

# Studies on Polyphenols Isolated from Branches of *Prunus spinosa* L. Species

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*The aim of this study was to investigate the crude extracts isolated from branches of Prunus spinosa L. (Rosaceae), in order to quantify polyphenols and tannins using instrumental methods (UV-Vis spectrometry and HPTLC densitometry). The qualitative analysis on biomass revealed the presence of valuable compounds such as polyphenols, flavonoids and tannins. HPTLC fingerprinting of crude methanol extract showed several peaks, with different R<sub>f</sub> values, corresponding to phytochemicals such as chlorogenic acid (R<sub>f</sub> 0.52), neochlorogenic acid (R<sub>f</sub> 0.58), caffeic acid (R<sub>f</sub> 0.95) and protocatechuic acid (R<sub>f</sub> 0.96). The quantitative analysis revealed that the branches of P. spinosa contain polyphenols (2.97±0.059% gallic acid equivalent/g dried vegetal material) and tannins (0.90±0.033% gallic acid equivalent/g dried vegetal material). The HPTLC densitometry analysis offers information about the amount of neochlorogenic acid in the vegetal material 0.12% g/g. The content in polyphenols and tannins suggests the possibilities to use this species to separate compounds with antioxidant potential. These results open perspectives for complex valorization of Prunus spinosa species, a bioresource known only for phytotherapeutic and nutritional potential of their fruits.*

*Keywords: blackthorn, polyphenols, phytochemical identification, HPTLC, UV-Vis, FTIR spectroscopy*

Polyphenols are secondary metabolites synthesized in all parts of plants: bark, leaves, stem, root, flower, fruits and seeds. Each matrix has a different polyphenol composition and concentration [1,2]. *Prunus spinosa* L. (blackthorn or sloe), which belongs to the rose family (*Rosaceae*) is a shrub widespread in the temperate regions. Different flavonoids have been isolated from the leaves of *P. spinosa* such as kaempferol, quercetin, their 3-O- $\alpha$ -L-arabinofuranosides, kaempferol 3- and 7-O- $\alpha$ -L-rhamnopyranosides [3]. The blackthorn constituents, such as flavonoid pentosides (arabinosides, xylosides, rhamnosides) and A-type procyanidin dimers with twice-bonded structures are quite rare in nature [4]. This unique composition suggests a distinctive profile of *P. spinosa*. The number of studies investigating the phytotherapeutic potential of branches of *P. spinosa* has grown in recent years, but there are still limited. Special attention has been given to the antioxidant activity of the extract from the branches, leaves and fruits, as one of the possible action mechanism of the blackthorn polyphenols [5, 6]. Thus, the chemical characterization of branches and bark is important for evaluating the entire potentiality of its use. This study was focused mainly on the bioactive compounds from branches of *P. spinosa* species, in order to highlight new sources of biomass containing valuable polyphenols. The aim of this study was to investigate the polyphenols composition of branches of *P. spinosa* in order to identify and quantify some bioactive compounds with antioxidant potential. The identification of the main constituents was done applying the phytochemical study and using High Performance Thin Layer Chromatography (HPTLC). To have a complete qualitative confirmation of polyphenolic and flavonoid composition the UV-Vis and FT-IR spectroscopy were used. The chemical compounds quantification was done using densitometry and UV-Vis spectroscopy.

## Experimental part

### Materials and methods

**Plant material:** Branches of *P. spinosa* were collected from a temperate continental climate area (Siret Valley, Bacau county, Romania), in September 2016. The biomass 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 particles with 0.8 mm medium size. The sample of *P. spinosa* (PS) was stored in a clean desiccator until were used for phytochemical study and HPTLC, UV-Vis, FT-IR evaluation. The weight loss during drying was measured with an infrared KERN MLS Thermobalance.

**Samples preparation:** 5g of vegetal material (PS) were dispersed separately in 100 mL of different solvents such as: methanol, ethanol 30% v/v, ethanol 50% v/v, and ethanol 70% v/v. A batch extraction was performed at room temperature 20-23°C, for 24 h. Then the extracts were filtered and used for phytochemical study. The obtained methanolic extract (PSMe) was used also for UV-Vis and HPTLC evaluation. 1 g of vegetal material (PS) as such was used for FT-IR investigation.

### Chemicals and Reagents

All the standards were of analytical grade or pure and the following were used: chlorogenic acid (min. 95% HPLC from Sigma-Aldrich, Switzerland), hyperoside (min. 97% HPLC, from Sigma-Aldrich, Switzerland), caffeic acid (min. 95% HPLC from Sigma-Aldrich, Switzerland), neochlorogenic acid (min. 98% HPLC, from Sigma-Aldrich, Switzerland), ferulic acid (min. 99% HPLC, from Sigma-Aldrich, Switzerland), rutin hydrate (min. 95% HPLC from Sigma-Aldrich, Switzerland), quercetin (min. 95% HPLC from Sigma-Aldrich, Switzerland), protocatechuic acid (analytical standard from Fluka, Switzerland), kaempferol

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(analytical standard from Fluka, Switzerland). All the reagents were of analytical grade or pure: ethyl acetate, min. 99.5% (Sigma-Aldrich), formic acid p.a. (Merck Germany), acetic acid p.a. (99%, Silal Trading, Romania), methanol p.a., min. 99.8% (Chempur Poland), polyethylene glycol 400 (PEG 400) (Ph. Eur, Fluka, Sigma-Aldrich, Switzerland), diphenylboric acid aminoethyl ester (min. 97%, Fluka, Switzerland). HPTLC plates G60 F254, 200x100mm (Merck, Darmstadt, Germany) were used as stationary phase for HPTLC identification and HPTLC densitometric assay.

### Qualitative analysis

**Phytochemical study:** Characterization of *P. spinosa* extracts for various phytochemical constituents was carried out using standard methods [7] as described in table 1.

**UV-Vis and FT-IR characterization:** Molecular absorption spectrophotometry in ultraviolet/visible light (UV/VIS) is an analytical method based on the property of an ion or molecular species to absorb at certain wavelengths the UV/VIS radiation. Spectroscopic analysis was performed as follows: UV-Vis absorption spectra of extract was recorded using a Cary 50 UV-Visible Varian spectrophotometer, in the wavelength range between 200-800 nm. The Fourier transform infrared spectrophotometer Cary 630 FT-IR Agilent was used to obtain the FT-IR spectra for the dried vegetal material in order to establish functional groups of the main bioactive compounds in the sample. FT-IR spectrum was recorded in 400-4000  $\text{cm}^{-1}$  range.

**HPTLC identification and densitometric assay:** HPTLC chromatographic study was done according to literature

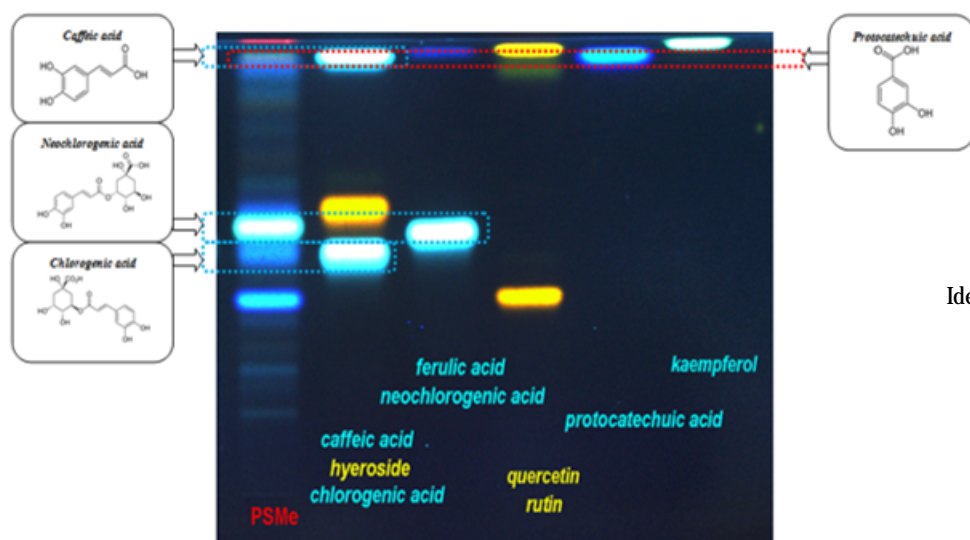
[8-10] using the equipment CAMAG LINOMAT IV, TLC 3 Scanner and as software WINCATS Planar Chromatography Manager.

Reference solutions: S1 (chlorogenic acid, hyperoside, caffeic acid), S2 (neochlorogenic acid, ferulic acid), S3 (rutin, quercetin), S4 (protocatechuic acid) and S5 (kaempferol) were prepared with a concentration of 0.2 mg/mL in methanol for each and stored at 4°C until use.

**Chromatographic procedure:** Methanolic extract (13  $\mu\text{L}$  - PSMe) and aliquots (5  $\mu\text{L}$  - S1; 5  $\mu\text{L}$  - S2; 6  $\mu\text{L}$  - S3; 6  $\mu\text{L}$  - S4; 7  $\mu\text{L}$  - S5) of reference solutions were applied separately as bands (start position - 31 mm from left side and 10 mm from the bottom, distance between bands - 6 mm, bandlength - 12 mm, delivery speed - 8  $\mu\text{L}/\text{s}$ ) to silica gel 60 F254 - pre-coated HPTLC plates, 20 x 10 cm, using Camag Linomat IV automatic sample applicator. For the samples, the plates were developed in a saturated vertical-developing chamber at room temperature (20-22°C) for 30 min, using as mobile phase a mixture of ethyl acetate: formic acid: acetic acid: distilled water (20:2:2:2:5,4). 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 detection as well as confirmation of the identity of the phytoconstituents. A 1% diphenylboric acid 2-aminoethyl ester methanolic solution and a 5% polyethylene glycol 400 ethanolic solution were used for spraying the plate, for visualising of polyphenols and flavonoids in the extract, followed by heating it at a temperature of 100°C for 10 min. The processing of the chromatogram was carried out

Phytoconstituents	Test	Observation
<b>Phenols</b>		
Folin-Ciocalteu Test	2mL extract+0.5mL Folin-Ciocalteu reagent+2mL 20% sodium carbonate	Blue coloration
Ferric chloride Test	2 mL extract+1mL 3% ferric chloride	Olive-brown coloration
Arnou Test	2.5mL extract+2.5mL hydrochloric acid 0.5N+2.5mL Arnou reagent+2.5mL sodium hydroxide	Red-brick coloration
<b>Tannins</b>		
The ferric chloride reaction	1mL extract+2mL distilled water+2-3 drops 1% ferric chloride	Blue-black coloration - gallic tannins Green-black coloration - condensed tannins
<b>Flavonoids</b>		
Aluminium chloride Test	2mL extract+5mL 10% sodium acetate+3mL 2.5% aluminium chloride	Yellow coloration
The Shibata Test	3mL extract evaporated +2mL 50% methanol+2-3pieces magnesium span+10 drops concentrated hydrochloric acid	Red coloration - flavonols Orange coloration - flavones

**Table 1**  
PRELIMINARY  
PHOTOCHEMICAL  
TESTS FOR  
PLANT EXTRACTS



**Fig.1.** Polyphenols and Flavonoids  
Identification Chromatogram by HPTLC

**Table 2**  
Rf VALUES OF PHYTOCHEMICALS IN *P. SPINOSA* METHANOLIC EXTRACT (PSMe)

Image of HPTLC plate	Rf value	Reference standard	Spot colour	
			Reference standard	<i>P. spinosa</i> extract (PSMe)
after derivatization	0.43	Rutin	Yellow band	-
	0.52	Chlorogenic acid	Blue fluorescent band	Blue fluorescent band (diffuse)
	0.58	Neochlorogenic acid	Blue fluorescent band	Blue fluorescent band (intense)
	0.62	hyperoside	Yellow band	-
	0.95	Caffeic acid	Blue fluorescent band	Blue fluorescent band (low intensity)
	0.96	Protocatechuic acid	Blue fluorescent band	Blue fluorescent band (low intensity)
	0.97	Ferulic acid	Blue band	-
	0.97	Quercetin	Yellow band	-
0.98	Kaempferol	Blue fluorescent band	-	

using CAMAG Reprostar 3 with digital video camera, on derivatized plate at 366 nm (Fig. 1). The *Rf* values of phytochemicals in *P. spinosa* extract (PSMe) are presented in table 2. 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 254 nm wavelength for polyphenols (neochlorogenic acid). The analysis were performed in air-conditioned room maintained at 22°C.

The densitometric evaluation of neochlorogenic acid spot in sample (PSMe) 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 percentage of neochlorogenic acid present in methanolic extracts of *P. spinosa* was calculated by comparison of the peak height measured for standard solution (Table 3).

#### Quantitative analysis

**Determination of total phenolic content:** The total phenolic concentration was determined according to Folin-

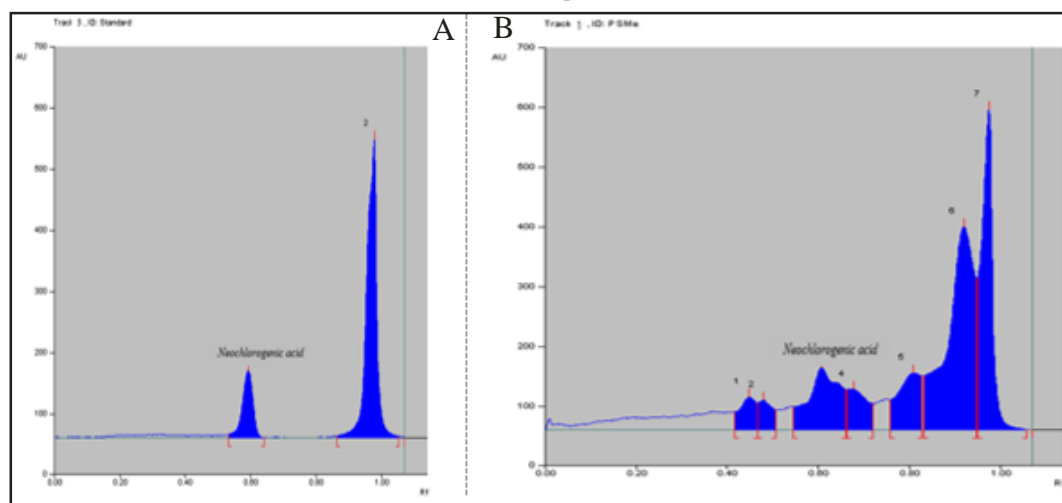


Fig. 2. Digital scanning profiles of the HPTLC fingerprint chromatogram (A) HPTLC chromatogram of reference standard neochlorogenic acid Legend: 2 - unknown polyphenol; (B) HPTLC chromatogram of *P. spinosa* methanolic extract (PSMe) Legend: 1, 2, 4, 5, 6, 7 - unknown polyphenols

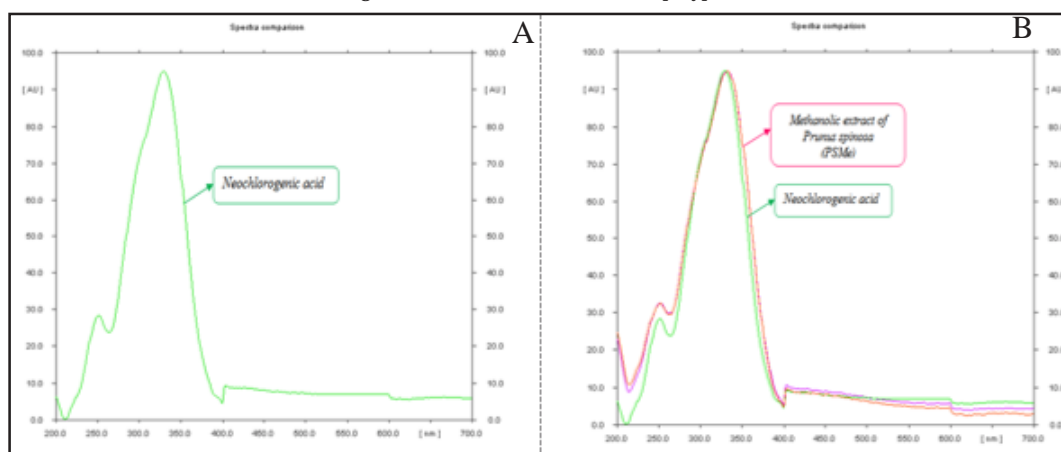


Fig. 3. Spectra of neochlorogenic acid in reference standard and *P. spinosa* methanolic extract (PSMe) (A) UV spectra of neochlorogenic acid as reference; (B) Spectral comparison of methanolic extract *P. spinosa* (PSMe) with reference standard neochlorogenic acid

Table 3

THE RESULTS OF DENSITOMETRIC DETERMINATION OF NEOCHLOROGENIC ACID IN *P. SPINOSA* METHANOLIC EXTRACT (PSMe)

Sample	Applied volume $\mu\text{L}$	Calculated quantity based on peak height, ng	Quantity of the compound in methanolic extract, mg/100 ml	Concentration (Neochlorogenic acid) (%)
				in dried vegetal material
Reference standard solution (neochlorogenic acid)	4	-	-	-
PSMe	13	758.65	5.8	0.12

Ciocalteu procedure [11], using Folin-Ciocalteu reagent. The same procedure was performed for the gallic acid standard curve. The results were expressed in mg of gallic acid equivalent (GAE) per gram of dried vegetal material (% g GAE/g) taking into account the sample dilution. The analyses were performed in triplicates.

**Determination of total tannins content:** The total tannins compound was determined according to Folin-Denis method [12], using Folin-Denis reagent. The absorbance was measured at 726 nm using a spectrophotometer (UV-Vis CARY50). A standard calibration curve was obtained for the tannic acid following the same procedure. The results were expressed in grams of tannic acid equivalent per gram of dried vegetal material (%g/g), taking into account the sample dilution. The analyses were performed in triplicates.

### Results and discussions

The chemical composition of branches of *P. spinosa* was less studied and this is the reason why the following chemical and instrumental methods of identification were used in order to confirm the presence of interesting polyphenolic compounds.

**Qualitative analysis:** The results of phytochemical study are presented in table 4. These results revealed that the various alcoholic and aqueous extracts of branches of *P. spinosa* contain polyphenols, tannins and flavonoids. The

phenols, tannins and flavonoids were detected in all type of extracts obtained from the vegetal species, with high, moderate and low intensity.

Phenolic compounds were detected in all type of extracts, with high and moderate intensity. The ferric chloride test showed a dark-green coloration, which has indicated the tannins presence in all extracts, with high intensity in those obtained with 70% ethanol. Flavonoids were detected in all type of extracts, with moderate content in methanolic extract. Thus, 50%v/v and 70%v/v ethanol were the most efficient extraction solvents.

UV-VIS analysis of phenolic compounds was performed on methanolic extract (PSMe) and the spectra is presented in figure 4. Depending on the specific absorption, flavonoids were identified at wavelength between 250-260 nm and condensed tannins in the range of 270-290 nm. The results proved that methanolic extract showed maximum absorption peaks for flavonoids such as rutin and quercetin at 255 nm and for condensed tannins such as catechin at 280 nm.

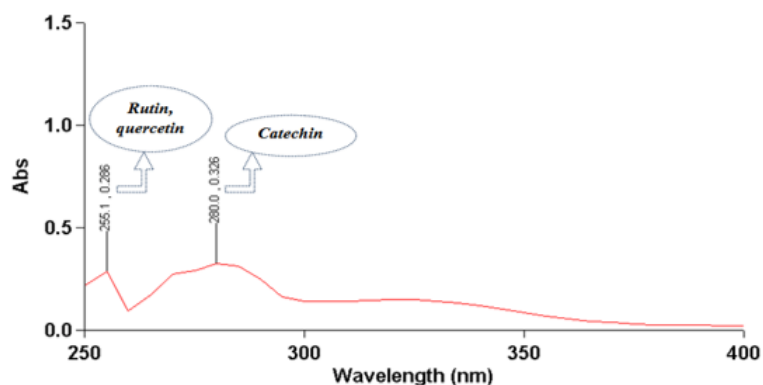
In the FT-IR spectra (Fig. 5) for the powdered vegetal material (*P. spinosa* - branches) there are some wavelengths which can be attributed to OH group at 3279  $\text{cm}^{-1}$  and to C-H stretch at 2903  $\text{cm}^{-1}$ . Specific absorption bands corresponding to stretching vibrations of the C-C

Table 4

QUALITATIVE PHYTOCHEMICAL ANALYSIS OF VARIOUS ALCOHOLIC AND AQUEOUS EXTRACTS OF *P. SPINOSA* BRANCHES.

Plant constituents/ Solvents		<i>Prunus spinosa</i> branches (PS)				
		Methanol (PSMe)	Water	Ethanol 70% w/v	Ethanol 50% w/v	Ethanol 30% w/v
Phenolic compounds	The Folin- Ciocalteu test	++	++	+++	++	++
	The ferric chloride test	++	+	+++	+++	++
	Arnow test	++	++	+++	+++	+++
Tannins	The ferric chloride test	++	+	+++	++	+
Flavonoids	The aluminium chloride test	++	+	+	+	+
	The Shibata test	+	+	+	++	++

Legend: +++: highly present, ++: moderately present, +: low present

Fig. 4. UV-Vis absorption spectra of *P. spinosa* methanolic extract (PSMe)

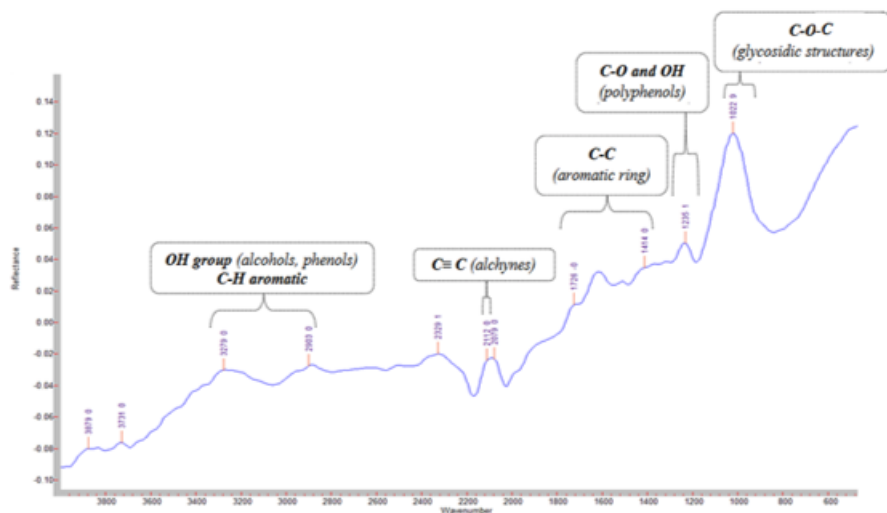


Fig. 5. FT-IR spectra of powdered vegetal material (branches) of *P. spinosa*

Parameters	<i>Prunus spinosa</i> (branches)
Weight loss of drying (%)	8.35±0.56
Total phenolic content (as gallic acid) (%g GAE/g)	2.97±0.059
Total tannins content (as tannic acid) (%g/g)	0.90±0.033

**Table 5**  
DETERMINATIONS OF THE MAIN CONSTITUENTS FROM *P. SPINOSA* BRANCHES

(aromatic ring) can be observed at  $1726\text{ cm}^{-1}$  and  $1414\text{ cm}^{-1}$ . Peaks at  $1235\text{ cm}^{-1}$  can be assigned to C-O and OH groups from polyphenols and at  $1022\text{ cm}^{-1}$  corresponding to C-O bond from glycosidic structures. FT-IR spectra of powdered vegetal material (branches) of *Prunus spinosa* is presented in figure 5.

**Interpretation of HPTLC fingerprint chromatogram:** The extract from *P. spinosa* (PSMe) was analysed by HPTLC according to the methods described in the experimental section. Methanolic extract was scanned under UV at 254 nm and 366 nm. The presence of polyphenols was detected as distinct blue fluorescent bands and the presence of flavonoids was visualised as distinct yellow bands. The HPTLC image (Fig. 1) indicates that the sample constituents were clearly separated. Blue fluorescent bands corresponding to chlorogenic acid ( $R_f$  0.52), neochlorogenic acid ( $R_f$  0.58), caffeic acid ( $R_f$  0.95) and protocatechuic acid ( $R_f$  0.96) were identified in methanolic extract (track 1 - PSMe), under specific chromatographic conditions and after derivatization at 366 nm.

**Interpretation of digital scanning profiles of the HPTLC fingerprint chromatogram:** The peak intensity of neochlorogenic acid was in accordance with those of blue fluorescent band. According to this, the neochlorogenic content in sample can be evaluated by quantitative comparison of peak intensity (maximum peak heights).

Figure 2 presents the digital scanning profile of *P. spinosa* methanolic extract (PSMe) at 254 nm. Neochlorogenic acid (at  $R_f$  0.58) fraction in extract track is represented as a specific peak with defined value of absorbance and height. The absorption spectra of the reference standard neochlorogenic acid overlaps the spectra of neochlorogenic acid separated from the vegetal material *P. spinosa* branches (fig. 3B).

**Densitometric analysis:** The screening at 254 nm has as result the quantitative determination of the phytochemical neochlorogenic acid in the *P. spinosa* methanolic extract. The calculated quantity of neochlorogenic acid in reference standard solution and extract was done based on the peak height using the WinCats software. The HPTLC densitometry analysis revealed the amount of neochlorogenic acid in extracts from branches of *P. spinosa* as 0.12% g/g reported at dried

vegetal material (fig 2, table 3). The HPTLC profile and the recorded  $R_f$  values could serve for the scientists in the research activity developed in the field of medicinal plants.

**Quantitative analysis:** In this work, the total polyphenolic content in branches of *P. spinosa* was established. The vegetal material (branches) has 8.35 % weight loss of drying. The obtained results showed a content in polyphenols ( $2.97\pm 0.059\%$  gallic acid equivalent/g) and tannins ( $0.90\pm 0.033\%$  tannic acid equivalent/g) (table 5).

## Conclusions

This study presents the results of investigating polyphenols and flavonoids separated from *P. spinosa* branches using different extraction solvents and applying various qualitative and quantitative methods. Phenols, flavonoids and tannins were evidenced using specific phytochemical tests. The flavonoids such as rutin and quercetin (maximum absorption peak at 255nm) and condensed tannins such as catechin (maximum absorption peak at 280nm) were detected using UV-VIS spectroscopy. Intense absorption bands corresponding to stretching vibration of functional groups specific to polyphenols and flavonoids (OH group  $3279\text{ cm}^{-1}$ , C-H aromatic  $2903\text{ cm}^{-1}$ , C-C from aromatic ring  $1726\text{ cm}^{-1}$ , C-O and OH group from polyphenols  $1235\text{ cm}^{-1}$ ) were detected using FT-IR spectroscopy. UV-VIS and FT-IR spectroscopy were applied to complete the qualitative confirmation of polyphenolic and flavonoid compounds in branches of *P. spinosa*. Phytochemicals such as chlorogenic acid ( $R_f$  0.52), neochlorogenic acid ( $R_f$  0.58), caffeic acid ( $R_f$  0.95) and protocatechuic acid ( $R_f$  0.96) were HPTLC fingerprinted from crude methanol extract. The quantitative analysis revealed the branches of *P. spinosa* contain polyphenols ( $2.97\pm 0.059\%$  gallic acid equivalent/g dried vegetal material), tannins ( $0.90\pm 0.033\%$  tannic acid equivalent/g dried vegetal material) and neochlorogenic acid  $0.12\%$  g/g dried vegetal material (determined by HPTLC densitometry). The obtained results revealed that *P. spinosa* species is an important source of biomass, rich in polyphenols and flavonoids, which can be exploited in order to obtain phytochemicals with biological activity.

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