NPC Natural Product Communications

2008
Vol. 3
No. 11
1885 - 1888

Characterization and Quantification of Triterpenes in the Neotropical Medicinal Plant *Souroubea sympetala* (Marcgraviaceae) by HPLC-APCI-MS

Martha Mullally[1], Kari Kramp[1], Ammar Saleem[1], Marco Otorola Rojas[2], Pablo Sanchez Vindas[2], Mario Garcia[2], Luis Poveda Alvarez[2], Tony Durst[3], Vance. L. Trudeau[1] and John. T. Arnason[1*]

[1]*Centre for Advanced Research in Environmental Genomics (CAREG), University of Ottawa, Ottawa, ON, Canada*

[2]*Department of Chemistry, University of Ottawa, Ottawa, ON, Canada*

[3]*Department of Chemistry, University of Ottawa, Ottawa, ON. Canada K1N 6N5*

**John.Arnason@uottawa.ca*

Received: December 15th, 2007; Accepted: October 3rd, 2008

A rapid, two-solvent, HPLC-APCI-MS method was developed to identify and quantify four pentacyclic triterpenes (betulinic acid, ursolic acid, α-amyrin and β-amyrin) in extracts of the neotropical medicinal plant *Souroubea sympetala*. Analysis of plant organs, wood, bark, leaves, immature fruit and flowers, indicated that the phytochemical distribution and quantity of triterpenes varies across the plant, with betulinic acid and ursolic acid the major constituents in the bark, wood, fruit and flowers and the amyrins the major constituents of the leaves.

Keywords: *Souroubea sympetala*, Marcgraviaceae, pentacyclic triterpenes, anxiolysis.

The Marcgraviaceae is a neotropical plant family, indigenous to tropical America, consisting of 5 genera and 125 species [1a] for which the phytochemistry is not well described. The genus *Souroubea* (Marcgraviaceae) was identified during a natural product discovery study in Costa Rica (C.R.) as an anxiolytic. *S. guianensis* may also be used to treat *susto* (fright) [1b]. *Susto* is a condition of folk etiology known throughout Latin America and understood to occur following a sudden frightening event that leads to the loss of "soul" or essence. The physiological characteristics of *susto* include diarrhea, loss of appetite, and restlessness [1c]. For diagnostic purposes, *susto* is considered a "culture-bound syndrome" linked to both anxiety and depression [1d,1e]. Preliminary *in vivo* evidence indicates that *Souroubea* sp. significantly reduces anxiety in a rodent behavioural assay of anxiety. Treatment of rats with 1 mg/kg of an ethanolic extract of *Souroubea* sp. exerted significant anxiolysis in the elevated plus maze (EPM), a standard behavioural assay of anxiety [1f]. Further, an ethanolic extract of *S. gilgii* inhibits rat gamma amino butyric acid-transaminase (GABA-T) activity (IC_{50} = 0.6 mg/mL) [2a], a major pharmacological target in the treatment of anxiety and epilepsy [2b,2c].

Currently there are no methods for the phytochemical analysis of Marcgraviaceae. From *S. sympetala* we have isolated a variety of known triterpenes and flavonoids [1f]. The anxiolytic activity is associated with a terpene fraction containing four pentacyclic triterpenes, betulinic acid (BA), ursolic acid (UA), α-amyrin (α-A) and β-amyrin (β-A), and a method for their analysis in extracts of this plant is described here.

HPLC method development

Preliminary HPLC study of BA in *S. sympetala* extracts (Figure 1A) was initiated using diode array detection (DAD) (UV, 205 nm). DAD sensitivity was low due to poor light absorbance. Detection was enhanced with the use of MS detection versus DAD (Figure 1B). The detection method was optimized for all four compounds by selected ion mode (SIM). The gradient was optimized to increase separation. Separation was complicated by the fact that BA and UA have the same molar mass and α-A and β-A are

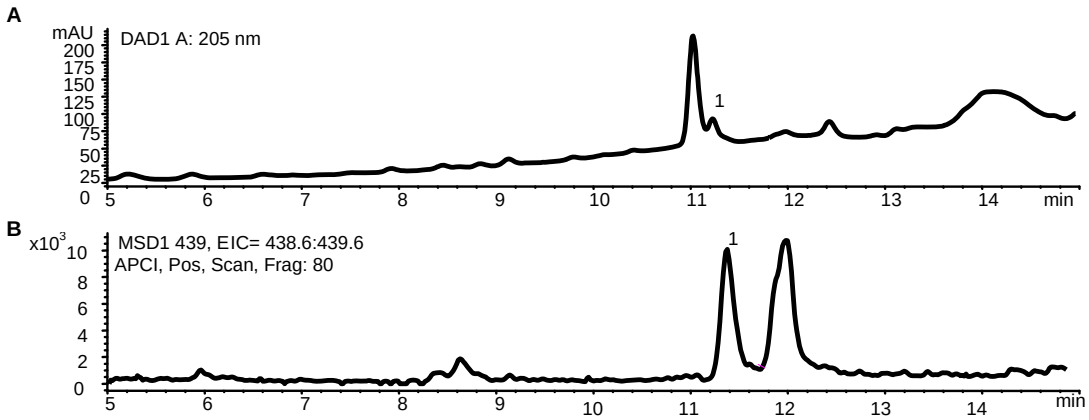

Figure 1: HPLC chromatogram of betulinic acid (1) detected via diode array detection (UV = 205 nm) in an ethanolic extract of *S. sympetala* (A), versus the same extract detected via mass spectrometry detection (B).

structural isomers. Initial isocractic conditions used caused co-elution of BA and UA. A 10 min gradient, followed by 8 min isocratic at 100% acetonitrile eliminated co-elution and resulted in two distinct peaks.

Chromatographic profiles of *S. sympetala* extracts and compound identification: The chromatograms (Figure 2) show the presence of the four triterpenes in all plant parts, but the profile varies across the plant parts, with BA and UA detected at highest levels in the bark and the wood and the amyrins detected at highest levels in the leaves. There is a differential distribution of amyrins in the leaves, with α-A most prevalent in the old leaves and β-A the major amyrin detected in the young leaves. Lower levels of triterpenes were detected in the immature fruit and flowers; in both, the major peak detected was BA.

Quantification of triterpenes in *S. sympetala* extracts: Extraction yields from the ASE extracts (Table 1) were highest in the flowers and lowest in the immature fruit. Quantification of the phytochemicals across the plant parts (Figure 3) showed more BA in the bark, with a mean value of 85.7 ± 30.3 μg/mg extract, than in the wood, 46.6 ± 11.7 μg/mg. There is significantly more BA in the bark versus the old leaves, young leaves, flowers and fruit ($p < 0.05$). The bark contains the greatest amount of UA, 0.50 ± 0.2 μg/mg, significantly higher than UA levels in the other plant organs ($p < 0.05$). There are higher levels of amyrins in the leaves than in the other plant organs. α-A is the major triterpene present in the old leaves, with a mean value of 11.5 ± 7.7 μg/mg, 3.3 times greater than that measured in the young leaves, 3.4 ± 3.1 μg/mg. There is more α-A in the old leaves than in the wood, bark, flowers and fruit, although a significant difference exists

Table 1: Percent yield for ASE extraction of each of the *S. sympetala* plant organs investigated.

Plant Organ	% Yield
Wood	3.4
Bark	5.0
Old Leaves	16.2
Young Leaves	15.5
Flowers	26.5
Immature Fruit	0.3

only between the α-A content of the old leaves, 11.5 ± 7.7 μg/mg, and α-A levels of the fruit, 0.04 ± 0.01 μg/mg ($p < 0.05$). Finally, β-A is the major triterpene in the young leaves, with a mean value of 12.2 ± 6.4 μg/mg versus 6.1 ± 3.0 μg/mg in the old leaves. There is significantly more β-A present in the young leaves than in the wood, flowers and fruit ($p < 0.05$). β-A levels in the old leaves are lower than in the young leaves, but significantly greater than in the flowers or fruit ($p < 0.05$).

This report provides the first method for identification of *S. sympetala* plant organs by HPLC-APCI-MS. The method is straightforward and allows for detection and quantification of the four triterpenes of *S. sympetala* from a biologically active fraction. The extraction method is also rapid and simple, with the added value that ASE extraction methods consume less solvent and are less labor intensive than conventional extraction approaches [2d]. The HPLC-APCI-MS method is similarly rapid (28 min) and employs a two solvent system that effectively accomplishes the challenging separation of a pair of molecules with identical molecular mass (BA and UA) and a pair of isomers (α-A and β-A).

While other methods to separate triterpenes exist [3a,3b], our method is, to the best of our knowledge, the first to separate this particular combination of molecules. Further, we have characterized the phytochemical profile of *S. sympetala* across the plant

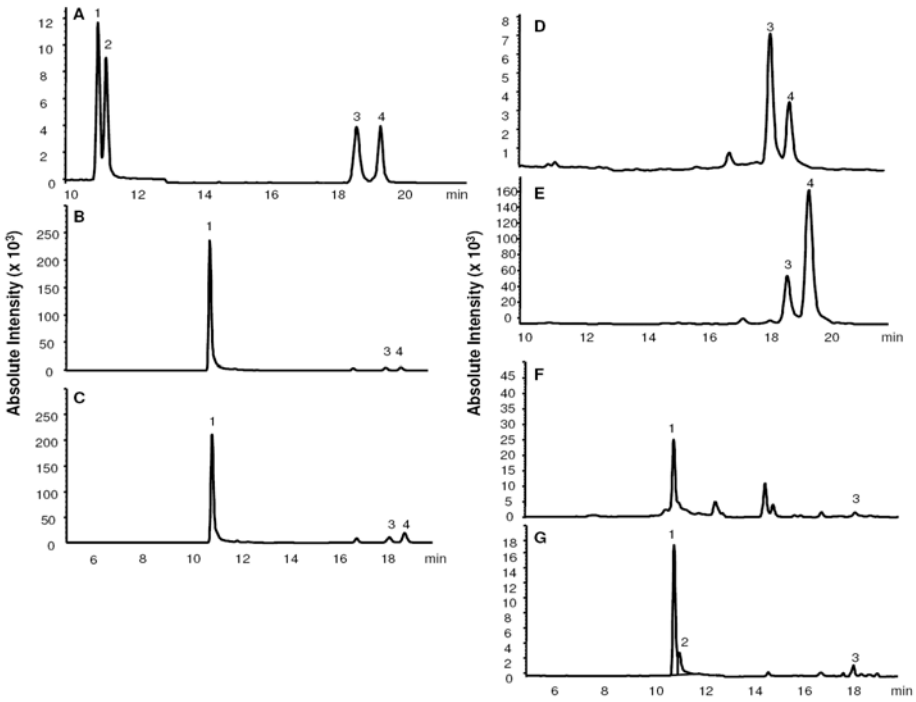

Figure 2: HPLC-APCI-MS profiles of *S. sympetala* extracts and standard mix of triterpenes. (A) standard mix, (B) wood, (C) bark, (D) young leaves, (E) old leaves, (F) flowers and (G) immature fruits. Marker triterpenes detected: **1**: betulinic acid, **2**: ursolic acid, **3**: β-amyrin, and **4**: α-amyrin. For each sample, 1 µL of a 20 mg/mL extract was injected into the autosampler.

organs. From this characterization, it is clear that the phytochemistry varies across the plant, with more BA and UA present in the wood and the bark versus the leaves, whereas the leaves contain more amyrins. The anxiety reducing properties identified in the traditionally used material, the leaves, may be due to the presence of the amyrins, which have been shown to exert anxiolysis [3c]. The phytochemical characterization described here will facilitate phytochemical identification of *S. sympetala* and on-going identification of plant organs that represent best candidates for medicinal application. The present study has addressed new strategic priorities in the characterization of *S. sympetala*. Future work will investigate the variability and phytochemical diversity of this species and compare it with other members of the same genus, particularly *S. gilgii*, a species also found in Costa Rica. Finally, due to the lipophilic nature of these triterpenes, emerging extraction technologies (for example, super critical CO_2) are under development to optimize complete extraction.

Experimental

Materials: Analytical grade HPLC solvents were purchased from J.T. Baker (USA). Standards of BA, UA, α-A and β-A were obtained from Sigma (St. Louis, MO).

Sample preparation and extraction: Fresh samples of wild *S. sympetala* wood, bark, early and late foliage (young & old leaves), flowers and immature fruits were collected in the area near Tortuguero, C.R., during the dry season (February & March) of 2006 and stored in 95% ethanol. Voucher specimens were placed in the University of Ottawa Herbarium (UOH No. 19915). Storage ethanol was removed, filtered and recombined later with ASE extracts of solids. Plant material was dried, weighed, coarsely ground via manual blender and extracted via pressurized liquid extraction with an Accelerated Solvent Extraction (ASE) 200 Extractor (Dionex, Sunnydale, USA). The extraction was conducted with 80% ethanol at 110ºC, a pressure of 120 bar, for two 5 min static cycles, parameters previously demonstrated to optimize triterpene extraction [2d]. The ASE extract was combined with the storage ethanol extract and dried down via speed vacuum at 40ºC and lyophilized. All extracts were stored in opaque glass vials at 4ºC.

HPLC-APCI-MS analyses: HPLC-APCI-MS analyses were conducted on wood, bark, young leaf, old leaf, flower and immature fruit extracts. Analyses were performed with a 1100 LC MSD VL APCI system consisting of an autosampler, quaternary pump, diode array detector (DAD) and an online APCI-MS with a mass range of 50 – 1500 a.m.u.

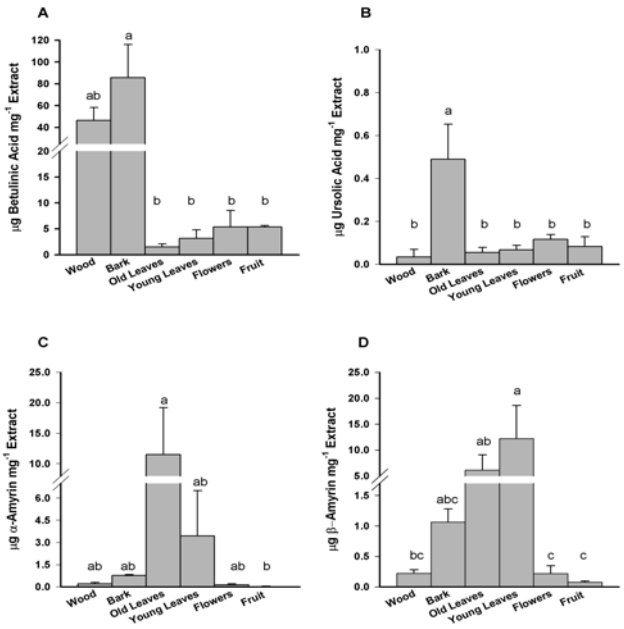

Figure 3: Quantitative comparisons of the four triterpenes, betulinic acid (A), ursolic acid (B), α-amyrin (C) and β-amyrin (D) in wood, bark, old leaf, young leaf, flower and immature fruit extracts of *S. sympetala*. Letters indicate significant differences ($p < 0.05$) as determined by a Tukey multiple comparison of means.

(Agilent, Palo Alto, CA, USA). A Waters YMC ODS-AM column (100 x 2 mm I.D.; 3 µm particle size, 120 Å), maintained at 45ºC was used at a flow rate of 0.4 mL/min. The elution conditions were optimized with a mobile phase of water (solvent A) and acetonitrile (solvent B) as follows: initial conditions: 70% A: 30% B, linear gradient to 100% B in 10 min, maintained at 100% B for 8 min and returned to 70% A: 30% B in 7 min, post-time 3 min, for a total run time of 28 min. One microlitre of each extract was injected through the autosampler for each run and the elution profiles monitored via MS.

Detection and quantification of triterpenes was conducted via MS. The mass spectrometer was tuned in positive ion mode at the beginning of all experiments. The optimized spray chamber conditions were: drying gas flow rate of 5.0 L/min; nebulizer pressure of 60 psi; drying gas temperature of 200ºC; vaporizer temperature of 325ºC; capillary voltage of 3200 V; and corona current of 5.0 µA. The MS was operated in SIM and tuned to detect ions with a mass/charge (m/z) ratio of 439.1 (BA), 439.2 (UA) and 409.2 (α-A and β-A), which correspond to the molecular mass of each triterpene following the loss of a hydroxyl group and hydrogen atom during fragmentation.

Calibration standards: Individual stock solutions of the four standards were dissolved in methanol at a concentration of 2 mg/mL. The stock solutions were diluted through the addition of the appropriate volume of methanol to a range of 1 µg/mL - 1 mg/mL to yield the solutions used to generate the calibration curve. The identities of the triterpenes in the extracts were determined by comparing the retention times and mass data with those of the calibration standards.

Statistical analysis: All statistical analyses were performed with S-PLUS software version 7.0 (Insightful Corp., Seattle, USA). Tukey multiple mean comparison tests were conducted on log-transformed raw data to compare phytochemical distribution across the plant.

Acknowledgments – Thanks very much to L. Kimpe for her technical assistance. Financial support to MM is provided by Natural Sciences and Engineering Research Council of Canada.

References

[1] (a) Heywood VH. (**1993**) *Flowering Plants of the World*. B T Batsford Ltd. London.; (b) Schultes RE, Raffauf RF. (**1990**) *The healing forest: Medicinal and toxic plants of the Northwest Amazonia.* Dioscorides Press, Portland, OR; (c) Klein J. (**1978**) Susto: The anthropological study of diseases of adaptation. *Social Science & Medicine. Part B: Medical Anthropology*, ***12***, 23-28; (d) WHO. (**1993**) *The ICD-10 classification of mental and behavioural disorders: Diagnostic criteria for research*. World Health Organization, Geneva; (e) APA. (**1994**) *Diagnostic and statistical manual of mental disorders (4th ed.).* American Psychiatric Association Washington, DC; (f) Puniani E. (**2004**) *Novel natural product based anti-anxiety therapy and natural insecticides*. PhD Thesis. Ottawa-Carleton Chemistry Institute. Ottawa, ON.

[2] (a) Awad R, Levac D, Cybulska P, Trudeau VL, Arnason, JT. (**2007**) Effects of traditionally used anxiolytic botanicals on enzymes of the γ-amino butyric acid (GABA) metabolism. *Canadian Journal of Physiology and Pharmacology*, ***85***, 933-942; (b) Ashton H, Young AH. (**2003**) GABA-ergic drugs: Exit stage left, enter stage right. *Journal of Psychopharmacology*, ***17***, 174-178; (c) Zwanzger P, Rupprecht R. (**2005**) Selective GABAergic treatment for panic? Investigations in experimental panic induction and panic disorder. *Journal of Psychiatry and Neuroscience*, ***30***, 167-175; (d) Huie CW. (**2002**) A review of modern sample-preparation techniques for the extraction and analysis of medicinal plants. *Analytical and Bioanalytical Chemistry*, ***373***, 23-30.

[3] (a) Schaaf O, Jarvis A, van der Esch SA, Giagnacovo G, Oldham N. (**2000**) Rapid and sensitive analysis of azadirachtin and related triterpenoids from Neem (*Azadirachta indica*) by high-performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry. *Journal of Chromatography A*, ***886***, 89-97; (b) Zaugg J, Potterat O, Plescher A, Honermeier B, Hamburger M. (**2006**) Quantitative analysis of anti-inflammatory and radical scavenging triterpenoid esters in evening primrose seeds. *Journal of Agricultural and Food Chemistry*, ***54***, 6623-6628; (c) Aragão GF, Carneiro LMV, Junior APF, Vieira LC, Bandeira PN, Lemos TLG, Viana GSd.B. (**2006**) A possible mechanism for anxiolytic and antidepressant effects of alpha- and beta-amyrin from *Protium heptaphyllum* (Aubl.) March. *Pharmacology Biochemistry and Behavior*, ***85***, 827-834.