Plant Cell Tiss Organ Cult
DOI 10.1007/s11240-010-9884-4

ORIGINAL PAPER

HPLC and GC analyses of in vitro-grown leaves of the cancer bush *Lessertia* (*Sutherlandia*) *frutescens* L. reveal higher yields of bioactive compounds

Shakira Shaik · Nisha Singh · Ashley Nicholas

Received: 27 August 2010 / Accepted: 12 November 2010

Abstract *Lessertia frutescens* L., commonly known as cancer-bush, is a medicinally reputed plant species indigenous to southern Africa. Field leaf extracts of this species are known to exhibit many curative properties. However, little is known about the bioactive compounds that are present in in vitro leaf extracts and seed extracts. The objective of this study was to verify the presence of and quantify L-canavanine, gamma amino butyric acid (GABA), arginine and D-pinitol in the seeds, field leaves and in vitro leaves of *L. frutescens* using gas and liquid chromatography. Methanolic extracts of in vitro leaves, field leaves and seeds were used. MRM chromatograms were recorded for L-canavanine and arginine using tandem mass spectrometry. GC chromatograms were recorded for GABA and D-pinitol using gas chromatography. D-Pinitol was found to be most abundant and was 14.75 and 18.17 mg/g in in vitro and field leaf extracts respectively, followed by GABA (7.29 and 3.48 mg/g), arginine (7.08 and 0.35 mg/g) and L-canavanine (0.55 and 0.08 mg/g). In the seed extracts, GABA content was found to be the highest (1.69 mg/g) followed by L-canavanine (0.37 mg/g), then D-pinitol (0.25 mg/g), and arginine (0.02 mg/g). In vitro leaves had higher quantities of all compounds, except for D-pinitol. This study therefore highlights the potential of bulking in vitro leaves for the extraction of the medicinal compounds, L-canavanine and GABA.

Keywords GABA · Gas chromatography · L-Canavanine · LC–MS/MS · Phyto-compounds

S. Shaik · N. Singh (✉) · A. Nicholas
School of Biological and Conservation Sciences, University of KwaZulu-Natal, Westville Campus, Private bag X54001, Durban 4000, South Africa
e-mail: Singhni@ukzn.ac.za

Introduction

Primary metabolism in plants includes essential chemical reactions that occur to maintain growth, reproduction and development. The products of these primary metabolic pathways are called primary metabolites e.g. carbohydrates, lipids, amino acids, nucleotides etc. Secondary or subsidiary chemical pathways are now also regarded as dynamic and essential components of plant metabolism since they produce a battery of secondary metabolites (Yazaki 2006) or phyto-compounds that are crucial for the survival of individuals in their natural environment (Hartmann 2007). These pathways produce numerous chemicals that perform a diverse range of functions within plants. Importantly, some of these compounds are considered to be potent chemical defense agents against viruses, microorganisms and insects (Wink 2003) and include a great variety of nitrogen-containing compounds (e.g. alkaloids, cyanogenic glucosides and non-protein amino acids) and also non-nitrogen compounds (e.g. phenolics, terpenoids and steroids).

Pharmacognosy has afforded adequate scientific evidence to the pharmaceutical industry that phyto-compounds derived from long used and purported medicinal plants have tremendous potential for the development of novel drugs (Phillipson 2007). In Africa, traditional medicinal plants are still being utilized by the vast majority of people in the healing of an assortment of diseases as they are considered more affordable, safer to consume, convenient, more accessible and, because of their long use, are also more acceptable (Vermani and Garg 2002). The bioactive phyto-compounds used in the management of diseases may be found in various organs of the plant; such as roots, leaves, stems, flowers, seeds or bark. These organs are mainly derived from the harvesting of natural populations or from field cultivated plants. However, chemical and genetic

Published online: 26 November 2010

variability in plants from different geographical biotopes complicates the standardization of the commercially available end product. Consequentially, biotechnologists in particular, have resorted to plant cell, tissue and organ cultures as alternative ways of deriving the desired phyto-compounds, under controlled conditions. Where plant organs have been used, these have usually been hairy roots and shoot cultures (Bourgaud et al. 2001).

Because *Lessertia frutescens*, commonly called the cancer-bush, is widely used in traditional medicine in Africa, we decided to investigate this species to demonstrate its potential as a source of medicinal phyto-compounds, using the tools of plant biotechnology. Numerous reports document the anti-oxidant (Tai et al. 2004), stress-relieving (Prevoo et al. 2008), hypoglycaemic (Chadwick et al. 2007), anti-mutagenic (Reid et al. 2006), and anti-tumour (Stander et al. 2007) properties of extracts of *L. frutescens*. Extracts are also reported to inhibit the action of HIV target enzymes (Hartnett et al. 2005). Furthermore, aqueous extracts of field leaves have been shown to contain L-canavanine, gamma amino butyric acid (GABA), D-pinitol, flavonoids and saponins (Fu et al. 2008; van Wyk and Albrecht 2008), but reports on their yields in seeds and in vitro cultures are lacking. The objective of this study was therefore to quantify the content of L-canavanine, arginine, GABA and D-pinitol, in seeds, field leaves and in vitro leaves of *L. frutescens* using modern analytical techniques such as gas and liquid chromatography (Cheng et al. 2007; Dias et al. 2009).

Materials and methods

Plant materials

Stock plants of *L. frutescens* used in this and previous studies in our laboratory were verified against specimens (W. J. Louw 2876 and R. Erasmus 198) in the Ward Herbarium, University of KwaZulu-Natal, South Africa.

Three different types of plant tissues were used for chemical profiling including in vitro leaves, field leaves and seeds. In vitro leaves (Sample A) were cut from 6 week old tissue-cultured *L. frutescens* plants in our laboratory where all cultures were maintained under diffuse white light of 55 µmol m/s, with an 18 h photoperiod at 25°C (Shaik et al. 2010). Commercially available powdered leaves (Bee-Med Natural Herbs, South Africa) were used as a source of field leaves (Sample B), and fresh seeds (Sample C) were obtained from Silverhill Seeds and Books, Kenilworth, South Africa.

Samples A and C were dried at 60°C for 48 h in a laboratory oven followed by grinding into fine powder using a pestle and mortar. Thereafter, 2 g each of samples A, B and C were sequentially extracted using organic solvents of increasing polarity including: hexane (98%), dichloromethane (DCM) (99.5%) and methanol (99.5%) via sonication for 15 min in a Branson 2210E-MT Ultrasonic Bath (USA). The hexane extracts (A1, B1, C1), DCM extracts (A2, B2, C2) and methanol extracts (A3, B3, C3) were vacuum filtrated and concentrated in a rotary evaporator (Buchi, Swtizerland) at 54°C. All extracts were stored at 4°C until experimental use. All analyses were done in triplicate.

Thin layer chromatography (TLC)

Preparation of standards and samples

L-Canavanine, GABA and D-pinitol standards were purchased from Sigma–Aldrich (South Africa). Half a mg of each standard was dissolved in 1 ml HPLC-grade methanol. Each solvent extract was reconstituted in 5 ml of hexane or DCM or HPLC-grade methanol for TLC analysis.

TLC analysis

Using a fine glass capillary tube, three drops of each standard and each extract were spotted onto aluminium plates (60 F254–Merck) pre-coated with silica gel and run in a solvent system consisting of DCM: methanol (50:50). The solvent front was marked with a pencil before air-drying the plates. Thereafter the plates were sprayed with 0.2% ninhydrin in acetone. Spots became visible after drying the plates in a laboratory oven at 110°C for 10 min. Methanol extracts of in vitro leaves (sample A3), field leaves (sample B3) and seeds (sample C3) were selected for further chemical analysis since each of these extracts indicated the presence of L-canavanine, GABA and D-pinitol after TLC analysis.

Gas chromatography (GC)

Preparation of standards and samples for GABA analysis

Two ml of HPLC-grade methanol was added to 10 mg of GABA standard in a reaction vial and then vortexed for 60 s. One hundred, 200, 400 and 600 µl of this solution was then diluted to a final volume of 1 ml using HPLC-grade methanol to prepare calibration curves ranging from 0.5 to 5 mg/ml. The calibration curves were plotted following linear regression of the peak area versus concentration. Three samples of A3, B3 and C3 each weighing 5 mg were dissolved separately in 1 ml of HPLC-grade methanol for GC analysis.

Pre-column derivatization of standards and samples for D-pinitol analysis

Derivatization of D-pinitol was carried out with the silylating reagent, trimethylsilylimidazole (TMSI)-pyridine

(Supelco). Briefly, 1 ml of TMSI-pyridine was added to each of 0.1, 0.5, 1, 2 and 5 mg of D-pinitol standard in separate reaction vials to create a series of dilutions for a calibration curve. The vials were capped and vortexed for 60 s. The calibration curves were plotted following linear regression of the peak area versus concentration. Three samples of A3, B3 and C3 each weighing 5 mg were separately subjected to silylation in the same way as the standard.

GC analysis

Aliquots of standards and samples were analyzed within 24 h of preparation using an Agilent 6820 GC system (China) equipped with a flame ionization detector (FID). Separation was carried out on a DB5 column (J & W Scientific, 30 m × 320 µm i.d. × 1 µm film thickness) using nitrogen as the carrier gas at a constant velocity of 0.7 ml/min. The injector temperature was set at 220°C, the FID temperature at 300°C and the split ratio was 50:1. In the run conditions for the GABA analysis oven temperature was initially 180°C held for 2 min, ramped at 10°C/min up to 250°C and then held for a further 2 min. The injection volume was 2 µl. Prior to injection, GABA samples were spiked with the 5 mg/ml standard using a 50:50 (v/v) ratio. In the run conditions for the D-pinitol analysis oven temperature was initially 180°C held for 1 min, ramped at 10°C/min up to 250°C and then held for 2 min. The injection volume was 0.5 µl. The phyto-compounds of interest were quantified using peak area and converted to compound mass using the calibration curves of external standards.

Liquid chromatography tandem mass spectrometry (LC–MS/MS)

Five mg each of samples A3, B3 and C3 were sent to the Central Analytical Facility, Stellenbosch University, South Africa for pre-column derivatization and analysis of L-canavanine and arginine. Samples were analyzed using a Micromass Quattro Micro API tandem mass spectrometer (Waters, Milford, MA, USA) according to the method of Colling et al. (2010).

Statistical analysis

Data were subjected to Duncan's multiple range test using the SAS program (Version 6.12, SAS Institute Inc., Cary, NC, USA).

Results and discussion

All plant samples were extracted by sonication as this is considered a powerful process for cell wall destruction which results in an increased yield of the sample (Pellati and Benvenuti 2008). In both the GABA and D-pinitol analyses, the calibration curves plotted following linear regression of the peak area versus concentration, showed a correlation coefficient of 0.99.

Quantitative analysis of L-canavanine, arginine, GABA and D-pinitol, was performed in triplicate and the results are presented in Table 1. LC–MS/MS analysis of L-canavanine and arginine, and GC analysis of GABA and D-pinitol are shown in Figs 1 and 2. Compound yields significantly differed with respect to source, i.e. in vitro leaves, field leaves or seeds. In the in vitro and field leaves respectively, D-pinitol content was highest (14.75 and 18.17 mg/g) followed by GABA (7.29 and 3.48 mg/g), arginine (7.08 and 0.35 mg/g) and L-canavanine (0.55 and 0.08 mg/g). In the seed extracts the distribution was slightly different, GABA was found to be the highest (1.69 mg/g) followed by L-canavanine (0.37 mg/g), then D-pinitol (0.25 mg/g) and finally arginine (0.02 mg/g). Importantly, the in vitro leaves had the highest quantities of L-canavanine and GABA. The higher accumulation of bioactive compounds in in vitro regenerated leaves of other medicinal plants was also demonstrated by Rathore and Shekhawat (2009) and Jain et al. (2010).

L-Canavanine is a toxic, non-protein amino acid, produced by more than 500 species of leguminous plants (Li et al. 2001). It is found mainly in the seeds of many legumes e.g. various *Canavalia* species (Sridhar and Seena 2006), but may also be present in the leaves (Natelson and Bratton 1984). The results of this study showed that L-canavanine was present in both seed and leaf extracts. In field materials, the seed extracts had a higher quantity (0.37 mg/g) of L-canavanine than the leaf extracts (0.08 mg/g). In seeds, L-canavanine is considered to have both primary and secondary functions (Hartmann 2007), firstly to operate as a nitrogen store in support of embryo development (Ekanayake et al. 2007) and, secondly, deterrence of predation because of its toxicity (Bell 2001). The similarity in structure of L-canavanine to the essential proteinogenic amino acid, arginine, has been postulated as the reason why the compound is lethal since it binds to

Table 1 Quantitative determination of compounds in extracts of *L. frutescens*

Extract	Quantity of compound (mg/g dry weight)			
	L-Canavanine	Arginine	GABA	D-Pinitol
In vitro leaves	0.55a*	7.08a	7.29a	14.75b
Field leaves	0.08c	0.35b	3.48b	18.17a
Seeds	0.37b	0.02c	1.69b	0.25c

*Mean separation within columns by Duncan's multiple range test ($P < 0.05$)

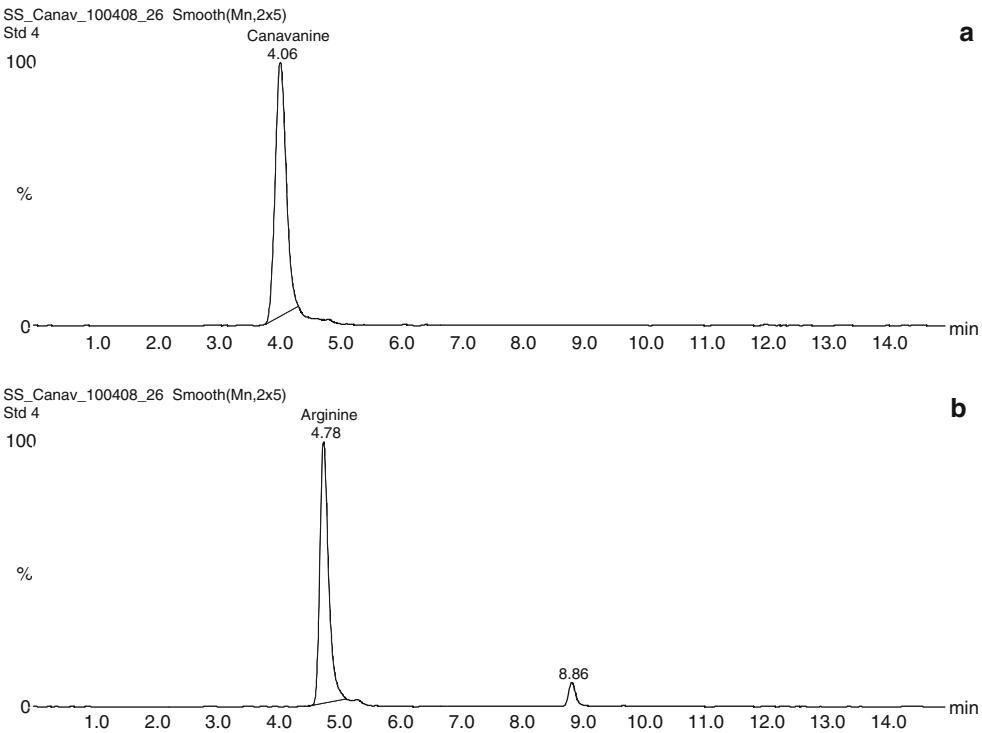

Fig. 1 Multiple reaction monitoring (MRM) chromatograms of *L. frutescens* showing retention times of **a** L-canavanine and **b** arginine

enzymes in place of arginine thereby causing certain proteins in viruses, bacteria and animals to malfunction (Shqueir et al. 1989). According to the literature, this antagonistic relationship only works when arginine concentrations are low (Swaffer et al. 1994). This investigation showed that the seed extracts contain low quantities of arginine (0.02 mg/g), and higher quantities of L-canavanine (0.37 mg/g). Therefore it may be assumed that the primary role of L-canavanine in the seeds is most likely that of a predator repellant.

In leaves, the role of L-canavanine is reported to be the repulsion of herbivores, either as a direct result of the toxicity of the compound, or indirectly, as a result of its acerbic taste (Udedibie and Carlini 1998). The latter has, however, not dissuaded humans from consuming the leaves as a bitter tea considered to be an efficacious and safe stimulant with no adverse effects (Xaba and Notten 2003). The results of this study show that the content of L-canavanine in field leaf extracts was low (0.08 mg/g), and it is assumed that at this level L-canavanine is not toxic to humans. The results revealed that when the content of L-canavanine was low, then arginine was high, and vice versa. We purport that the enzyme arginase, catabolizes both L-canavanine and arginine in *L. frutescens*; as has been shown in other legumes (Kavanaugh et al. 1990). When L-canavanine is cleaved by arginase to mobilize nitrogen, arginine is not, and vice versa. This inhibitory action of L-canavanine has also been observed by Hrabák et al. (1994).

The higher content of L-canavanine (0.55 mg/g) in in vitro leaf extracts compared to field leaf extracts could be attributed to favourable culture conditions such as pH, temperature, and nutrient supply, especially in respect of the high nitrogen content in MS medium. Hence this study has revealed a high yielding source of L-canavanine and the prospect of bulking, through tissue culture, for the purpose of commercialization. Other studies have indicated that L-canavanine has potential as an anti-cancer agent, especially in pancreatic cancer (Sridhar and Seena 2006).

GABA, like L-canavanine is a non-protein amino acid. GABA content in plants is characteristically low (Shelp et al. 1999), but was found in relatively high levels, especially in the leaves of *L. frutescens* (7.29 and 3.48 mg/g in in vitro and field leaves respectively). However, GABA levels were lower in the seeds (1.69 mg/g), and therefore it may be assumed that the role of GABA is firstly, that of nitrogen storage in seeds, and then a nitrogen source at germination (Wink 2003). Kuo et al. (2004) reported that legume seed levels of GABA are initially low or absent but dramatically increase after germination to aid in growth and development. Rapid and large accumulation of GABA occurs in response to mechanical manipulation e.g. during sampling of plant material (Wahid et al. 2007). This may explain the high levels of GABA found in the in vitro (7.29 mg/g) compared to field leaves (3.48 mg/g) in this investigation. The GABA levels of field leaves in this study are in contrast to that found by van Wyk and Albrecht (2008) who recorded commercial leaf samples to contain

between 0.23 and 0.85 mg/g. This difference may be attributed to the more acidic pH of the culture medium than field soil, resulting in the activation of glutamate decarboxylase, the main enzyme involved in GABA biosynthesis (Wahid et al. 2007). Furthermore, the biosynthesis of GABA, known as the GABA shunt, is said to be activated by light and nitrogen status (Fait et al. 2007). The in vitro plants used in this study were exposed to a longer photoperiod, compared to those found in natural habitats, and the culture medium contained an adequate supply of nitrogen (Shaik et al. 2010). Ojewole (2008) reported that GABA, in *L. frutescens* shoot extracts, may act as an inhibitory neurotransmitter causing an anti-seizure effect in mice. The findings of this study, therefore highlights the use of in vitro biology for the extraction of GABA for medicinal use.

D-Pinitol, a carbohydrate cyclitol, and purported hypoglycaemic agent (Chadwick et al. 2007), is commonly found in the leaves of the Leguminosae/Fabaceae, Pinaceae and Caryophyllaceae (Murakeözy et al. 2002) and is found in low levels in other plant families (Ghias-Ud-Din et al. 1981). D-Pinitol is less abundant in seeds (Horbowicz and Obendorf 1994) as was evident in the seeds of *L. frutescens* (0.25 mg/g) where it possibly serves as an ephemeral component, as in other legumes such as soybean (Kuo et al.

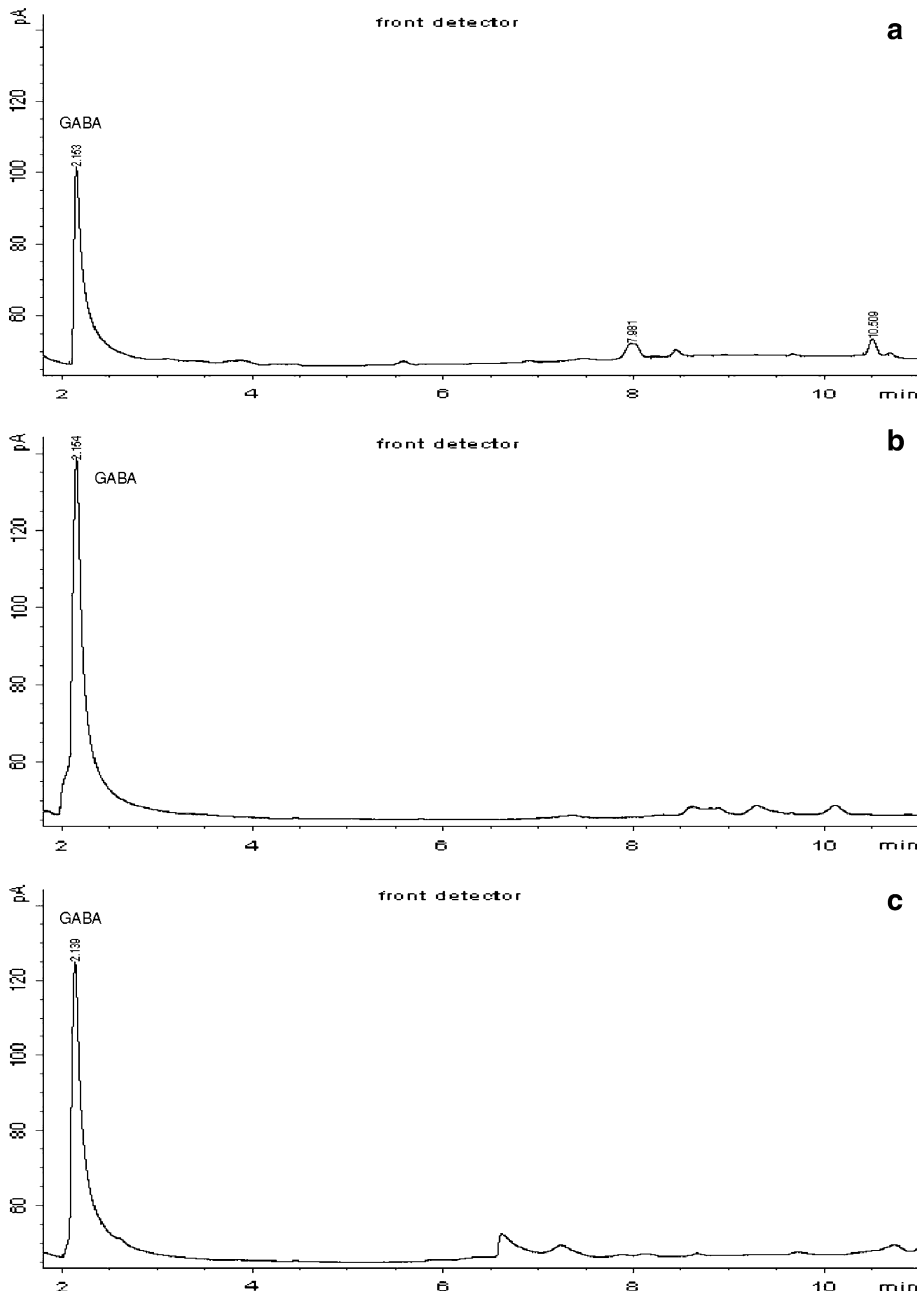

Fig. 2 GC chromatograms of *L. frutescens* showing retention times of GABA in **a** in vitro leaves **b** field leaves and **c** seeds and D-pinitol in **d** in vitro leaves **e** field leaves and **f** seeds

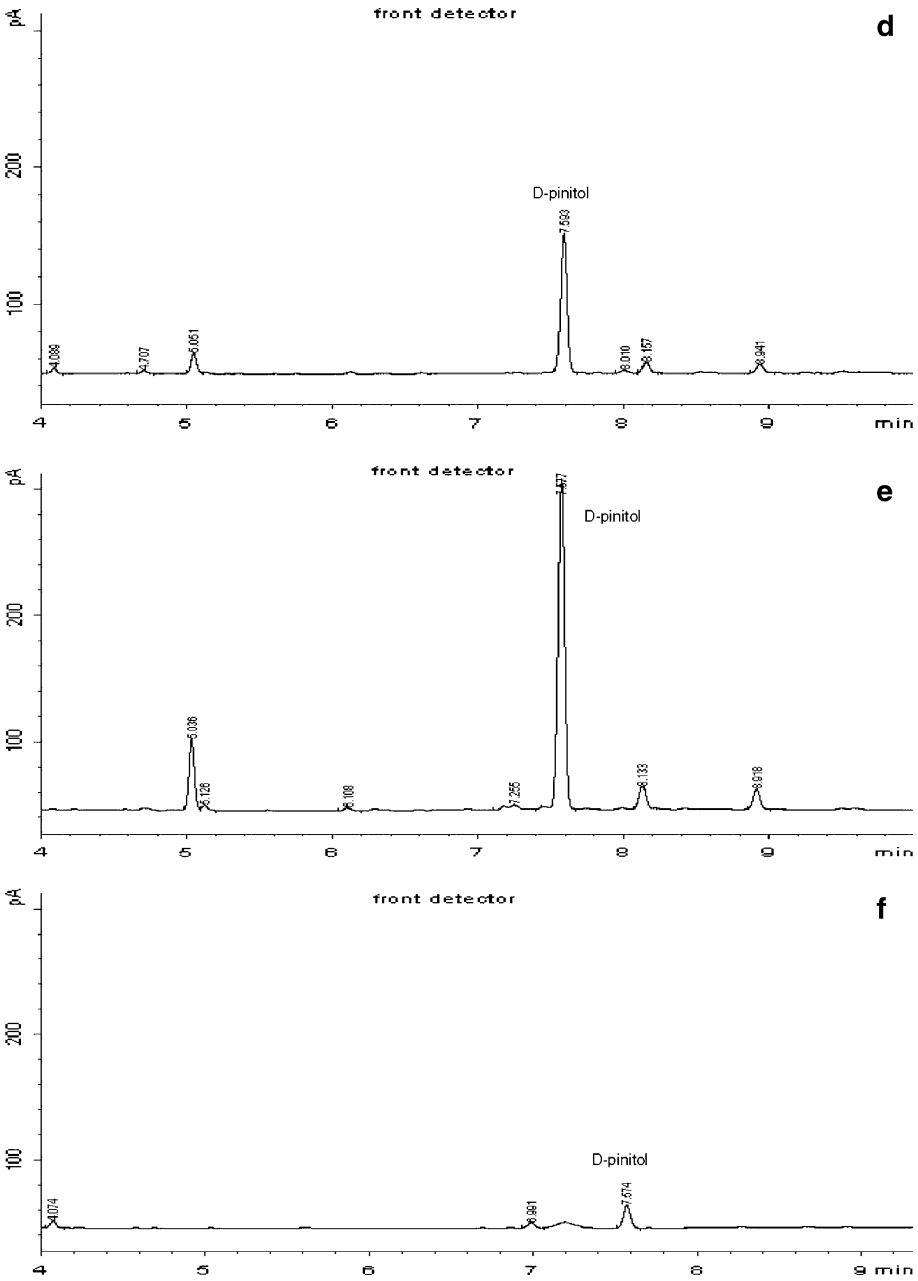

Fig. 2 continued

1997). D-Pinitol content in the leaf extracts was significantly higher (14.75 and 18.17 mg/g in vitro and field leaves respectively), where it may exist as a stored form of photosynthetically derived sugar (McManus et al. 2000), and therefore less likely to have been produced in response to abiotic stresses (Gomes et al. 2005). Seasonal variation of D-pinitol in leaves has also been recorded (Murakeözy et al. 2002), and this too may account for the difference between the in vitro and field leaf extracts in this study, as the effect of seasons are obliterated during in vitro culture. Other studies have demonstrated the effect of seasonal variation on phyto-compound yield (Mostafa et al. 2010). The quantity of D-pinitol detected in the field leaves of this study was comparable to that found by Moshe (1998).

The main aim of the present study was to verify the presence and quantify selected biological compounds in in vitro leaves, field leaves and seeds of *L. frutescens* using modern and accurate methods of analysis. The results reveal that the quantities of L-canavanine, arginine, GABA and D-pinitol significantly differ with the source. The major finding was that in vitro leaves had higher quantities of all compounds, except for D-pinitol. This study therefore

highlights the use of in vitro biology for the extraction of medicinal compounds of *L. frutescens*.

Acknowledgments Research funds were made available by the University of KwaZulu-Natal and the National Research Foundation.

References

Bell EA (2001) Ecological biochemistry and its development. Phytochemistry 56:223–227

Bourgaud F, Gravot A, Milesi S, Gontier E (2001) Production of plant secondary metabolites: a historical perspective. Plant Sci 161: 839–851

Chadwick WA, Roux S, van de Venter M, Louw J, Oelofsen W (2007) Antidiabetic effects of *Sutherlandia frutescens* in Wistar rats fed a diabetogenic diet. J Ethnopharmacol 109:121–127

Cheng L, Xu Y, Grotewold E, Jin Z, Wu F, Fu C, Zhao D (2007) Characterization of anthocyanidin synthase (ANS) gene and anthocyanidin in rare medicinal plant-*Saussurea medusa*. Plant Cell Tissue Organ Cult 89:63–73

Colling J, Stander MA, Makunga NP (2010) Nitrogen supply and abiotic stress influence canavanine synthesis and the productivity of in vitro regenerated *Sutherlandia frutescens* microshoots. J Plant Physiol 167:1521–1524

Dias LLC, Santa-Catarina C, Ribeiro DM, Barros RS, Floh EIS, Otoni WC (2009) Ethylene and polyamine production patterns during in vitro shoot organogenesis of two passion fruit species as affected by polyamines and their inhibitor. Plant Cell Tissue Organ Cult 99:199–208

Ekanayake S, Skog K, Asp NG (2007) Canavanine content in sword beans (*Canavalia gladiata*): analysis and effect of processing. Food Chem Toxicol 45:797–803

Fait A, Fromm H, Walter D, Galili G, Fernie AR (2007) Highway or byway: the metabolic role of the GABA shunt in plants. Trends Plant Sci 13:14–19

Fu X, Li XC, Smillie TJ, Carvalho P, Mabusela W, Syce J, Johnson Q, Folk W, Avery MA, Khan IA (2008) Cycloartane glycosides from *Sutherlandia frutescens*. J Nat Prod 71:1749–1753

Ghias-Ud-Din M, Smith AE, Phillips DV (1981) Separation of pinitol and some other cyclitols by high-performance liquid chromatography. J Chromatogr 211:295–298

Gomes CI, Obendorf RL, Horbowicz M (2005) Myo-inositol, D-chiro-inositol, and D-pinitol synthesis, transport, and galactoside formation in soybean explants. Crop Sci 45:1312–1319

Hartmann T (2007) From waste products to ecochemicals: fifty years research of plant secondary metabolism. Phytochemistry 68: 2831–2846

Hartnett SM, Oosthuizen V, van de Venter M (2005) Anti-HIV activities of organic and aqueous extracts of *Sutherlandia frutescens* and *Lobostemon trigonus*. J Ethnopharmacol 96:113–119

Horbowicz M, Obendorf RL (1994) Seed desiccation tolerance and storability: dependence on flatulence-producing oligosaccahrides and cyclitols—review and survey. Seed Sci Res 4:385–405

Hrabák A, Bajor T, Temesi A (1994) Comparison of substrate and inhibitor specificity of arginase and nitric oxide (NO) synthase for arginine analogues and related compounds in murine and rat macrophages. Biochem Biophys Res Commun 198:206–212

Jain R, Sinha A, Jain D, Kachhwaha S, Kothari, SL (2010) Adventitious shoot regeneration and in vitro biosynthesis of steroidal lactones in Withania coagulans (Stocks) Dunal. Plant Cell Tissue Organ Cult. doi:10.1007/s11240-010-9840-3

Kavanaugh D, Berge MA, Rosenthal GG (1990) A higher plant enzyme exhibiting broad acceptance of stereoisomers. Plant Physiol 94:67–70

Kuo TM, Lowell CA, Nelsen TC (1997) Occurrence of pinitol in developing soybean seed tissues. Phytochemistry 45:29–35

Kuo YH, Rozan P, Lambein F, Frias J, Vidal-Valverde C (2004) Effects of different germination conditions on the contents of free protein and non-protein amino acids of commercial legumes. Food Chem 86:537–545

Li X, Atkinson RN, King SB (2001) Preparation and evaluation of new L-canavanine derivatives as nitric oxide synthase inhibitors. Tetrahedron 57:6557–6565

McManus MT, Bieleski RL, Caradus JR, Barker DJ (2000) Pinitol accumulation in mature leaves of white clover in response to a water deficit. Environ Exp Bot 43:11–18

Moshe D (1998) A biosystematic study of the genus *Sutherlandia* R.Br. (Fabaceae, Galgeae). M.Sc. Thesis (Botany), University of Johannesburg

Mostafa SE, Karam NS, Shibi RA, Alali FQ (2010) Micropropagation and production of arbutin in oriental strawberry tree (*Arbutus andrachne* L.). Plant Cell Tissue Organ Cult 103:111–121

Murakeözy EP, Smirnoff N, Nagy Z, Tuba Z (2002) Seasonal accumulation pattern of pinitol and other carbohydrates in *Limonium gmelini* subsp. *hungarica*. J Plant Physiol 159:485–490

Natelson S, Bratton GR (1984) Canavanine assay of some alfalfa varieties (*Medicago sativa*) by fluorescence: practical procedure for canavanine preparation. Microchem J 29:26–43

Ojewole JAO (2008) Anticonvulsant property of *Sutherlandia frutescens* R.BR. (variety *incana* E.MEY.) [Fabaceae] shoot aqueous extract. Brain Res Bull 75:126–132

Pellati F, Benvenuti S (2008) Determination of ephedrine alkaloids in *Ephedra* natural products using HPLC on a pentafluorophenyl-propyl stationary phase. Journal of Pharmaceut Biomed Anal 48:254–263

Phillipson JD (2007) Phytochemistry and pharmacognosy. Phytochemistry 68:2960–2972

Prevoo D, Swart P, Swart AC (2008) The influence of *Sutherlandia frutescens* on adrenal steroidogenic cytochrome P450 enzymes. J Ethnopharmacol 118:118–126

Rathore MS, Shekhawat NS (2009) Micropropagation of Pueraria tuberose (Roxb. Ex Willd.) and determination of puerarin content in different tissues. Plant Cell Tissue Organ Cult 99: 327–334

Reid KA, Maes J, van Staden J, De Kimpe N, Mulholland DA, Verschaeve L (2006) Evaluation of the mutagenic and antimutagenic effects of South African plants. J Ethnopharmacol 106: 44–50

Shaik S, Dewir YH, Singh N, Nicholas A (2010) Micropropagation and bioreactor studies of the medicinally important plant *Lessertia (Sutherlandia) frutescens* L. S Afr J Bot 76:180–186

Shelp BJ, Bown AW, McLean MD (1999) Metabolism and functions of gamma-aminobutyric acid. Trends Plant Sci 4:446–452

Shqueir AA, Brown DL, Klasing KC (1989) Canavanine content and toxicity of *Sesbania* leaf meal for growing chicks. Anim Feed Sci Technol 25:137–147

Sridhar KR, Seena S (2006) Nutritional and antinutritional significane of four unconventional legumes of the genus *Canavalia*—a comparative study. Food Chem 99:267–288

Stander BA, Marais S, Steynberg TJ, Theron D, Joubert F, Albrecht C, Joubert AM (2007) Influence of *Sutherlandia frutescens* extracts on cell numbers, morphology and gene expression in MCF-7 cells. J Ethnopharmacol 112:312–318

Swaffer DS, Ang CY, Desai PB, Rosenthal GA (1994) Inhibition of the growth of human pancreatic cancer cells by the arginine antimetabolite L-canavanine. Cancer Res 54:6045–6048

Tai J, Cheung S, Chan E, Hasman D (2004) In vitro culture studies of *Sutherlandia frutescens* on human tumor cell lines. J Ethnopharmacol 93:9–19

Udedibie ABI, Carlini CR (1998) Questions and answers to edibility problem of the *Canavalia ensiformis* seeds—a review. Anim Feed Sci Technol 74:95–106

van Wyk BE, Albrecht C (2008) A review of the taxonomy, ethnobotany, chemistry and pharmacology of *Sutherlandia frutescens* (Fabaceae). J Ethnopharmacol 119:620–629

Vermani K, Garg S (2002) Herbal medicines for sexually transmitted diseases and AIDS. J Ethnopharmacol 80:46–66

Wahid A, Gelani S, Ashraf M, Foolad MR (2007) Heat tolerance in plants: an overview. Environ Exp Bot 61:199–223

Wink M (2003) Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. Phytochemistry 64:3–19

Xaba PM, Notten A (2003) *Sutherlandia frutescens*. South African National Biodiversity Institute (SANBI). Available online http://www.plantzafrica.com/plantqrs/sutherfrut.htm. Accessed 12 Feb 2007

Yazaki K (2006) ABC transporters involved in the transport of plant secondary metabolites. FEBS Lett 580:1183–1191