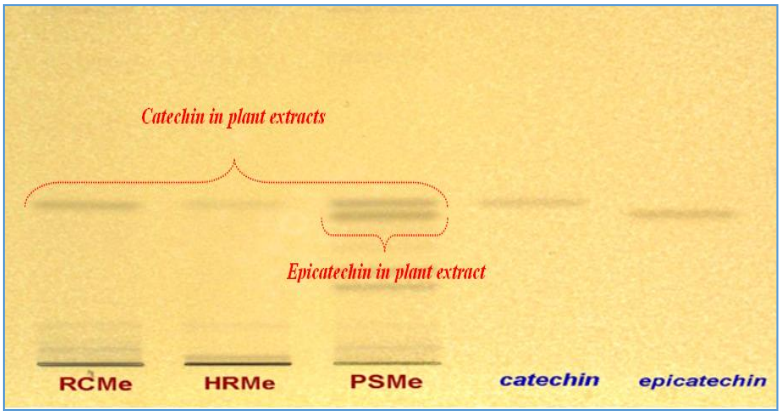
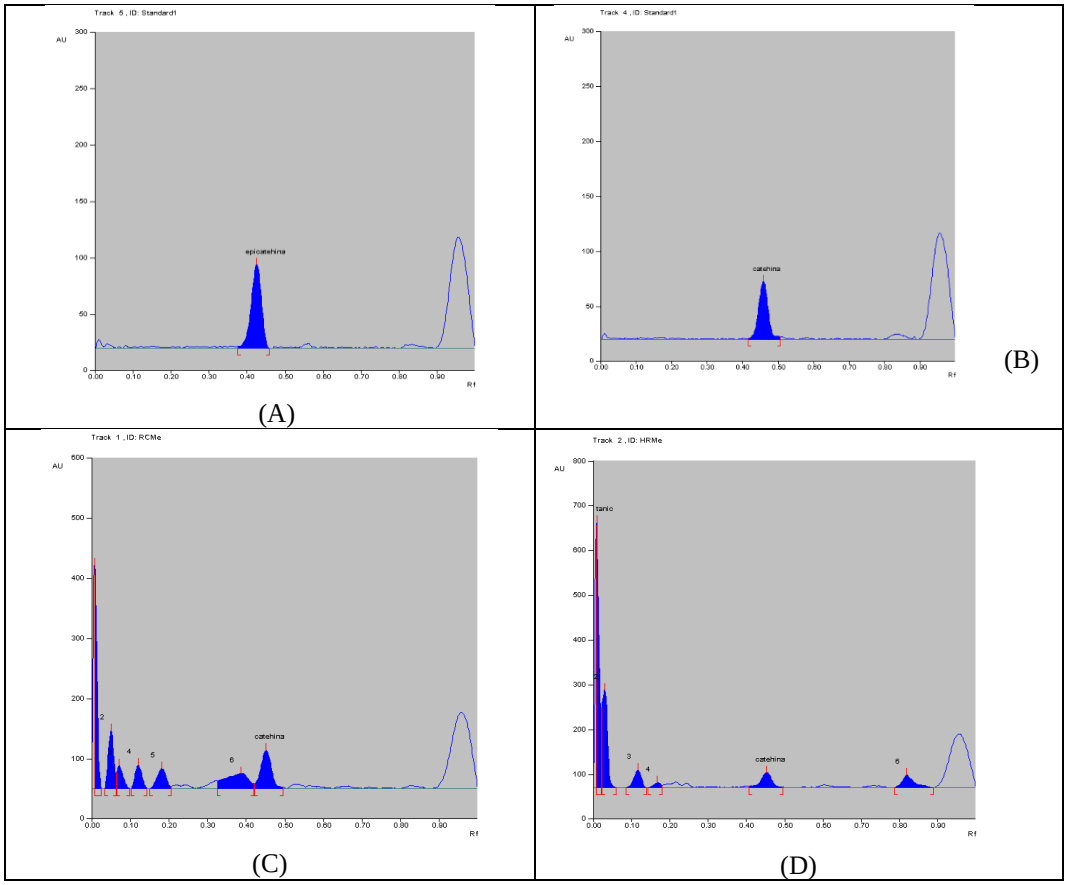


Fig. 1. HPTLC fingerprint of *Rosa canina*, *Hippophae rhamnoides* and *Prunus spinosa* branches extracts, after derivatization/visible light; Legend: RCMe – methanolic extract of *Rosa canina*; HRMe – methanolic extract of *Hippophae rhamnoides*; PSMe – methanolic extract of *Prunus spinosa.*

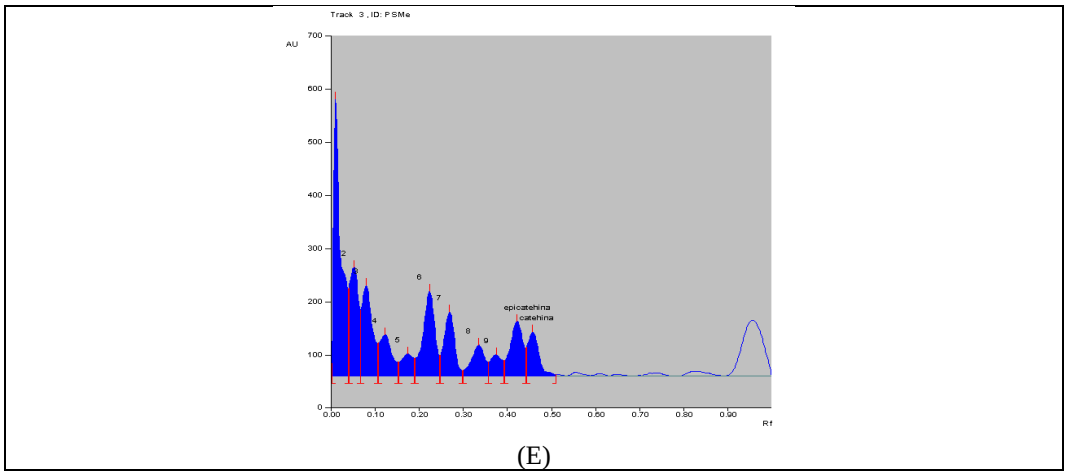
Table 1. *Rf* values of phytochemicals in plant material of *Rosa canina*, *Hippophae rhamnoides* and *Prunus spinosa* branches. Legend: RCMe – methanolic extract of *Rosa canina*; HRMe – methanolic extract of *Hippophae rhamnoides*; PSMe – methanolic extract of *Prunus spinosa*.

Image of HPTLC plate	***Plant material extract***			
	***Rf* value**	**RCMe**	**HRMe**	**PSMe**
		Spot colour		
White light, after derivatization	0.02	Black-grey	Black-grey	Black-grey
	0.43	Black-grey	Black-grey	Black-grey
	0.46	Black-grey	Black-grey	Black-grey
	0.54	Black-grey	Black-grey	Black-grey

The corresponding digital scanning profiling (Fig. 2) was carried out with a Camag TLC Scanner III fitted with WinCATS software (Camag, Switzerland), used for the densitometric measurements, spectra recording and data processing. Densitograms were recorded at the wavelength of 254 nm for condensed tannins (catechin and epicatechin). The analysis were performed in air-conditioned room maintained ar 22°C.



(A) (B) (C) (D)



(E)

Fig. 2. Digital scanning profiles of the HPTLC fingerprint chromatogram - (A) HPTLC chromatogram of standard epicatechin; (B) HPTLC chromatogram of standard catechin; (C) HPTLC chromatogram of methanolic extract from *R. canina* (RCMe);(D) HPTLC chromatogram of methanolic extract from *H. rhamnoides* (HRMe); (E) HPTLC chromatogram of methanolic extract from *P. spinosa* (PSMe).

The densitometric evaluation of catechin and epicatechin spots in samples (plant material) was carried out using a Camag TLC Scanner III. The scanner was combined with WinCATS software for the evaluation of densitometry results. Scan settings: slit dimension 12x0.4 mm, scanning speed 20 mm/s, data resolution 100 μm/step. Spectral detection was carried out between 200 and 700 nm wavelengths (Fig. 3). The retention factor (*Rf*) was calculated by the WinCats software. The percentages of catechin and epicatechin present in methanolic extracts of *R. canina*, *H. rhamnoides* and *P. spinosa* were calculated by comparison of the peak height measured for standard solution.

3. Results and discussion

3.1. Interpretation of HPTLC fingerprint chromatogram

The plant material extracts from *R. canina*, *H. rhamnoides* and *P. spinosa* were analysed by HPTLC according to the methods described in the experimental section. Methanolic extracts from branches were developed in the mobile phase of toluen: ethyl acetate: formic acid (12:12:2) and scanned under UV at 254 nm and under visible light. The presence of catechin and epicatechin was visualised as distinct black-grey bands with 5% alcoholic $FeCl_3$ as visualising agent. The HPTLC image (Fig. 1) indicates that the sample constituents (condensed tannins – catechin and epicatechin) were clearly separated. Black-grey bands corresponding to catechin (track 4) at *Rf* 0.46 and epicatechin (track 5) at *Rf* 0.43 were identified in plant material extracts (track 1 – RCMe, track 2 – HRMe, track 3 – PSMe), under specific chromatographic conditions and after derivatization in visible light.

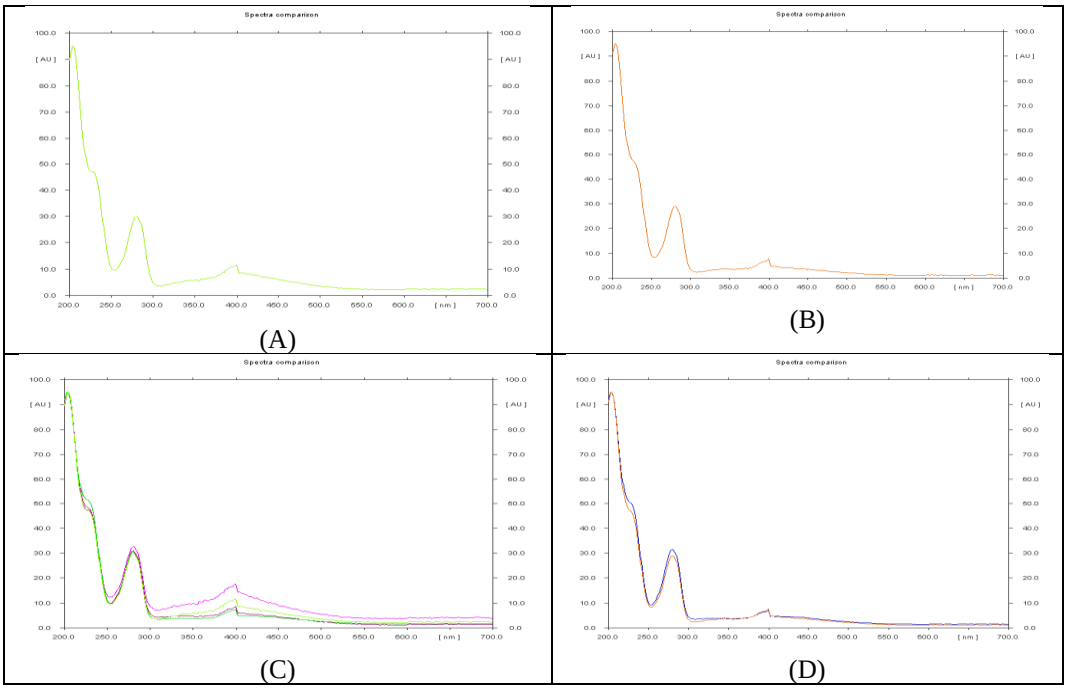


Fig. 2. Spectra of catechin and epicatechin in various extracts
(A) UV spectra of standard catechin; (B) UV spectra of standard epicatechin; (C) Spectral comparison of methanolic extracts of *R. canina* (RCMe), *H. rhamnoides* (HRMe) and *P. spinosa* (PSMe) with reference standard catechin; (D) Spectral comparison of methanolic extract of *P. spinosa* (PSMe) with reference standard epicatechin
catechin; epicatechin; RCMe; HRMe; PSMe.

Catechin bands were identified in samples of *R. canina* (RCMe), *H. rhamnoides* (HRMe) and *P. spinosa* (PSMe) according to the reference substance, while epicatechin band was identified only in *P. spinosa* (PSMe), also according to the specific reference substance.

3.2. Interpretation of digital scanning profiles of the HPTLC fingerprint chromatogram

The peak intensities of catechin and epicatechin were in accordance with those of black-grey bands. According to this, the catechin and epicatechin content in plant samples was evaluated by quantitative comparison of peak intensity (maximum peak heights). Figure 3 presents the digital scanning profile of *R. canina* (RCMe), *H. rhamnoides* (HRMe) and *P. spinosa* (PSMe) extracts at 254 nm. Catechin (at *Rf* 0.43) and epicatechin (at *Rf* 0.43) fractions in sample tracks are represented a a specific peaks with defined values of absorbance and height. The absorption spectra of the reference catechin overlaps the spectra of catechin separated from the plant material belonging to the three species. The absorption

spectra of the reference epicatechin overlaps the spectra of epicatechin separated from plant material of *P. spinosa*.

3.3. Densitometric analysis

The screening at 254 nm has as result the quantitative determination of the biomarker catechin in the samples of the three species. The calculated quantities of catechin and epicatechin in reference solutions and plant extracts were done based on the peak hight using the WinCats software. The HPTLC densitometry analysis reveal the amount of catechin in branches of *R. canina* (0.17% g/g), *H. rhamnoides* (0.10% g/g) and *P. spinosa* (0.25% g/g) and the amount of epicatechin in branches of *P. spinosa* (0.22% g/g) (Fig. 2, Table 2).

Table 2. The results of densitometric determination of catechin (C) (in samples RCMe – *R. canina* methanolic extract, HRMe – *H. rhamnoides* methanolic extract, PSMe – *P. spinosa* methanolic extract) and epicatechin (EC) (in sample PSMe - *P. spinosa* methanolic extract)

Sample	***Calculated quantity based on peak height, µg***		***Quantity of the compound in extracts solution , mg/100 ml***		***Concentration (%) in dried plant material***	
	C	***EC***	***C***	***EC***	***C***	***EC***
Reference solution	1.2	1.2	-	-	-	-
RCMe	1.454	-	8.55	-	***0.17***	-
HRMe	0.773	-	5.1	-	***0.1***	-
PSMe	1.878	1.659	12.5	11.06	***0.25***	***0.22***

Conclusions

The qualitative study of phytochemicals by HPTLC analyses evidenced that all three vegetal species *R. canina, H. rhamnoides* and *P. spinosa* contain condensed tannins. The chromatographic identification of condensed tannins, in visible light indicated the presence of black-grey bands with different intesities of colour, corresponding to catechin (at *Rf* 0.46) and epicatechin (at *Rf* 0.43).

The quantitative analysis by HPTLC densitometry highlighted the content in condensed tannins (catechin and epicatechin) in branches of *R. canina, H. rhamnoides* and *P. spinosa*. Thus, catechin is present in high concentration in the dried material of *P. spinosa* (0.22% g/g), followed by *R. canina* (0.17% g/g) and *H. rhamnoides* (0.10% g/g). Epicatechin is present only in dried branches of *P. spinosa* (0.22% g/g). These results open perspectives for advanced valorization of the branches of *R. canina, H. rhamnoides* and *P. spinosa* species, bioresources known only for phytotherapeutical and nutritional potential of their fruits.

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