

Contributions on Spruce Bark Polyphenols Identification Using Instrumental (UV-VIS Spectrometry), Qualitative (Thin Layer Chromatography) and Quantitative (HPTLC Densitometry) Methods

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This work assessed the qualitative and quantitative polyphenols profile in a crude ethanol extract separated from spruce bark (two fractions with <0.25 and 1 mm diameter) using ultrasound assisted extraction (UAE) as a green extraction method. The evaluation of phenolic acids and condensed tannins profile was performed using instrumental (UV-VIS spectrometry), qualitative (Thin Layer Chromatography, TLC) and quantitative (HPTLC densitometry) methods. Using ultrasound assisted extraction (UAE) technique under specific parameters, the higher total phenolic content (TPC) was 29.785 mg GAE/g¹ for spruce bark fraction with particle size <0.25 mm and 14.448 mg GAE/g¹ for fraction with 1 mm diameters. The TLC assay of the crude ethanol extract was performed considering the standards: gallic, sinapic, p-coumaric and vanillic acids, catechin, epicatechin and tannic acid. Smaller material particle sizes lead to higher yield of polyphenols in ethanol extract. The quantitative evaluation by HPTLC densitometry revealed following amounts: sinapic acid 0.84 mg/g, p-coumaric acid 0.61 mg/g, catechin 1.03 mg/g, tannic acid 2.81 mg/g. Considering the chemical composition, the spread and availability, spruce bark could be considered as a resource with environmental and economic benefits.

Keywords: spruce bark, ultrasounds assisted extraction, polyphenols, phytochemical screening, HPTLC (High Performance Thin Layer Chromatography).

The aim of biorefining is a complete and sustainable use of biomass with minimal energy and processing costs. From biomass conversion fine chemicals, platform molecules and added-value co-products could be obtained in a sustainable approach [1]. Phenolic compounds, one of the most widely occurring groups of phytochemicals, are of considerable physiological and morphological importance in plants [2]. Moreover, polyphenols present important bioactive properties for human health, reported in treatment of vascular, pulmonary, neurological diseases, having antitumor, anti-allergenic, anti-inflammatory, anti-microbial, antioxidant and cardioprotective effects [3, -6].

The bark, resulted as a waste from wood processing industry is a renewable raw material [7], considered as a source of polyphenols. Currently, more than half of the bark is burned and/or landfilled and the remainder is mainly used as a source of energy [8]. Its valorisation is a key of economic lignocellulosic biorefinery [9, 10].

In recent years new techniques for bioactive compounds extraction have become efficient (quick and selective extraction with less amount of solvent) cleaner (where products are devoid of organic solvents) and cheaper [11]. Ultrasound assisted extraction (UAE) is a non-conventional extraction technique, less expensive and a simple efficient alternative [12] to extract phenolics from biomass [13].

In this paper the results of a qualitative and quantitative polyphenols profile, separated from spruce bark (the fractions of a mean particle diameter of <0.25 and 1 mm diameter) using a green extraction method (ultrasound assisted extraction) are presented.

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Table 1

STANDARDS USED IN HPTLC QUALITATIVE ASSESSMENT PERFORMED ON SPRUCE BARK CRUDE ETHANOL EXTRACTS

Standards	Rf values	Synonym/Molecular formula	Purity %, Provider
1. Investigation of POLYPHENOL CARBOXYLIC ACIDS			
Gallic acid	0.40	3,4,5-Trihydroxybenzoic acid/ $C_7H_6O_5$	95%, Fluka
p-Coumaric acid	0.63	4-Hydroxycinnamic acid/ $C_9H_8O_3$	98%, Fluka
Sinapic acid	0.57	3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid/ $C_{11}H_{12}O_5$	98%, Fluka
Vanillic acid	0.64	4-Hydroxy-3-methoxybenzoic acid/ $C_8H_8O_4$	98%, Fluka
2. Investigation of TANNINS			
Catechin	0.44	(2R,3S)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol / $C_{15}H_{14}O_6$	99%, HPLC, Fluka
Epicatechin	0.40	(-)- <i>cis</i> -3,3',4',5',7'-Pentahydroxyflavane, (2R,3R)-2-(3,4-Dihydroxyphenyl)- 3,4-dihydro-1(2H)-benzopyran-3,5,7-triol/ $C_{15}H_{14}O_6$	Rotichrom TLC, Roth
Tannic acid	0.01	2,3-dihydroxy-5-(((2R,3R,4S,5R,6R)-3,4,5,6-tetrakis((3,4-dihydroxy-5- [(3,4,5-trihydroxyphenyl)carbonyloxy]phenyl)carbonyloxy)oxan-2- yl)methoxy)carbonyl)phenyl 3,4,5-trihydroxybenzoate/ $C_{76}H_{52}O_{46}$	86%, ACS
Procyanidin B1	0.12	(2R,2'R,3R,3'S,4R)-2,2'-bis(3,4-dihydroxyphenyl)-3,3',4,4'-tetrahydro- 2H,2'H-4,8'-bichromene-3,3',5,5',7,7'-hexol/ $C_{30}H_{26}O_{12}$	90%, HPLC, Fluka

Experimental part

Materials and methods

Feedstock: Spruce bark was obtained as waste from a wood processing company (Vatra Dornei, România). Prior to extraction, the bark was dried at room temperature (8.8% humidity), under normal aeration conditions. After drying, the spruce bark was milled in a GrindMix GM 2000 equipment and the fractions of a mean particle diameter of <0.25 mm (P1) and 1 mm diameter (P2) were selected for investigation.

Chemicals and Reagents: The chemicals used were of analytical grade or pure: acetic acid (p.a., min. 99.0%; Silal Trading), methanol (99.98%; Chempur), toluene (99.7%; Sigma Aldrich), methyl alcohol (p.a., min. 99.3% Consors); ethyl acetate (99.5%; Sigma-Aldrich), formic acid (p.a. 98-100%; Merck), diphenylboric acid 2-aminoethyl ester (NP) (p.a. min. 97%; Fluka), polyethyleneglycol 400 (PEG 400) (Ph.Eur.; Fluka), ferric chloride (>98.5%; Roth), ethanol (96%; Chemical Company). Also, a number of standards have been used in Thin Layer Chromatography (TLC) procedure (Table 1).

Extraction procedure

Crude ethanol extracts were obtained using an UAE extraction following a protocol previously described [14]. 5 g of spruce bark and 50 mL ethanol-water (70 % v/v) as solvent, in a solid/liquid (S/L) ratio of 1:10 g sample/mL solvent were used.

Qualitative assays

Chromatographic identification of polyphenolic acids

The HPTLC chromatographic assay was done according to literature [15, 16] using a CAMAG LINOMAT IV, TLC 3 Scanner and a WINCATS Planar Chromatography Manager software. A HPTLC plate G60 F254, 200x100 mm (Merck, Germany), saturated in methanol, was used as stationary phase. The length of the band was 8 mm, application

rate: 8 $\mu\text{L/s}$ and the application volume were 6 μL for samples and 3 μL for standards. The plates were examined at 254 and 366 nm before and after spraying the homogenized reagents (followed by air drying). The mobile phase used to separate simple phenols was toluene: ethyl acetate:formic acid in a ratio of 12.5:10:1.25 (v/v/v). A NP 1% methanolic solution + PEG 400 ethanolic solution was used for spraying the plate followed by heating at 100°C for 10 minutes. The analysis was performed in an air-conditioned room maintained at 22°C.

Chromatographic identification of tannins

HPTLC chromatographic assay for tannins was similar excepting the application volume of 10 μL for samples and 5 μL for standards. The mobile phase used in this case was toluene: ethyl acetate:formic acid in a ratio of 12:12:2 (v/v/v). A 5% ethanol ferric chloride solution was used for spraying the plate, followed by heating at 100°C for 10 minutes.

Quantitative analysis

Determination of total phenolic content (TPC)

The total phenolic content (TPC) was determined using the Folin-Ciocalteu procedure following the protocol reported in literature [17, 18]. Results were expressed as milligrams of gallic acid equivalents (GAE) per gram of spruce bark (mg GAE/g^{-1}). The analyses were performed in triplicate.

Results and discussions

The ultrasound assisted extraction is considered a green intensification process. Ultrasounds incites the formation of tiny bubbles subjected to fast adiabatic compression and expansion, provoking a fast-local increase of temperature and pressure, which leads to a breakage of spruce bark cell wall, the release of intracellular substances, increasing the diffusion across the cell walls and absorption into the solvent [14, 19]. The power of ultrasounds, temperature, solvent and particle size are the parameters that influence the extraction process. In this study UAE was conducted at 50°C for 45 minutes using as solvent an ethanol-water (70% v/v) solution in a solid/liquid (S/L) ratio of 1:10 g sample/mL solvent. The crude ethanol extracts were subjected to a HPTLC evaluation.

Thin Layer Chromatographic identification of simple phenolic acids in the crude ethanol extracts from spruce bark

The crude ethanol extract was scanned at 366 nm and 254 nm. For scanning at 366 nm, the appearance of a blue/fluorescent band indicated the presence of simple polyphenols at different values of the R_f (Fig. 1).

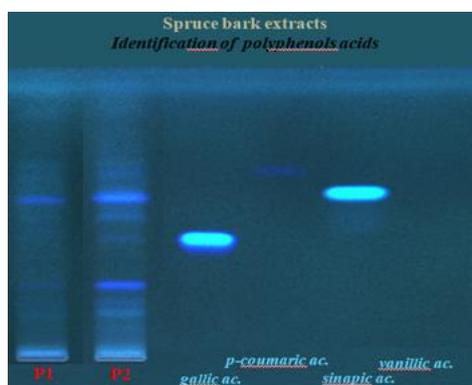


Fig. 1. Thin Layer Chromatogram for the identification of polyphenolic acids in crude ethanol extracts obtained from spruce bark at 366 nm after derivatization
Tracks: 1- P1; 2 - P2; 3 - gallic acid standard; 4 - p-coumaric acid standard; 5 - sinapic acid standard; 6 - vanillic acid standard

The HPTLC image indicates the separation of standards and of samples constituents. The standards were identified at the following R_f values: gallic acid - 0.40; sinapic acid - 0.57; p-coumaric acid - 0.63; vanillic acid - 0.64. Also, some constituents of the spruce bark separated in ethanol extracts have been identified (Fig. 1). p-coumaric and sinapic acids for P1 track and p-coumaric, sinapic and gallic acids for P2 track. As well, some other polyphenols appear as distinct bands with low intensity but were not identified. The scanning at 254 nm revealed also vanillic acid for both tracks (Fig. 2).

The HPTLC densitometry assay highlight the amounts of sinapic and p-coumaric acids in ethanol extract separated in P1 (0.84 mg/g, respectively 0.61 mg/g) and in P2 (0.53mg/g and 0.56 mg/g) (Fig. 3).

Thin Layer Chromatographic identification of tannins in the ethanol extracts

In HPTLC scanning at 366 nm, a blue-grey or green-grey colour, depending on the compounds structure, appears and allow the identification of tannins. The standards were identified at the following R_f values: catechin - 0.44; epicatechin - 0.40; tannic acid - 0.01, procyanidin B₁ - 0.12. In visible light some specific constituents of the spruce

bark separated in ethanol extracts have been identified: catechin, epicatechin and tannic acid (Fig. 4). The procedure did not reveal the presence of procyanidin B₁.

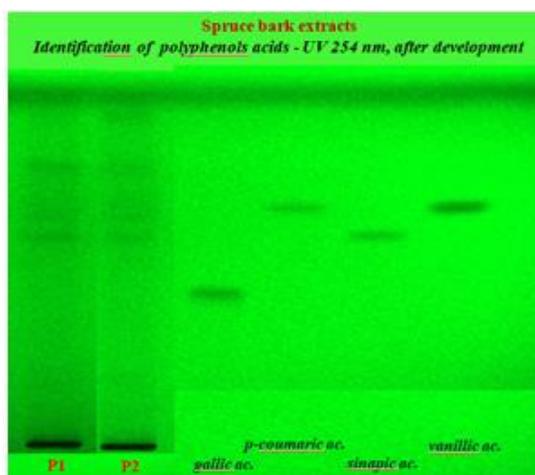


Fig. 2. Chromatogram for the identification of polyphenolic acids in crude ethanol extracts obtained from spruce bark at UV 254 nm, after development
Tracks: 1 - P1; 2 - P2; 3 - gallic acid standard; 4 - p-coumaric acid standard; 5 - sinapic acid standard; 6 - vanillic acid standard

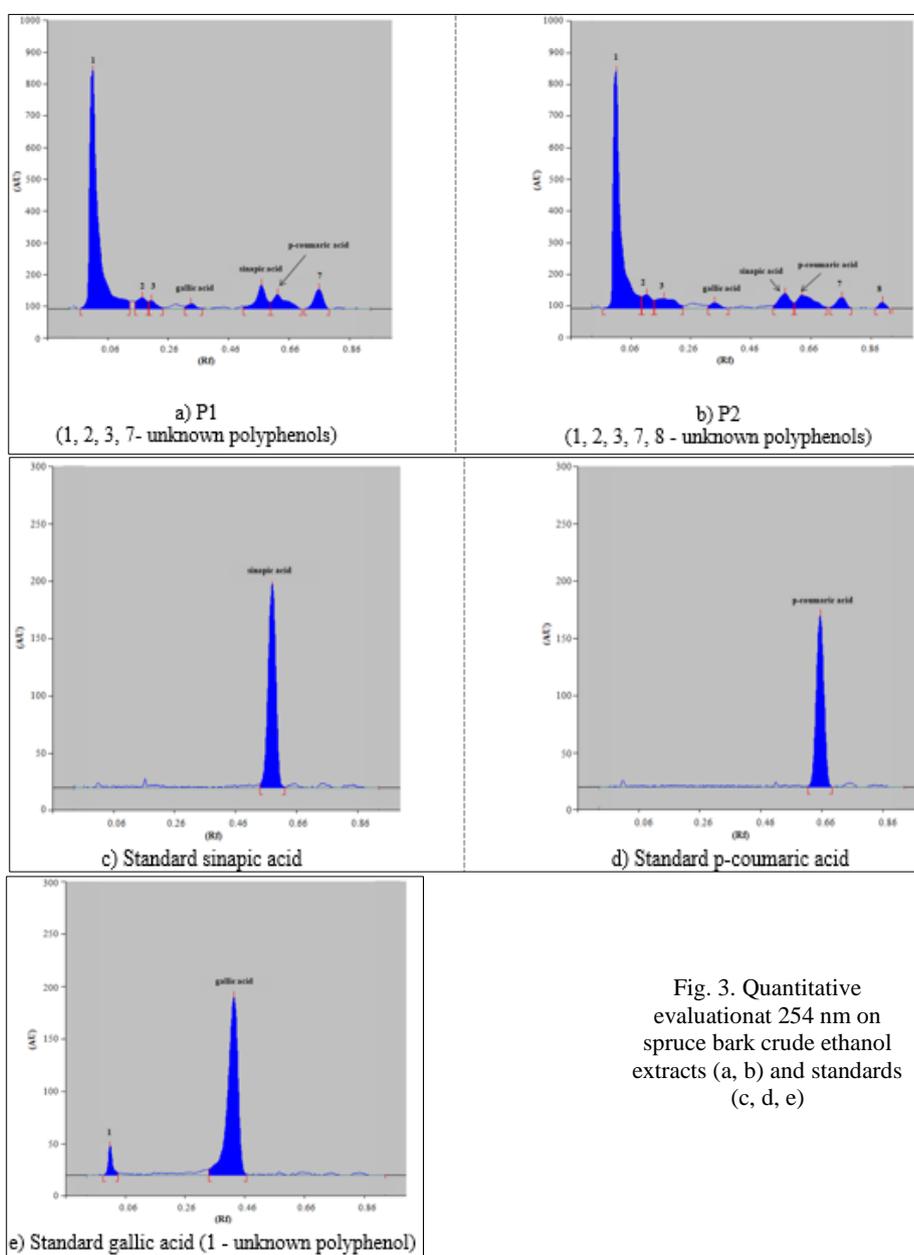


Fig. 3. Quantitative evaluation at 254 nm on spruce bark crude ethanol extracts (a, b) and standards (c, d, e)

The HPTLC scanning in UV at 254 nm assigned the same compounds (Figure 5).

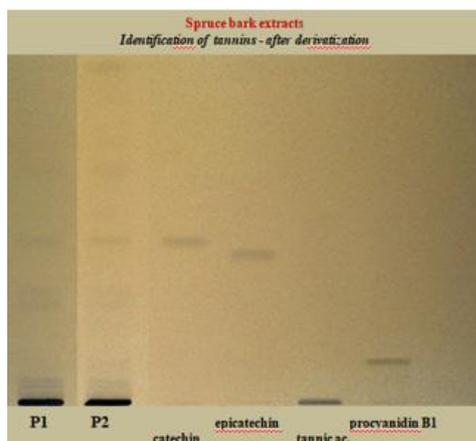


Fig. 4. Thin Layer Chromatogram (366 nm) for the identification of tannins in crude ethanol extracts obtained from spruce bark. Tracks: 1- P1; 2 - P2; 3 - catechin standard; 4 - epicatechin standard; 5 - tannic acid standard; 5 - procyanidin B₁ standard



Fig. 5. Thin Layer Chromatogram (254 nm) for the identification of tannins in crude ethanol extracts obtained from spruce bark. Tracks: 1- P1; 2 - P2; 3 - catechin standard; 4 - epicatechin standard; 5 - tannic acid standard; 5 - procyanidin B₁ standard

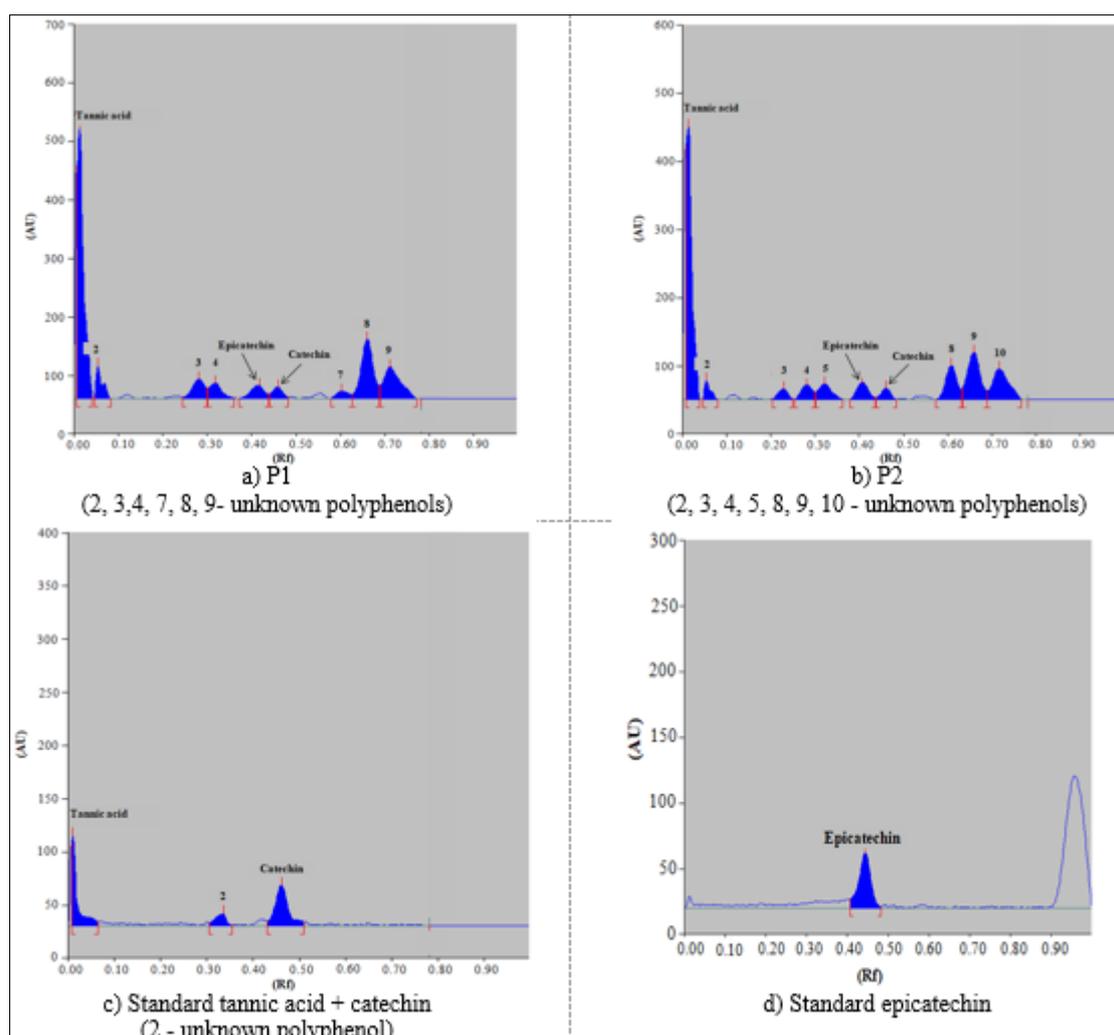


Fig. 6. Quantitative evaluation at 254 nm on spruce bark crude ethanol extracts (a, b) and standards (c, d)

For the quantitative evaluation of the tannins, a densitometric assay was performed. The assay revealed the amounts of catechin (1.03 mg/g), epicatechin (0.94 mg/g) and tannic acid (2.81 mg/g) in P1 sample and catechin (0.86 mg/g), epicatechin (1.07 mg/g) and tannic acid (2.43 mg/g) in P2 sample (Fig. 6).

For assessment of polyphenolic profile of spruce bark other evaluation methods such as HPLC (High Pressure Liquid Chromatography) are usually used. For the same feedstock, literature revealed mainly phenolic acids (mg/g) such as: p-coumaric (13.44), vanillic (1.55), syringic (2.48), ferulic (1.1), and sinapic acids (1.8) and important amount of catechin (7.72) [14]. This reference is in consensus with the findings of HPTLC assay. However, HPLC offer a qualitative and quantitative evaluation while HPTLC only provide useful acquaintance in the first step of a phytochemical screening

Total phenolic content (TPC) in the spruce bark ethanol crude extract

The spruce bark crude ethanol extract obtained by ultrasound-assisted extraction showed an important quantity of total polyphenols 29.785 mg GAE/g⁻¹ for <0.25 mm particles diameter and 14.448 mg GAE/g⁻¹ for 1 mm particles diameter. The significant difference, determined by particle size reducing is a consequence of the total surface of bark matrix increasing. A larger contact surface enhances the access of solvent to the soluble compounds, the mass transfer and the improvement of the extraction yield.

Conclusions

The HPTLC method provides useful information in the first step of a phytochemical screening. The evaluation of polyphenols profile separated from spruce bark using a green extraction method had successfully confirmed the presence of sinapic acid, p-coumaric acid, catechin, epicatechin and tannic acid.

The particle size of spruce bark significantly affects the yield of polyphenols separated in crude ethanol extracts. Thin Layer Chromatograms have highlighted the following amounts: sinapic acid (0.84 mg/g), p-coumaric acid (0.61 mg/g), catechin (1.03 mg/g), tannic acid (2.81 mg/g) in sample P1. The particle size reduction offers greater surface area for mass transfer and enhance the transfer of active compounds to the solvent. Also, the permeability of cellular wall increases and facilitates the extraction process.

The spruce bark, resulted in high amounts as a waste from wood processing industry could be considered as a resource of phytochemicals (especial polyphenols) and subjected to a complex and integrate valorisation.

References

- 1.HERRERO, M., IBAÑEZ, E.J.,*Supercrit Fluids*, **134**, 2018, p. 252-259.
- 2.VOLF, I., IGNAT, I., NEAMȚU, M., POPA, V.I.,*Chem. Pap.*, **68**, no. 1, 2014.
- 3.GHIȚESCU, R.E., VOLF,I., CĂRĂUȘU, C., BÜHLMANN, A.M., GÎLCĂ, I.A., POPA, V.I., *Ultrason. Sonochem.*, **22**, 2015, p. 535-541.
- 4.PREDESCU, N.C., PAPUC,C., NICORESCU,V.,GAJAILA, I., GORAN, G.V., PETCU, C.D., STEFAN,G.,*Rev. Chim. (Bucharest)*, **67**, no. 10, 2016, p.1922-1927.
- 5.JABLONSKY, M., NOSALOVA, J., HAZ, A., SKULCOVA, A., KREPS, F., *Int. J. Recent. Sci. Res.*, **8**, 2017, p. 16075-16079.
- 6.PROCHAZKOVA, D., BOUSOVA, I.,WILHELHELMOVA, N., *Fitoterapia*, **82**, 2011, p. 513-523.
- 7.SLÁDKOVÁ, A., BENEDEKOV, M., STOPKA, J., ŠURINA, I., HÁZ, A., STRIŽINCOVÁ, P., ČIŽOVÁ, K., ŠKULCOVÁ, A., BURČOVÁ, Z., KREPS, F., ŠIMA, J., JABLONSKÝ,M.,*BioRes.*, **11**, no. 4, 2016, p. 9912-9921.
- 8.FENG, S., CHENG, S., YUAN, Z., LEITCH, M., XU, C., *Renew. Sust. Energ. Rev.*, **26**, 2013, p. 560-578.
- 9.JABLONSKY, M., SKULCOVA, A., KAMENSKA, L., VRSKA, M., VRSKA, M., *BioRes.*, **10**, no 4, 2015, p. 8039-8047.
- 10.SURINA, I., JABLONSKY, M., HAZ, A., SLADKOVA, A., BRISKAROVA, A., KACIK, F., SIMA, J., *BioRes.*, **10**, no 1, 2015, p. 1408-1423.
- 11.PANJA, P., *Current Opinion in Food Science*, In Press, Corrected Proof , 2017.
- 12.ALUPULUI,A., LAVRIC,V., *Rev. Chim. (Bucharest)*, **60**, no. 5, 2009, p.1922-1927.
- 13.STOICA, R.,SENIN, R.M., ION, R.M.,*Rev. Chim. (Bucharest)*, **64**, no. 6, 2013.
- 14.LAZĂR, L., TĂLMACIU, A.I., VOLF, I., POPA, V.I., *Ultrason. Sonochem.*, **32**, 2016, p. 191-197.
- 15.PATIL, S., WANKHADE, S., KAMBLE, V., *Int. J. Drug. Dev. & Res.*, **6**, no. 3, 2014, p. 53-62.
- 16.ȚEBRENCU, C.E., CRETU, R.M., MITROI, G.R., IACOB, E., IONESCU,I., *Phytochem. Rev.*, **14**, no. 4, 2015, p. 613-621.
- 17.SINGLETON, V.L., ROSSI,J.A., *Am. J. Enol. Vitic.*, **16**, 1965, p. 144-158.
- 18.TĂLMACIU, A., VOLF, I., POPA, V.I., *Environl. Eng. Manag. J.*, **14**, no. 3, 2015, p. 615-623.
- 19.GOULA, A.M., *J. Food Eng.*, **117**, 2013, p. 492-498.

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