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

OANA T. CIUPERCA1,2, CARMEN E. TEBRENCU1,2, ELENA IONESCU1,2, ELENA IACOB2, IRINA VOLF1*

1Gheorghe Asachi Technical University, Faculty of Chemical Engineering and Environmental Protection, 73 Dimitrie Mangeron Blvd., 700050, Iasi, Romania
2Research and Processing Centre for Medicinal Plants PLANTAVOREL S.A., 46 Cuza Voda, 610019, Piatra Neamt, Romania
3Academy of Romanian Scientists, 54 Splaiul Independentei, 050094, Bucharest, Romania

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 RF values, corresponding to phytoconstituents such as chlorogenic acid (RF 0.52), neochlorogenic acid (RF 0.58), caffeic acid (RF 0.95) and protocatechuic acid (RF 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% tannic 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. 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 phytotherapeutical 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-α-L-arabinofuranosides, kaempferol 3- and 7-O-α-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 mechanisms 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 Micronot 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 (PSMe) 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

*email: iwolf@tuiasi.ro
(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 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 (kaempherol) 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 (13µL - PSMe) and aliquots (5µL - S1; 5µL - S2; 6µL - S3; 6µL - S4; 7µL - 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, band length - 12 mm, delivery speed - 8 µL/s) to silica gel 60 F254 - precoated 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 (20:2,2,2,2,5,4). 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 phyto-constituents found in the preliminary phytochemical screening tests. The visualising reagent helps in detection as well as confirmation of the identity of the phyto-constituents. 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 |
|------------------|------|------------|
| Phenols          |      |            |
| Folin-Ciocalteu  | 2mL  | Blue coloration |
| extract + 0.5mL  |      |             |
| 20% sodium carbonate |      |             |
| Folin-Ciocalteu reactant + 2mL |      |             |
| Ferric chloride | 2mL  | Olive brown coloration |
| extract + 1mL 5% ferric chloride |      |             |
| Arrow Test       | 2.3mL| Red brick coloration |
| extract + 2.3mL hydrochloric acid 0.5N + 2.3mL |      |             |
| Arrow reactant + 2.3mL sodium hydroxide |      |             |
| Tannins          |      |            |
| Ferric chloride reaction | 1mL | Blue-black coloration – gallic tannins |
| extract + 2mL distilled water + 2 drops 1% ferric chloride |      | Green black coloration – condensed tannins |
| Flavonoids       |      |            |
| Aluminum chloride | 2mL | Yellow coloration |
| extract + 5mL 10% sodium acetate + 3mL 2.5% aluminum chloride |      |             |
| The Shiba test   | 3mL  | Red coloration – flavonols |
| extract evaporated + 2mL 50% methanol + 2-3 drops magnesium solution + 10 drops concentrated hydrochloric acid |      | Orange coloration – flavonols |

Table 1 PRELIMINARY PHOTOCHEMICAL TESTS FOR PLANT EXTRACTS

Fig.1. Polyphenols and Flavonoids
Identification Chromatogram by HPTLC
using CAMAG Reprostar 3 with digital video camera, on derivatized plate at 366 nm (Fig. 1). The $R_f$ 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 ($R_f$) 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-

---

### Table 2

| Image of HPTLC plate | $R_f$ value | Reference standard | Spot colour |
|-----------------------|-------------|--------------------|-------------|
| after derivatization  | 0.43        | Rutin              | Yellow band |
|                       | 0.52        | Chlorogenic acid   | Blue fluorescent band |
|                       | 0.58        | Neochlorogenic acid| Blue fluorescent band |
|                       | 0.62        | Hyperoside         | Blue fluorescent band (intense) |
|                       | 0.95        | Caffeic acid       | Yellow band |
|                       | 0.96        | Protopaucaric acid | Blue fluorescent band (low intensity) |
|                       | 0.97        | Ferulic acid       | Blue band   |
|                       | 0.97        | Quercetin          | Yellow band |
|                       | 0.98        | Kaempherol         | Blue fluorescent band |

---

**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**
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 methanol 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 cm⁻¹ and to C-H strech at 2903 cm⁻¹. Specific absorption bands corresponding to stretching vibrations of the C-C

| Sample | Applied volume μL | Calculated quantity based on peak height, ng | Quantity of the compound in methanolic extract, mg/100 ml | Concentration (Neochlorogenic acid) (%) |
|--------|-------------------|---------------------------------------------|--------------------------------------------------------|----------------------------------------|
| Reference standard solution (neochlorogenic acid) | 4 | - | - | - |
| PSMe | 13 | 758.65 | 5.8 | 0.12 |

Table 3

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

Table 4

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

| Plant constituents/Solvents | *Punica spinosa* branches (PS) | Methanol (PSMe) | Water | Ethanol 76% v/v | Ethanol 56% v/v | Ethanol 39% v/v |
|----------------------------|--------------------------------|----------------|-------|----------------|----------------|----------------|
| Phenolic compounds         | *The Folin-Ciocalteu test*     | ++             | ++    | +++            | ++             | ++             |
|                            | *The ferric chloride test*     | ++             | +     | +++            | +++            | ++             |
|                            | *The arnon test*               | ++             | ++    | +++            | +++            | ++             |
| Tannins                    | *The ferric chloride test*     | ++             | +     | +++            | +              | ++             |
|                            | *The aluminium chloride test*  | ++             | +     | +              | ++             | ++             |
|                            | *The Sishata test*             | +              | +     | +              | ++             | ++             |

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

Fig. 4. UV-Vis absorption spectra of *P. spinosa* methanolic extract (PSMe)
(aromatic ring) can be observed at 1726 cm$^{-1}$ and 1414 cm$^{-1}$. Peaks at 1235 cm$^{-1}$ can be assigned to C-O and OH groups from polyphenols and at 1022 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 (Rf 0.52), neochlorogenic acid (Rf 0.58), caffeic acid (Rf 0.95) and protocatechuic acid (Rf 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 Rf 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 phytocompound 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 Rf 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±0.059%g gallic acid equivalent/g) and tannins (0.90±0.033%g 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 cm$^{-1}$, C-H aromatic 2903 cm$^{-1}$, C-C from aromatic ring 1726 cm$^{-1}$, C-O and OH group from polyphenols 1235 cm$^{-1}$) were detected using FT-IR spectroscopy. UV-VIS and FT-IR spectroscopy were applied to complete the qualitative confirmation of polyphenolic and flavonoidic compounds in branches of P. spinosa. Phytocompounds such as chlorogenic acid (Rf 0.52), neochlorogenic acid (Rf 0.58), caffeic acid (Rf 0.95) and protocatechuic acid (Rf 0.96) were HPTLC fingerprinted from crude methanol extract. The quantitative analysis revealed the branches of P. spinosa contain polyphenols (2.97±0.059%g gallic acid equivalent/g dried vegetal material), tannins (0.90±0.033%g 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 phytocompounds with biological activity.
References
1. BUJOR, O.C., LE BOURVELLEC, C., VOLF, I., POPA, V.I., DUFOUR, C., Food chem., 213, 2016, p. 58-68.
2. VOLF, I., POPA, V.I., Vitis, 2004, p. 707-710.
3. OLSZEW SKA, M., AND WOLBI’ S, M., Acta Pol. Pharm., 59, 2002a, p. 133-137.
4. PINACHO, R, CAVERO, R.Y., ASTIASARAN, I., ANSORENA, D., CALVO, M.I., J Funct Foods, 19, 2015, p. 49-62
5. BARROS, L., CARVALHO, A. M., MORAIS, J. S., FERREIRA, I. C. F. R., Food Chem., 120, 2010, p. 247-254, doi: 10.1016/j.foodchem.2009.10.016
6. IGNAT, I., VOLF, I., POPA, V.I., Food chem., 126 (6), 2011, p. 1821 - 1835.
7. CIULEI, I., GRIGORESCU, E., STANESCU, U., Plante medicinale, fitochimie și fitoterapie - tratat de farmacognozie, 1. Edit. Medicala, București, 1993, p. 226-237
8. DHARMENDER, R., MADHAVI, T., REENA, A., SHEETAL., A., Pharm. Anal. Acta., 1, 2010, p. 1-9.
9. TEBRENCU, C.E., CRETU, R.M., MITROI, G.R., IACOB, E., IONESCU, I., Phytochem. Rev, 2015; DOI 10.1007/s11101-015-9410-8.
10. STEFANACHE, C.P., PETER, S., MEIER, B., DANILA, D., TANASE, C., WOLFRAM, E., Rev. Chim. (Bucharest), 66, no. 5, 2015, p. 784-787.
11. SINGLETON, VL, ROSSI, J., Am J Enol Viticult, 16, 1965, p. 144-158.
12. KATCOH, R., Analytical techniques in biochemistry and molecular biology, Springer, 2011, p. 301-303.

Manuscript received: 17.07.2017