JOURNAL OF OPTOELECTRONICS AND ADVANCED MATERIALS Vol. 9, No. 3, March 2007, p. 783 - 787

Characterization of birch tree leaves, buds and bark dry extracts with antitumor activity

C. A. DEHELEAN, S. CÎNTĂ PÎNZARU[a*], C. I. PEEV, C. SOICA, D. S. ANTAL

"Victor Babeş" University of Medicine and Pharmacy, Eftimie Murgu Square 2, RO- 300041, Timişoara, România.
[a]Babeş-Bolyai University, Molecular Spectroscopy Dept., Kogălniceanu 1, RO- 400084 Cluj-Napoca, Romania.

The main compounds from *Betula pendula Roth* birch tree leaves bark and buds extracts are the saponins and especially triterpenes, which proved an antitumor activity. In order to monitor the presence of the pentacyclic triterpene within the extracts content, vibrational FTIR and FT-Raman spectra of the betulinic acid, betulin and lupeol have been recorded and assigned. The aim of the present study was to characterise the vegetal samples obtained from birch tree employing vibrational spectroscopy and TLC densitometry techniques. The FT-vibrational spectra of the extracts exhibit the characteristic spectral feature of the skeletal triterpene modes, betulin species being considered as dominant. The extract compounds present an increasing interest because they can be applied on therapy if they are rich in the specific compounds. The description of the differences between the vegetal parts in the dry extracts is important in correlating with the differences linked to antitumor/toxic activity. The results indicate differences between the tested samples with higher content of betulin in the bark ones.

(Received November 15, 2006; accepted December 21, 2006)

Keywords: Antitumor activity, Birch tree leaves, FT-IR, FT-Raman

1. Introducion

Betulinic acid (3β-hydroxy-20(19)-lupaen-28-oic acid) and its hydrolysed form, the alcohol betulin, are pentacyclic triterpenes found in the outer bark of the birch tree (*Betula pendula* Roth); of these compounds, betulin is the major one as its content surpasses 20% [1,2]. The structures of the main constituents in birch bark extracts were elucidated by UV, IR, ^{1}H-NMR, GC-MS [3,4], and for the first time the Raman spectra, IR or SERS were reported [5].

Betulin, betulinic acid, and lupeol exhibit important anticancer, anti-HIV and anti-inflammatory therapeutic activities. For betulinic acid, the most important activity resides in the antimelanoma, being characterized by a selective mechanism of action on melanoma cells (depending on the pH of cells - 6.4 being the most favourable one) and the proapoptotic activity of the compound. The mixture of compounds existing in birch tree bark has an outstanding therapeutic potential in skin cancer and melanoma, when administered in standardized extracts. The lupans are also present in the plane tree bark [1,5,6,7].

In this concept, the present research aims at developing new pre-standardization methods for birch tree extracts, concerning their content in triterpenic compounds with therapeutic activity. The study also targets to find the optimum solvent for the extraction of active principles, as the ratio in which the main compounds will be extracted depends on the solvent used for the procedure. Subsequently, we intend to perform further analytical investigations of birch extracts with view to the standardization of active principles, and to evaluate the therapeutic value of the extracts by biologic tests on melanoma and skin cancer cell lines (MCF7, A431, etc.).

2. Materials and methods

Vegetal material

Outer bark and buds of birch (*Betula pendula Roth*), and plane tree (*Platanus acerifolia*) were harvested from the Aninei Mountains (Banat region, Romania) and positively identified at the Department of Pharmaceutical Botany of the Faculty of Pharmacy, University of Medicie and Pharmacy, Timisoara, Romania. Voucher samples were deposed in the Herbarium of the mentioned Faculty.

Extraction procedures

Five grams dry vegetal material were degreased with n-hexane (150 ml) 2x10 minutes in a SONOMATIC ultrasound bath and dried to solid residue. The residues were subsequently extracted with 200 ml solvent, in fourfold repetition (4x10 minutes). Following the named coded extract, the employed solvents were ethyl acetate (C2), chloroform (C3), dichlormetan (C4), methanol (C5), dichloromethane and further purification in n-hexane (C6), as well as C7 (plane tree in dichloromethane), and C8 (plane tree in methanol). The using solvents were evaporated easily with a Büchi rotavapor. The extractions were made in duplicate [7].

Apparatus

Following the extractions, TLC-densitometry analysis was performed using a densitometer Schimadzu CS-9301 PC/IBM) at 540 nm, zigzag reading mode. Determinations were made in triplicate and the standard deviation was measured.

FTIR and FT-Raman spectra have been recorded using an Equinox 55 Bruker spectrometer with an integrated FRA 106 S Raman module. A Nd:YAG laser operating at 1064 nm line was employed for the excitation

of the powdered sample extracts. The power was 400 mW and the spectral resolution 4 cm^{-1}.

An attenuated total reflectance (ATR) module has been coupled to the Equinox 55 FTIR Bruker spectrometer for the recording of the FTIR absorbance spectra in the 4000-650 cm^{-1} range. The spectral resolution was 4 cm^{-1} and 40 scans were accumulated for each powder sample.

Chemicals

Betulin and betulinic acid were purchased from Extrasynthese, France. Analytical-grade solvents were purchased from Merck (Germany).

The ox blood samples were kindly provided by University of Agricultural and Veterinary Sciences of Banat. The saponine content determination followed the F.R.X procedure and the literature data [8]. The extract were mixed with washed ox erythrocytes with the same isotonic-buffered solution, and left for 20-24 hours at room temperature. The complete haemolysis was indicating by the presence of haemoglobin in the supernatant, no deposits of erythrocytes being observed. Sample haemolysis was compared to the standard solution of haemolytic saponin. Concentration of haemolytic saponins is expressed in grams of saponin per 100 grams of dried vegetal material. The test was performed for the most active in vitro samples [9].

3. Results and discussion

TLC measurement was applied for preliminary determinations of betulinic acid and betulin, as main triterpenes. The spots were visible after anysaldehide reagent spraying (the same formula as in the literature [12]). The amount of betulin was found much higher than the betulinic acid

FT-Raman spectra of the reference compounds, betulinic acid (BA) and betulin are presented in Fig 1. Significant differences are observed in the high wavenumbers spectral range and at the 1675 cm^{-1} band assigned to the C=O vibrational mode of the carboxylic group, characteristic for the acid species.

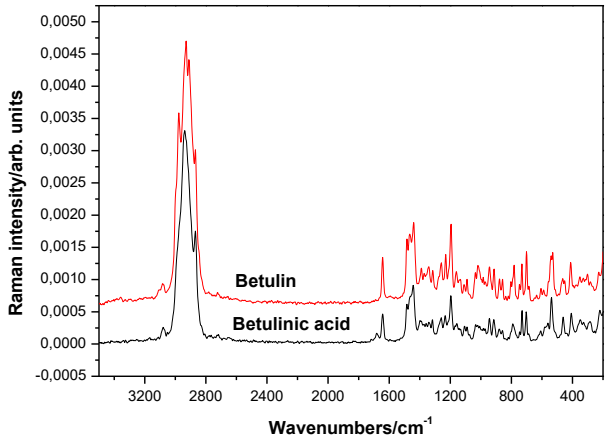

Fig. 1. FT-Raman spectra of the reference compounds, betulinic acid and betulin.

The high wavenumber spectral range clearly displays the differences corresponding to the –OH stretching modes from the –COOH and -CH_2-OH groups, characteristic for the pure betulinic acid and betulin, respectively.

FT-Raman spectra collected from the obtained vegetal extracts are presented in the Fig. 2, in comparison with the reference spectra of BA and betulin. Figs. 3 and 4 present the corresponding FT-IR spectra of the BA, betulin and the extract samples.

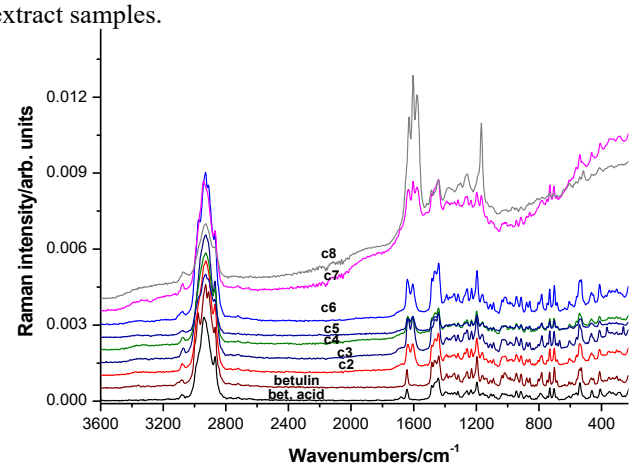

Fig. 2. FT-Raman spectra of the extracts C_2-C_8 in comparison with the spectra of betulinic acid and betulin, (C2 - ethyl acetate extract, C3-chloroform extract, C4 – dichlormetan extract, C5 – methanol extract; C6 –dichlormetane extract, C7- plane tree dichlormethane extract , C8-plane tree methanol extract).

Taking into account the strong intensity of the C=O vibrational mode of betulinic acid, observed at 1684 cm^{-1} that is missing in the IR spectrum of betulin, the presence of the acid species could be easier monitored in the C2-C8 samples.

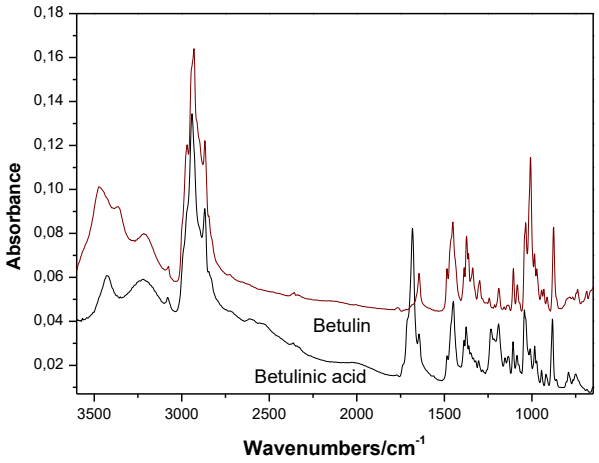

Fig. 3. FTIR (ATR) spectra of the reference compounds, betulinic acid and betulin.

As a general characteristic, the investigated extracts exhibit rather similar vibrational IR behaviour, the small differences observed being attributable to the presence of

other molecular species, excepting the two references. The C2-C8 present similar absorption bands at 1606 cm^{-1} (dominant in the C8 spectrum), 1578, 1513 and 820 cm^{-1}. These bands were not attributable to the betulinic acid or betulin, respectively (Fig. 3).

Comparing each spectrum of the C2-C8 samples with those of the references molecules, one can affirm that C2, C3, C4, C5 exhibit spectral feature more characteristic to betulin, suggesting that those content is higher than that of betulinic acid. In the C7 sample clearly dominates the betulinic acid content, besides other new species that were not observed in the C2-C6 samples.

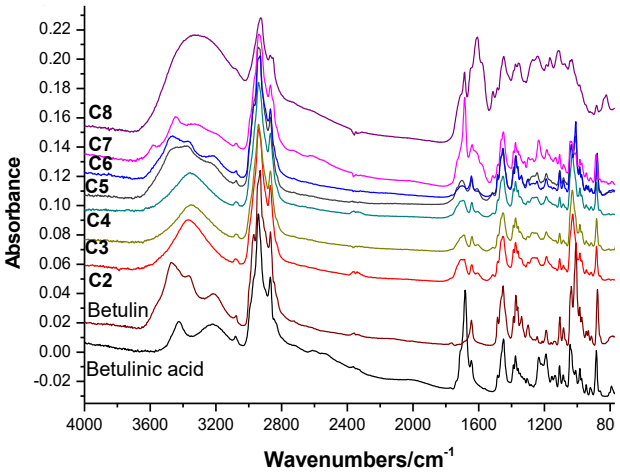

Fig. 4. FTIR (ATR) spectra spectra of the extracts C_2-C_8 in comparison with the spectra of betulinic acid and betulin (C2 - ethyl acetate extract, C3-chloroform extract,C4 – dichlormetan extract, C5 – methanol extract; C6 –dichlormetan extract, C7- plane tree dichlormethane extract , C8-plane tree methanol extract)

The C8 species displays weak bands characteristic for the betulinic acid or betulin, suggesting the abundant presence of other species or impurities. The C7 and C8 were used for comparing the analysed ones with these 2 more concentrate in betulinic acid (Fig. 4).

The content in betulinic acid and betulin of the sample is situated within the range of concentration indicated in the literature [8,10,11]. The differences between the indicated amounts are due to the different solvents used during the extractions. The differences between the total quantities of obtained dry extracts are presented in the Table 1, and it seems that dichloromethane leads to the highest total amount of dry powder. The highest yield for betulinic acid could be obtained with chloroform or dichloromethane (C3, C4 extract), whereas betulin can be extracted best with dichloromethane (C4 extract - Table 2). The two steps extraction with the degrease process do not change too much the betulinic acid quantity but in the case of betulin could be important because of the reducing of its total amount in the extract.

The ratio between the two main compounds, betulin and betulinic acid in the C3-C6 extracts is situated around 10/1, close to the data in the literature [8, 10, 12], (Table 3.). This indicates that betulin is less extractable with the solvent used in C2 ethyl acetate. The birch tree leaves and plane tree leaves extracts saponins content was around 3,5 and 4,25% (saponines techniques).

The FT-IR spectra of the betula and plane tree buds are displayed in the Fig. 5, whereas Fig. 6 presents their absorbance in comparison with the spectra of pure reference compounds. The major bands characteristic to the skeletal triterpene species were observed in the spectra of the buds. Additionally, a characteristic band at 1604 cm^{-1} which was observed in all the spectra of the extracts is not attributable to the reference compounds. This band is believed to be related to other organic extraction residues.

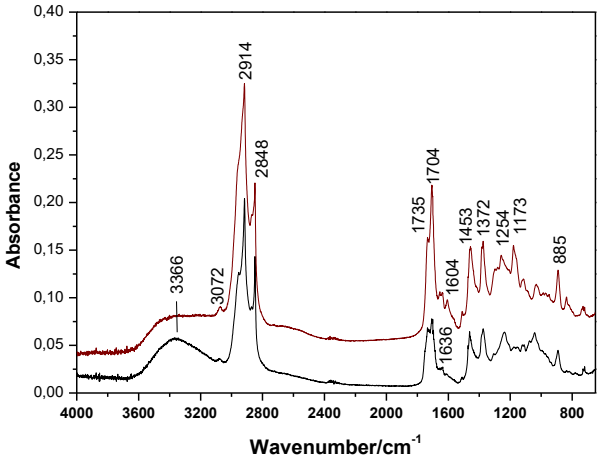

Fig. 5. ATR-FTIR spectra of the betula and plane tree buds.

FT-Raman spectra of the corresponding buds extracts are displayed in the Fig. 7, together with the spectra of the reference compounds, in order to illustrate the high similarity of the band positions and relative intensities of the molecular components obtained.

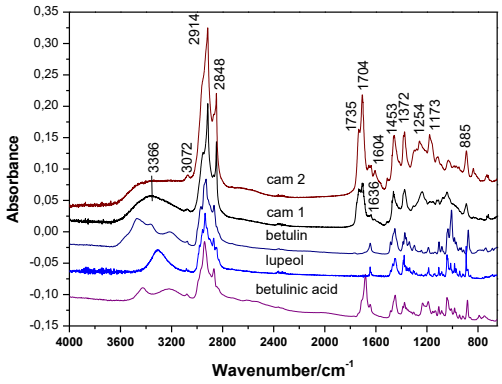

Fig. 6. ATR-FTIR spectra of the betula (cam 1) and plane tree buds (cam 2) in comparison with the pure betulinic acid, betulin or lupeol spectra, respectively, as indicated.

The plane tree buds spectrum (Fig. 7e) exhibit less Raman signal intensity in comparison with the references

fingerprint (a, b, c), suggesting a smaller content of the triterpene species than the betula extract (d).

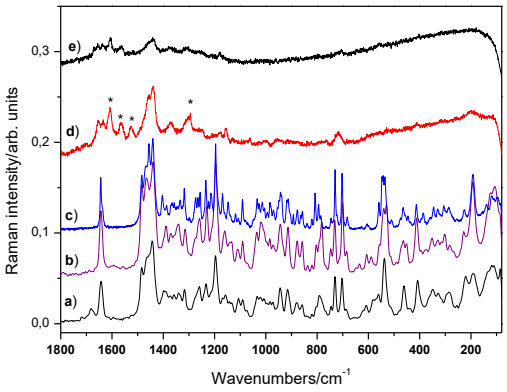

Fig. 7. FT-Raman spectra of betula (d) and plane tree buds (e) in comparison with the spectra of pure betulinic acid (a), betulin (b) and lupeol (c).

In order to differentiate the characteristic functional groups of each triterpene reference species, the 1600-1750 cm^{-1} spectral range is the most relevant. The accurate assignment of the functional group modes present in the spectra could also further allow to monitor the presence of betulinic acid, betulin, lupeol or other compounds from birch and perform quantitative measurements on these valuable extracts.

Table 1. The total amount of dry extract from 5 g of vegetal dry sample for C2-C6.

Sample code	Amount of dry extract (g)
C2	1.35
C3	2.20
C4	2.52
C5	2.07
C6	2.38

Table 2. TLC densitometry measurements for the birch tree bark extracts. (C2-C6)

SAMPLE 0CODES	BETULINIC ACID		BETULIN	
	content (mg/g dry bark)	%	content (mg/g dry bark)	%
C2	17.60 ± 0.61	1.76	120 ± 6.55	12
C3	20.12 ± 0.36	2.01	216 ± 5.03	21.6
C4	21.50 ± 0.30	2.15	230 ± 5.00	23
C5	16.90 ± 0.31	1.69	183 ± 4.04	18.3
C6	20.01 ± 0.50	2.00	195 ± 6.50	19.5

Table 3. The betulin/betulinic acid ratio in the analysed extracts (C2-C6).

Sample codes	Betulin/Betulinic acid Ratio
C2	7/1
C3	11/1
C4	11/1
C5	11/1
C6	10/1

4. Conclusions

Different extraction products of the birch three have been obtained and analysed.

A high selectivity of the FT-vibrational techniques was observed for evidencing betulin and betulinic acid in the analysed natural extracts. Due to the high sensitivity, without sample preparation requirements and non-destructive character, Raman spectroscopy could be successfully employed for the standardization procedure of birch tree extracts.

Dichlormetahne and chloroform are the best solvents for the extraction of betulinic acid, whereas dichloromethane extracts the highest amount of betulin. The extraction yield for betulinic acid and betulin through the present method, as well as the relative proportions of the two compounds from the extract are similar to literature data reported so far. An accurate analysis of the product extracts showed great promise for obtaining pharmaceutical derivatives of birch and plane tree compounds with antitumor activity. The procedure is relatively simple and the solvents are cheap and easy to evaporate and remove from the vegetal sample. These preliminary results confirmed the presence of betulin and betulinic acid species in the natural extracts obtained with different applied solvents. The birch tree and plane tree bud extracts showed great promises for a high content of the tested triterpenes. However, the birch tree and plane tree leaves contain higher quantities of saponins.

References

[1] J. Patocka, Journal of Applied Biomedicine **1**, 7 (2003).
[2] W. H. E. Hayek, U. Jordis, W. Moche, F. Sauter, Phytochemistry, **28** (9), 2229 (1989).
[3] T. Fujioka, Y. Kashiwada, R. E. Kilkuskie, L. M. Cosentino, L. M. Ballas, J. B. Jitang, W. P. Janzen, L. S. Chen, K. H. Lee, J. Nat. Prod., **57**(2), 243 (1994).
[4] E. I. Seedi, R. Hesham, A. M. Sahar Sobaith, Rev. Latioam. Quim. **27**(1), 17 (1999).
[5] S. Cinta Pinzaru, N. Leopold, Kiefer W., Talanta **57**, 625 (2002).
[6] Cristina Dehelean, D. S. Antal, A. Kaycsa, A. Dragomirescu, C. Soica, Timisoara Medical J., 55, suppl. 5, 2005, 191.
[7] G. Janicsak, K. Veres, M. Kallai, I. Mathe, Chromatographia, **58**(5/6), 295 (2003).

[8] G. Pavanasasivam, M. U. S. Sultanbawa, Phytochemistry **13**, 1974 (2002).
[9] ***Farmacopeea .Romana, ed a X-a, Ed. Medicala, Bucuresti, 1993, 1060-1063.
[10] G. Bringmann, W. Saeb, A. L. Assi, G. Francois, A. S. Narayanan Sankara, K. Peters, E. A. Peters, Planta Med. **63**, 255 (1997).
[11] L. Voutquenne, C. Kokougan, C. Lavaud, I. Pounz, M. Litaoudon, Phytochemistry **59**, 825 (2002).
[12] T. Galgon, D. Hoke, B. Drager, Phytochem. Anal. **10**, 187 (1999).

*Corresponding author: scinta@phys.ubbcluj.ro