COMPARISON OF THE SELECTED SECONDARY METABOLITE CONTENT PRESENT IN THE CANCER-BUSH *LESSERTIA (SUTHERLANDIA) FRUTESCENS* L. EXTRACTS

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Abstract

Extracts of *in vitro* leaves, field leaves and seeds of the leguminous plant *Lessertia frutescens* were analyzed using spectrophotometric and gravimetric methods, to the effect of quantitative comparison of their phenolic, flavonoid, alkaloid and saponin contents. As compared to the field leaves and seeds, saponins were found to be most abundantly represented in *in vitro* leaves, followed by phenolics, flavonoids and alkaloids. The extracts were also qualitatively analyzed so as to evaluate the presence of other phytochemicals of medicinal interest. This qualitative analysis indicated the presence of tannins, phlobatannins and cardiac glycosides. Having in mind the documented therapeutic use of these phytochemicals, the results of this study offer a strong rationale for further animal and clinical investigations of *L. frutescens* extracts.

Key words: alkaloids, flavonoids, phenolics, phytochemicals, saponins, spectrophotometry

Introduction

Complex secondary metabolic pathways seen in higher plants eventually result in the production of a vast assortment of chemical complexes known as the secondary metabolites (Yazaki, 2006) or phytochemicals. Although these natural compounds may not be essential for the growth and reproduction (Starmans and Nijhuis, 1996), recent technological advancements, particularly those in the fields of Molecular Biology and Biochemistry, have provided evidence of their allelochemical functions. If sessile, a plant's ability to avoid herbivore, bacterial, viral and fungal attacks is limited. Consequently, adaptive traits such as compounds capable of providing chemical-based defence have evolved in these plants so as to aid in their survival by vitue of warding off, inhibiting and destroying predators and diseases. According to Verpoorte and Memelink (2002), the primary function of these secondary metabolites is plant defence.

Secondary metabolites are structurally diverse; their classification is mainly derived from their biosynthetic pathways (Harborne, 1999). In pharmacognosy, phenolics (comprising flavonoids, tannins, coumarins, quinones and anthocyanins) are regarded as the widest spread phytochemical group, while alkaloids (containing one or more nitrogen atoms) are viewed upon as a more genera- and species- specific (Bourgaud, 2001); terpenoids (comprising triterpenes, steroids, saponins and cardiac glycosides) are considered to be the phytochemicals having the most diverse chemical structure (Yazaki, 2006).

Phenolic compounds may assume a wide range of structures; from simple ones containing one aromatic ring only, to very complex polymeric forms (Trease and Evans, 1996). These compounds have been documented to inhibit UV and carcinogenic tumours (Scalbert et al, 2005) and to exhibit anti-mutagenic, anti-bacterial, anti-viral and anti-inflammatory effects (Middelton et al, 2000). Flavonoids contain free hydroxyl groups attached to the aromatic rings. Flavonoids such as rutin, present in certain buckwheat species, are known to inhibit lipid oxidation by virtue of radicals' scavenging (Jiang et al, 2007). These compounds protect against coronary heart ailments and have anti-microbial, anti-tumour and anti-inflammatory effects (Harborne and Williams, 2000). Since capable of inhibiting intestinal α-glucosidase, phenolics and flavonoids found in eggplant also aid glucose absorption control in type 2 diabetes (Kwon et al, 2008).

Alkaloids are heterocyclic compounds with powerful physiological effects on mammals (Shamsa et al, 2008). Pyridine alkaloids like trigonelline found in *Trigonella foenum-graecum*, have been reported to be useful in diabetes management (Liu, et al, 2010). Saponins (a class of terpenoids) are amphipathic compounds composed of saccharide attached to a steroid or tripterpene. Saponins such as quercetine and soyasaponin, present in certain *Trifolium* species, are beneficial to human nutrition (Oleszek and Stochmal, 2002). Other representatives of this class, like bidesmosidic saponins present in *Mimusops laurifolia*, have found use in cosmetics and detergents because of their foaming ability (Eskander et al, 2006).

For the purposes of this study, *Lessertia frutescens*, a plant of medicinal importance, was selected for phytochemical profiling. This plant has been used in traditional medicine for centuries by diverse cultural groups residing in southern Africa in order to manage a wide variety of ailments including gynaecological, gastrointestinal, urogenital and musculoskeletal disorders; insofar, no adverse treatment outcomes have been recorded (Xaba and Notten, 2003). Numerous reports have documented an

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 *L. frutescens* extracts. These extracts have also been reported to inhibit HIV target enzymes (Hartnett et al, 2005). However, little is known about the bioactive compounds that facilitate the acting mechanisms of these remedies. Aqueous and methanol extracts of *L. frutescens* field leaves have been shown to contain flavonoids and saponins (Van Wyk & Albrecht, 2008; Avula et al, 2010). Nevertheless, quantitative and qualitative assessments of alkaloids, phenolics, saponins and flavonoids in the seeds or *in vitro* cultures of this species are very rare. Spectrophotometry has become a useful crude plant extract screening technique enabling the detection and verification of various compound classes. Data contributing to the elucidation of *L. frutescens* medicinal properties and its health-enhancing components shall vastly enhance the commercial value of these extracts. Therefore, the objective of this study was to compare phenolic, flavonoid and alkaloid content present in *in vitro* leaves, field leaves and seeds of *L. frutescens* using spectrophotometry, as well as to compare their saponin content using gravimetric analysis. In addition, qualitative tests were carried out so as to identify the presence of tannins, phlobatannins and cardiac glycosides.

Materials and Methods

Plant materials

Three different types of material, viz. *in vitro* leaves, field leaves and seeds were used for chemical profiling. *In vitro* leaves (Sample A) were obtained from the cultures grown in our laboratory (Shaik et al, 2010), where stock plants of *L. frutescens* were verified against the specimens (W.J. Louw 2876 and R. Erasmus 198) deposited into the Ward Herbarium, University of KwaZulu-Natal, South Africa. Commercially available powdered leaves (Bee-Med Natural Herbs, South Africa) were used as the field leaf source (Sample B), while the seeds (Sample C) were obtained from Silverhill Seeds and Books, Kenilworth, South Africa. Samples A and C were dried at 60ºC for 48 h in a laboratory oven and then grinded into a fine powder using a pestle and mortar.

Quantitative determination of phenolics, flavonoids, alkaloids and saponins

The extracts were concentrated using a Buchi rotary evaporator (Switzerland), while spectrophotometric measurements were completed using a Beckman DU 530 UV/VIS spectrophotometer. All analyses were done in duplicate.

Phenolics

An adaptation of the method published by Biglari et al. (2008) was used to determine phenolic content. Five grams of the samples A, B and C were separately extracted in 15 ml of methanol at the room temperature for 5 h using a mechanical shaker (Labcon 3100 E, South Africa). The extracts were then filtered through a Whatman No 1 filter paper and centrifuged for 10 min using an Eppendorf 5810 R (Germany) centrifuge at the rate of 5400 x *g*. The supernatant was concentrated for 15 min under a reduced pressure at 40 ºC using the rotary evaporator so as to obtain a crude methanol extract. Following the evaporation, dry weights of the extracts A, B and C were 0.51, 1.00, and 0.30 g, respectively. Each evaporated extract was diluted with 5 ml of methanol. Forty µl of each sample were mixed with 1.8 ml Folin-Ciocalteu reagent 10-fold pre-diluted with distilled water, and allowed to rest at the room temperature for 5 min. Thereafter, 1.2 ml of 7.5 %-sodium bicarbonate was added to each mixture. After resting for 60 min at the room temperature, the absorbance of these solutions was measured at 765 nm. Gallic acid (Sigma) was used as the standard. The concentration of total phenolic compounds was expressed as mg of gallic acid equivalents per g of dry plant weight using a Gallic acid-based calibration curve. The range covered by the calibration curve was 1-20 µg/ml. All of the Gallic acid solutions were assayed the same way as the samples.

Flavonoids

An adaptation of the method published by Wang et al. (2008) was used to determine flavonoid content. Two and a half grams of the samples A, B and C were separately extracted in 8 ml of methanol in a GFL 1083 water bath (Germany) and shaken under reflux for 12 h at 70 ºC. The extracts were then filtered through a Whatman No 1 filter paper and evaporated to dryness in the rotary vacuum evaporator for 10 min at 40 ºC. Following the evaporation, dry weights of A, B and C extracts were 0.26, 0.50, and 0.15 g, respectively. This was followed by the addition of 20 ml of methanol so as to dissolve each of the extracts. Thereafter, 1 ml of each methanol solution was transferred into 10 ml-volumetric flasks to which 0.3 ml of 5 % sodium nitrite was added, and left at the room temperature for 6 min. After the addition of 0.3 ml of 10 %-aluminium nitrate to each flask, the mixtures were incubated at the room temperature for another 6 min. Following the addition of 4 ml of 1N sodium hydroxide to each flask, the final volume was attained using methanol. Further 15-min incubation at the room temperature, enabling the colouration to develop, was followed by the measurement of absorbance at 510 nm. Rutin (Sigma) was used as the standard. Total flavonoid content was expressed as mg of rutin equivalents per g of dry plant weight using a rutin-based calibration curve. The calibration curve covered for the range of 10-100 µg/ml. All rutin solutions were assayed the same way as the samples.

Alkaloids

An adaptation of the method published by Shamsa et al. (2008) was used to determine alkaloid content. Five grams of the samples A, B and C were separately extracted in 15 ml of methanol for 24 h using a mechanical shaker (Labcon 3100 E, South Africa). The extracts were filtered through a Whatman No 1 filter paper, with methanol subsequently vacuum-evaporated to dryness at 45 ºC for 15 min. Following the evaporation, dry weights of A, B and C extracts were 0.55, 0.80, and 0.20 g, respectively. Thereafter, 20 mg of each residue was dissolved in 10 ml of 2N hydrochloric acid and then filtered as described before. One ml of the resulting solutions was transferred into separator funnels and washed three times using 10 ml of chloroform per washing session. The pH value of each solution was then adjusted to neutral using 0.1 N sodium hydroxide followed by the addition of 5 ml of bromocresol green solution (69.8 µg/ml) and 5 ml of phosphate buffer (pH 4.7). After a vigorous shaking, each solution was extracted separately in 1, 2, 3 and 4 ml of chloroform. Each extract was collected into a 10 ml-volumetric flask and diluted to the final volume using chloroform. The absorbance was measured at 470 nm. Atropine (Sigma) was used as the standard. The concentration of total alkaloid compounds was expressed as mg of atropine equivalents per g of dry plant weight using an atropine-based calibration curve. The calibration curve covered for the range of 1-20 µg/ml. All atropine solutions were assayed the same way as the samples.

Saponins

An adaptation of the method published by Shiau et al. (2009) was used to determine saponin content. Five grams of A, B and C samples were separately extracted by virtue of maceration in 50 ml of methanol at 70 ºC for 6 h. The cooled extracts were filtered through a Whatman No 1 filter paper, with methanol vacuum-evaporated to dryness at 45 ºC for 30 min. Following the evaporation, dry weights of A, B and C extracts were 0.20, 0.30, and 0.03 g, respectively. The residues were suspended in 50 ml of distilled water each and extracted in three successive sessions using 100 ml of ethyl ether per session. After removing the remaining ethyl ether from the aqueous layer by virtue of evaporation, the solutions were further extracted using 100 ml and 50 ml of *n*-butanol, respectively. The *n*-butanol fractions were dried by means of evaporation. The resultant products represented the crude saponin content and were expressed as mg of saponin per g of dry plant material weight.

Qualitative determination of other phytochemical compounds

Samples A, B and C were tested for tannins, phlobatannins and cardiac glycosides using phytochemical tests described by Trease and Evans (1978, 1996). Each test result was qualified as negative (-) or positive (+).

Ferric chloride test for tannins

Half a gram of dried samples A, B and C was placed into separate test tubes. Twenty ml of distilled water were added to each test tube and boiled for 10 min. After cooling, each extract was separately filtered through a Whatman No 1 filter paper. Thereafter, 3 drops of 0.1% - ferric chloride were added to each extract and observed for colouration.

Hydrochloric acid test for phlobatannins

Half a gram of dried samples A, B and C was placed into separate test tubes. Twenty ml of distilled water were added to each test tube and boiled for 10 min. After cooling, each extract was separately filtered through a Whatman No 1 filter paper. Thereafter, 2 ml of 1%- aqueous hydrochloric acid was added to each extract and observed for colouration.

Keller-Killiani test for cardiac glycosides

Half a gram of dried samples A, B and C was placed into separate test tubes. Twenty ml of distilled water were added to each tube. After 24 h, each extract was separately filtered through a Whatman No 1 filter paper. Thereafter, 5 ml of each extract were treated with 2 ml of concentrated glacial acetic acid and 2 drops of 0.1% ferric chloride solution. This mixture was then carefully added to 1 ml of the concentrated sulphuric acid. The interface was observed for colouration.

Statistical analysis

Data are represented as the means plus/minus standard deviations of the duplicate determinations. 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

Quantitative analyses of the total saponin, alkaloids, phenolic and flavonoid contents present in different *L. frutescens* extracts were performed in duplicate; the results are presented in Table 1. In all phytochemical groups profiled, the share of

saponins in *in vitro* leaves, field leaves and seeds was found to be the highest (53.34, 60.00, and 6.00 mg/g, respectively), followed by phenolics (30.18, 15.09, and 4.89 mg/g, respectively) and then alkaloids (4.52, 1.58, and 0.47 mg/g, respectively). As for flavonoids, only smaller amounts were found in the above samples (7.18, 5.55, and 1.87 mg/g, respectively).

The differences in the quantity of studied phytochemicals found in *in vitro* and field leaf extracts may be attributed to genetic differences in the plant material, environmental conditions, harvesting season and storage conditions (Santos-Gomes et al, 2002; Biglari et al, 2008; Jiang et al, 2007). It has been reported that metabolic cell reactions may become repressed prior to phytochemical analysis (Bourgaud et al, 2001). However, the *in vitro* leaf extract was shown to contain significantly higher quantities of phenolics, flavonoids and alkaloids. Bourgaud et al. (2001) reported this effect to be potentially attributed to the slowdown in the allocation of carbon needed for cell built-up; should so happen, the available carbon is used for an increased phytochemical production. Furthermore, all extraction parameters were kept constant throughout the study,, but the drying method applied on the field leaves by the commercial company remains unknown.

Table 1 Total content[y] of the selected secondary metabolites present in different *L. frutescens* extracts

Extract	Quantity of phytochemical compounds (mg/g dry weight)			
	Phenolics	Flavonoids	Alkaloids	Saponins
In vitro leaves	30.18 a[z] ± 1.16	7.18 a ± 0.05	4.52 a ± 0.31	53.34 a± 1.89
Field leaves	15.09 b ± 0.56	5.55 b ± 0.08	1.58 b ± 0.07	60.00 a ± 2.83
Seeds	4.89 c ± 0.00	1.87 c ± 0.38	0.47 c ± 0.08	6.00 b ± 1.41

[y]Values expressed as means ± standard deviations ($n = 2$)
[z] Mean separation within columns established by Duncan's Multiple Range Test ($P < 0.05$)

Table 2 Qualitative determination of the selected secondary metabolites in different *L. frutescens* samples

Sample	Tannin test for the brownish-green colour	Phlobatannin test for the bright red colour	Cardiac glycoside test for the brown ring expected to emerge at the interface
In vitro leaves	+	+	+
Field leaves	+	+	+
Seeds	+	+	+

Saponins are widespread in all legume cells and often occur in aerial parts of healthy plants in large quantities (Palazón et al, 2006). Saponin content established in this study (Table 1) mirrors these conclusions. Saponins present in *L. frutescens* appear to be stable, do not degrade easily and were therefore found in large quantities in *in vitro* (53.34 mg/g) and field leaves (60.00 mg/g) despite environmental and harvesting differences.

Phenolic content was higher in the leaf extracts (30.18 in *in vitro* and 15.09 mg/g in the field leaves) as compared to the seed extract (4.89 mg/g). Shoots and leaves were also reported to have higher phenolic content in comparison to other plant parts (Bernardi et al, 2008). The results of this study revealed the *in vitro* leaf extract to contain twice as much phenolics as the field leaf one. Reasons for the variations in phenolic quantity in *in vitro* and field leaves are said to be the result of endogenous degradation of some of the phenolic compounds, occuring after an air exposure, as well as to be the result of an increase in temperature or light exposure during field sampling (Santos-Gomes et al, 2002). Phenolic composition is also dependent on cultivation season, contamination by insects and other agents, different physiological phases and genetic profile (Bernardi et al, 2008), which may explain the lower phenolic yield from the field leaves.

Flavonoid content present in seeds is characteristically low (Oleszek and Stochmal, 2002), as was evident in this study (1.87 mg/g). Flavonoids in the *in vitro* leaf extract (7.18 mg/g) were significantly higher than in the field leaf extract (5.55 mg/g), which may be attributed to differences in light exposure (Hernandez et al, 2008) and to the warm temperature characteristic of growth chamber conditions of *in vitro* plant cultivation (Shaik et al., 2010). As indicated in the literature, it is likely that abiotic stresses arising due to the low light intensity and temperature differences during *in vitro* cultivation may have triggered the response mechanism in terms of an increased flavonoid production (Hernandez et al, 2008). Furthermore, the synthesis and accumulation of flavonoids can be influenced by other factors such as genotype (species and variety) and ecological conditions such as locality and harvesting period (Jiang et al, 2007).

In this study, alkaloid yields were the lowest since this group of compounds is sparsely distributed and more specific of genera and species (Bourgaud et al., 2001). The seed alkaloid content (0.47 mg/g) was significantly lower than that of the *in vitro*

(4.52 mg/g) and field leaves (1.58 mg/g), probably because alkaloids are biosynthesized in leaves and, consequential to the decreased translocation, get to be found in seeds in very small amounts. Out in the wild, plants are susceptible to a variety of pathogens; this too has contributed to the reduced alkaloid production (Zehra et al, 1998). An increased alkaloid production has been reported to be the result of favourable cultivation conditions such as pH, temperature and nutrient supply (Liu et al, 2010), coupled with specialized spatial and temporal controls (Yazaki, 2006) characteristic of an *in vitro* culture.

Qualitative analyses of tannins, phlobatannins and cardiac glycosides present in *L. frutescens* extracts were performed according to the standard phytochemical screening protocols; the results are presented in Table 2. All three phytochemical classes were found in all extracts studied. Tannins occur widely in higher plants and are represented by two subtypes: condensed tannins (or proanthocyanidins) and hydrolysable tannins. In this study, tannins determined qualitatively were found to be of the condensed type, as verified by the formation of the red insoluble compounds called phlobatannins (or phlobaphenes) that emerge whenever condensed tannins are treated with acid (Trease and Evans, 1996). This finding supports those of other studies which have shown the condensed tannins to be commonly present in woody plants (Trease and Evans 1978, 1996). The results also revealed the presence of cardenolides, a type of cardiac glycosides that has been used to treat congestive heart failure (Braga et al, 1997). Both tannins and cardiac glycosides are found in plants and are used as protection against herbivores (Chavan et al., 2001). The literature (mentioned above) is full of reports that validate the importance of *L. frutescens* extracts' medicinal use. Anecdotal information (Xaba and Notten, 2003) outlines the usefulness and efficacy of these extracts. This study provides evidence of some of the compounds that may contribute to the medicinal value of *L. frutescens* extracts.

The aim of the present study was to validate and quantify phenolics, flavonoids, alkaloids and saponins and to authenticate the presence of tannins, phlobatannins and cardiac glycosides in *in vitro* leaves, field leaves and seeds of *L. frutescens*. Due to the variability of many factors including environmental conditions, harvesting season, sampling techniques, nutrient disparity and genetic differences, the extracts studied showed great differences in phytochemical yield. One of the major findings was that the *in vitro* extracts contained the largest quantities of all studied phytochemicals except for saponins. In addition, the presence of tannins, phlobatannins and cardiac glycosides was confirmed as well. Because of the concerns about the depletion of indigenous plants witnessed in the wild, extracts of *in vitro*-grown plants provide a possible alternative for bio-production. Further research is required to evaluate the potential of *in vitro* biomass production; an optimized yield of medicinal compounds was proven dependent of environmental, nutritional and growth factors that can be easily maintained in the laboratory conditions. The establishment of these types of medicinal plant cultivation protocols that result in the consistent yields of bioactive compounds will render invaluable commercial and research applications possible. Indisputable presence and apparent stability of phenolics, flavonoids, alkaloids and saponins in the *in vitro* extracts indicated that they could be biotechnologically produced in suitable quantities.

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