Research Letters

South African Journal of Science 102, May/June 2006 267

Bioactive components of *Rhoicissus tridentata*: a pregnancy-related traditional medicine

K.B. Brookes* and L.C. Katsoulis

*Rhoicissus tridentata* subsp. *cuneifolia*, or wild grape (Vitaceae), is one of the most commonly selected plant species for South African traditional medicines used during pregnancy and childbirth. Twenty compounds novel to the species were isolated from the plant, which has received little prior chemical investigation. Most of these compounds have documented health-promoting properties. Water extracts of roots show notable *in vitro* activity on isolated rat uterine smooth muscle tissue. Extracts exhibiting the highest activity were found to contain proanthocyanidin monomers: (-)-epigallocatechin, (+)-gallocatechin, (+)-catechin hydrate, (+)-mollissacacidin, (+)-epicatechin, (-)-fisetinidol and epicatechin-3-O-gallate; and dimers: procyanidin B3, procyanidin B4, fisetinidol-(4c:8)-catechin and fisetinidol-(4j:8)-catechin, as well as gallic acid and 74% polymeric proanthocyanidins. The relative amounts of proanthocyanidins, determined colorimetrically, were higher in summer than in winter, and roots harvested in summer also produced greater uterine activity. Glucose, and a partially identified hydrogel of glucose which also greatly stimulated uterine muscle contraction, were isolated in addition. Sitosterol and sitosterolin exhibited only slight oestrogenic activity. Oleanolic acid was isolated from a chloroform extract, and two further triterpenoids, 20(29)-lupen-20(29)-lupene, were isolated in addition. Sitosterol and sitosterolin exhibited only slight oestrogenic activity. Oleanolic acid was isolated from a chloroform extract, and two further triterpenoids, 20(29)-lupen-3-one and 20-e-pi-taraxastananol, and a sterol, 5,7-sitostol, were identified by gas chromatography–mass spectrometry. The plant growth hormone, triacontanol, was purified from an extract of young branches. The reported paralysis of the central nervous system attributed to *Rhoicissus tridentata* preparations is possibly linked to the action of sitosterol, sitosterolin and proanthocyanidins present in extracts.

Materials and methods

**Plant root extraction**

*Rhoicissus tridentata* roots were harvested in July (winter) from Tresaure Beach Grasslands, KwaZulu-Natal (voucher specimen number 086931, C.E. Moss Herbarium, University of the Witwatersrand, Johannesburg). Plant roots were milled or shredded and successively extracted by boiling for approximately one hour in solvents of increasing polarity, i.e. chloroform (or methylene chloride), methanol and water. Solvents were removed under vacuum below 50°C or by freeze-drying. Typical yields, based on the dry root mass, were: methylene chloride extract 0.5%; methanol extract 2.1%; water extract 3.8%. The yield of methanol-soluble material was highest in the summer season (~3.5%). The percentages of compounds isolated are also based on the dry root mass.

**Isolated organ preparations**

Virgin Sprague-Dawley rats weighing about 250 g were oestrogenized with stilboestrol i.p. 24 h before being killed with CO₂. Portions of uterus were mounted in Tyrode solution maintained at 26°C to decrease spontaneous contractility. Isotonic contractions of the organs were recorded electronically using labographs. The organs were pre-treated for 5 min with the herbal extract, or purified component, before cumulatively adding the reference drug, acetylcholine. The stock solution contained 2 g of dried plant extract in 15 ml water solution; the maximum dose administered was usually 0.7 ml. The maximal responses to the plant extract are expressed as a percentage of the maximal response to acetylcholine.

**Colorimetric determination of total proanthocyanidin content**

A rapid, reliable colorimetric method, adapted from that of the U.S. Grape Seeds Methods Evaluation Committee (http://www.nnfa.org/...
Proanthocyanidins were roots or branches, with leaves always selected. For comparable samples the richest sources of such as region and season of collection as well as part of the plant.

Purified components were generally characterized by spectroscopic techniques although some non-polar components were identified by GC-MS analysis. Isolation and identification of components

Column chromatography using silica gel 60 was routinely used for purification of components. In addition Amberlite XAD7 and Sephadex LH-20 were used to partially purify the proanthocyanidin Fraction A. Purified components were generally characterized by spectroscopic techniques although some non-polar components were identified by GC-MS analysis.

The isocratic HPLC system of 1:4 methanol:1% aqueous acetic acid at 1 ml/min was selected as the best method for separation of proanthocyanidins, using a Hichrom reversed phase C-18 column and a 254-nm fixed wavelength detector. Components in the methanol extract were identified by co-elution with the relevant proanthocyanidin standard. Agreement between the retention time for the unknown compound and the standard was generally within 0.1 min. Compounds with very long retention times were also run with less water in the system to give shorter retention times. Several runs were carried out in order to match a particular unknown compound with a standard.

Results and discussion

Twenty compounds novel to the species, many showing bioactivity, were isolated from extracts of *Rhoicissus tridentata*.

Total proanthocyanidin content

Complex oligomeric proanthocyanidins (OPCs) have presented a formidable analytical challenge. In 2001, after three years of investigation, the Grape Seeds Methods Evaluation Committee, which falls under the U.S. National Nutritional Foods Association, proposed methods for the quantification of OPCs. The recommended Porter and Procyanidin methods involve colorimetric measurement of the red anthocyanidin chromophore after HCl/butanol or HCl/isopropanol hydrolysis of proanthocyanidin dimers or larger polymers.

Our analytical method, based on those recommended above, involved hydrolysis with methanolic hydrochloric acid for estimating the relative proanthocyanidin content of extracts, or even the finely milled root samples themselves. Table I shows results of hydrolysis of a methanol extract at 80°C using ethanol as solvent. Reaction was incomplete even after 2 hours. Hydrolysis was found to be more rapid in methanol than in ethanol. At 90–95°C hydrolysis in methanol was complete in 1 hour, so these conditions were used for all subsequent hydrolyses. Porter and co-workers have also noted that the thiolysis reaction, used for cleavage of complex procyanidins, proceeded more readily in methanol than in ethanol as solvent.

The colour absorbance after hydrolysis, measured at 540 nm, varied greatly (from 918 to 40) and was influenced by factors such as region and season of collection as well as part of the plant selected. For comparable samples the richest sources of proanthocyanidins were roots or branches, with leaves always being a poor source. Branches could therefore be an alternative choice to roots for healers, thereby avoiding sacrificing the whole plant when harvesting.

The absorbance measured after colour development using milled roots collected in KwaZulu-Natal in summer was 73.5 and that for roots harvested in winter from the same area was only 37.1. Uterine activity is greatest for extracts of plants collected in the summer and autumn seasons, indicating that the concentrations of uterineactive chemicals is highest in the warmer months. The proanthocyanidin content, as shown by the red colour development, is also greatest in summer, implying that proanthocyanidins are the uterine active agents. Uterine activity induced by extracts also varied greatly, according to the geographical source of the plants.

Table 1. Hydrolysis of root extract in ethanolic HCl at 80°C.

<table>
<thead>
<tr>
<th>Hydrolysis time (min)</th>
<th>Absorbance at 540 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>1.65</td>
</tr>
<tr>
<td>45</td>
<td>2.22</td>
</tr>
<tr>
<td>60</td>
<td>2.58</td>
</tr>
<tr>
<td>90</td>
<td>2.80</td>
</tr>
<tr>
<td>120</td>
<td>2.89</td>
</tr>
</tbody>
</table>

Proanthocyanidin Fraction A

Roots harvested in October in KwaZulu-Natal were successively extracted with methylene chloride, methanol and finally water. The phenolic compounds extracted with methanol were also water soluble, but methanol is a more efficient solvent for their extraction from plant material. Column chromatography on silica gel 60 of 66 g of a methanol extract of roots harvested in July yielded a sugar fraction, mass 21 g (1.21%) eluted with chloroform:methanol 1:1. This fraction was identified as glucose by HPLC on a carbohydrate analysis column and also by thin-layer chromatography (TLC), by comparison with an authentic standard.

A second major component, Fraction A, was then eluted from the column with methanol, and further purified by dissolving in methanol and precipitating with ether to give 21 g (1.21%) of a reddish-brown powder after drying. Although Fraction A appeared fairly pure by HPLC and TLC, neither Fraction A nor its acetylated or methylated derivatives gave good NMR spectra, and indicated that it was actually a mixture of closely related phenolic compounds. Attempts to further purify Fraction A acetylated or methylated material via semi-preparative HPLC or silica gel chromatography were unsuccessful. A sample of 1.2 mg of the freeze-dried methanol extract was tested for the presence of ellagitannins. No red colour developed and no absorbance maximum was detected in the 600 nm region. In addition no carbonyl absorption was detected in the infrared spectrum of Fraction A.

Amberlite XAD7 effected a crude preliminary separation of the freeze-dried methanol extract. Sugars and salts (26%) were eluted with 0.1% aqueous HCl, and proanthocyanidin material (20%) with 40:60 or 60:40 methanol:water containing 0.1% HCl. Methanol containing 0.1% HCl was used to remove the remaining polymeric material (54%) from the resin. Alpha Laboratories, California, estimated this oligomeric proanthocyanidin content for the same methanol extract to be 55%, confirming the high polymeric content. Excluding sugars and salts, proanthocyanidin material constitutes 74% of the methanol extract, based on results from the Amberlite XAD7 separation. Column chromatography using Sephadex LH-20 (Fluka) was only partially successful in purifying the proanthocyanidin mixture,
with (+)-catechin being eluted by 1:1 ethanol:water in a fairly pure state, and identified by comparison with an authentic standard by HPLC and TLC. HPLC analysis showed approximately 25 components in Fraction A. HPLC is the recommended method for estimating relative amounts of proanthocyanidin monomers, oligomers and polymers (http://www.nnfa.org/services/science/grape seed 09-01.htm), and has been used by many researchers.15,18–21 HPLC was found useful for identifying 10 monomers and dimers, common in Vitaceae, by co-elution with the relevant standard (Table 2). In addition, HPLC analysis at 280 nm by Alpha Laboratories showed four common monomers: catechin (0.85%), epicatechin (0.17%), gallic acid (0.20 %) and epicatechin-3-O-gallate (0.08%). The latter two standards were not available for our analyses. The former two results confirmed our own findings, although percentages were lower. Structures are shown in Fig. 1.

Hydrogel Fraction B

Fraction B was obtained by water extraction of roots which had been extracted previously with methylene chloride and methanol. It was obtained as a brown syrup after removal of water, and purified by adding methanol and stirring to produce a white, gum-like solid, m.p. 260° (decomp.), which shrank in size remarkably while drying, and was approximately 2% by mass of the roots extracted. Solutions of this material were viscous and jelly-like. TLC of Fraction B in methanol:acetic acid 10:1, giving a pale green spot on the origin with anisaldehyde reagent, whereas sucrose gave a green spot with \( R_f \) 0.64. The only product identified after hydrolysis of 1 g of Fraction B in 50 ml of 2 M HCl for 3 h at 90° was glucose, recrystallized from water/acetone, and identified by comparison with a standard by HPLC and TLC. The dried hydrolysis product was also acetylated with pyridine/acetic anhydride and recrystallized from methanol to form white crystals, m.p. 118–120°C, identified22 as \( \alpha \)-D-penta-O-acetylglucopyranose, m.p. 119–120°C.

Non-polar fraction

Sitosterol (0.009%) and its glucoside, sitosterolin (0.013%), were obtained from methanol extracts by elution from silica gel with hexane:ether (1:1) and chloroform:methanol (10:1), respectively. They were identified from comparison of melting points, TLC and spectral data with those of an authentic standard in each case. GC–MS analysis of the non-polar fraction also revealed the presence of \( \gamma \)-sitosterol and two related triterpenoids, lupen-3-one and 20-epi-\( \psi \)-taraxastanol.

Oleanolic acid (0.003%) was purified from a chloroform extract of shredded roots and branches. It was eluted from silica gel with 20:1 chloroform:methanol and was identified by comparing IR, \(^1\)H NMR and \(^13\)C NMR data for the acid, as well as its acetate and methyl ester derivatives, with literature data.23–25 Sterols and triterpenoids are shown in Fig. 2.

Triacontanol (0.026%) was eluted with chloroform:methanol (5:1) from a silica gel column of a chloroform:methanol extract of branches. Recrystallization from methanol yielded a white powder, m.p. 93–95°C, identified as triacontanol, together with sitosterol, lupeol and taraxerol.26 The IR spectrum indicated a primary alcohol.27 NMR and MS data for the alcohol and its acetate confirmed the structure \( CH_3(CH_2)_{29}OH \) for triacontanol.

Effects on the uterus

The stimulatory effects of Fraction A on uterine muscle tissue are shown in Fig. 3. The response of the uterus to extracts prepared with different solvents is shown in Fig. 4. The marked seasonal variation in the effects of these uterotropic proanthocyanidins should be considered in the preparation of herbal medicines.

Proanthocyanidin polymers constituted 74% of the methanol extract, excluding sugars and salts.

---

### Table 2. HPLC of proanthocyanidins in methanol extract. Mobile phase: methanol:1% aqueous acetic acid (1:4), 1 ml/min.

<table>
<thead>
<tr>
<th>( R_t ) (min)</th>
<th>Quantity (%)</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>1.04</td>
<td>(-)-Epigallocatechin</td>
</tr>
<tr>
<td>7.4</td>
<td>0.54</td>
<td>(+)-Gallocatechin</td>
</tr>
<tr>
<td>10.3</td>
<td>0.61</td>
<td>Procyanidin B3</td>
</tr>
<tr>
<td>15.3</td>
<td>0.88</td>
<td>Procyanidin B4</td>
</tr>
<tr>
<td>16.8</td>
<td>2.03</td>
<td>(+)-Catechin hydrate</td>
</tr>
<tr>
<td>18.5</td>
<td>1.79</td>
<td>(+)-Mollisacacidin</td>
</tr>
<tr>
<td>22.5</td>
<td>0.88</td>
<td>(+)-Epicatechin</td>
</tr>
<tr>
<td>33.8</td>
<td>0.10</td>
<td>Fisetinidol-(4e-8) catechin</td>
</tr>
<tr>
<td>35.6</td>
<td>0.33</td>
<td>(+)-Fisetinidol</td>
</tr>
<tr>
<td>50.9</td>
<td>0.24</td>
<td>Fisetinidol-(4β-8)catechin</td>
</tr>
</tbody>
</table>
These polymeric proanthocyanidins, or condensed tannins, produce astringent effects, which can account for their stimulatory action on uterine tissue. There are many references to the effect of tannins on female organs, particularly on the uterus during pregnancy. Tannins in various plant preparations such as raspberry extracts are credited with strengthening and toning the muscles of the uterus. Polymeric proanthocyanidins can be distinguished from hydrolysable tannins, which contain gallate or hexahydroxybiphenyl ester moieties. The latter exhibit a carbonyl absorption in their infrared spectra, which is absent for the former compounds. The absence of a carbonyl absorption in the IR spectrum of the uteroactive Fraction A eliminates the possibility of hydrolysable tannins being present in significant amounts. In addition, a colorimetric test for ellagitannins was negative. We therefore concluded that the principal spasmogens in this fraction are the proanthocyanidins.

Calcium ions play a critical role in muscle activity, with sudden changes in concentration initiating contraction. Potassium ions are also involved because they affect the electrical excitability of muscle cells. However, the calcium and potassium ion concentrations in the plant extract, after dilution in the organ bath, were 0.0039 and 0.0250 mmol/l, respectively, and were too low to have any direct effect on the isolated smooth muscle preparation, as established with standard solutions. This implies that contractions were stimulated by pharmacologically active compounds within the plant extract, and not by the action of ions. However, the presence of these ions in healers’ decoctions is likely to enhance uterine contractility, in conjunction with the effects of the uteroactive chemicals.

Fraction B, a partially characterized hydrogel polymer of glucose, stimulated uterine contractions over the full range of acetylcholine concentrations (Fig. 3). Various hydrogels, such as the natural gels lining the intestine and uterus, play a direct role in muscle contraction. Cervical mucus, for example, is a complex hydrogel which is important in sperm transport. A common property of all gels is the ability to undergo abrupt large changes in volume in response to small changes in external conditions, e.g. chemical or electrical changes. The rates of contraction and consequent forces generated by certain synthetic gel fibres approximate those of human muscle. A water-soluble glycoside from Cissus quadrangularis, also from the Vitaceae family, is reported as non-toxic when administered orally to mice, rats and guinea-pigs. However, it caused convulsions and death within five minutes of intravenous administration. This glucoside is possibly similar to Fraction B. Whereas it might over-stimulate muscle tissue when directly entering the bloodstream, it could be non-toxic when taken orally due to partial hydrolysis and dilution during digestion. Fraction B warrants further investigation.

Oestrogen levels increase during pregnancy and contribute to greater uterine contractility during labour. Structurally related compounds, such as certain stilbene derivatives, e.g. trans diethylstilbestrol, are potent oestrogenic substances. Two stilbene glycosides, combretastatin A-1 and B-1 glucosides, isolated from Combretum kraussii roots, contributed to its...
stimulatory effect on uterine tissue.\textsuperscript{24} Sitosterol and its glucoside sitosterolin, with structural similarities to the oestrogens, were isolated from \textit{Rhoicissus tridentata} extracts as well as several other, unidentified sterols. Both sitosterol (Fig. 3) and its glucoside sitosterolin showed only slight oestrogenic activity, increasing the response of the uterus by about 2\% at concentrations of acetylcholine below $10^{-4}$M and $10^{-3}$M, respectively. However they both inhibit this response at higher acetylcholine concentrations. The methylene chloride extract, containing several such non-polar sterols, also slightly stimulated the uterus at low concentrations, but showed a marked inhibition (64\%) of uterine activity at higher acetylcholine concentrations. This contrasts with the water extract used by healers, which enhances the response of the uterus at all concentrations. Figure 2 shows the response of uterine muscle tissue to extracts prepared by successive extraction of branches with methylene chloride, methanol and, finally, water.

There is some disagreement in the literature about the role of sitosterol, which is reported to exert oestrogenic effects, possibly even causing abortion.\textsuperscript{77} However, sitosterol and its glucoside are also claimed to be inactive on uterine tissue or to inhibit spasmogens.\textsuperscript{30-32} Our results suggest that the concentration of acetylcholine used for tests is the deciding factor as to whether there is a slight stimulation or inhibition. Possibly at higher acetylcholine concentrations there is competition for receptor sites.

Oleanolic acid, identified in another extract, is structurally related to the utoactive compounds oleanonic acid and 3-epi-oleanolic acid isolated from \textit{Ekebergia capensis},\textsuperscript{36} so is possibly also utoactive.

\subsection*{Health effects}
Proanthocyanidins are amongst the most powerful antioxidants in nature and have well-documented health benefits for the heart, cardiovascular system and immune system. These findings support the traditional healers’ claims\textsuperscript{6} that the species used in decoctions, promote general good health during pregnancy.

Some of the non-polar constituents have interesting physiological features that could also contribute to the health-promoting properties of \textit{isihlambezo} decoctions. Sitosterol and sitosterolin are reported to boost the immune system.\textsuperscript{79} Powerful antioxidant as well as antioxidant activity has been attributed to lupenone, identified in several plant species, one being the Korean mushroom, \textit{Daedaleopsis tricolour}.\textsuperscript{40} Lupane, oleanolic and taraxastane triterpenoids arise from a common baccarane precursor. The three triterpenoids identified in \textit{Rhoicissus tridentata}, lupen-3-one, oleanolic acid and 20-epi-\textit{t}-tara-xastanol, are thus biogenetically related. Triterpenoids such as these are well-known for their anti-inflammatory properties.\textsuperscript{41} The fatty alcohol triacontanol, found in young branches, is a naturally occurring plant growth hormone which also has cholesterol-lowering properties. A mixture of hexacosanol (C26), octacosanol (C28) and triacontanol (C30), three higher primary aliphatic alcohols from sugar cane (\textit{Saccharum officinarum} L.), induced cholesterol-lowering effects in rabbits, by affecting the rate-controlling enzyme.\textsuperscript{42}

Moreover, there are isolated reports that \textit{Rhoicissus tridentata} preparations cause central nervous system (CNS) depressant paralysis and respiratory arrest.\textsuperscript{7} This is possibly due to the action of sterols such as sitosterol or sitosterolin and proanthocyanidins, which are known to affect nerve signal transmission and receptor binding.

Sitosterol and sitosterolin were also found in leaf extracts of \textit{Byssomima crassifolia}, which cause CNS depressant and hypotensive effects.\textsuperscript{20} In a search for the CNS-active ingredients in the root bark of \textit{Alangium plantanifolium}, five compounds were isolated,\textsuperscript{43} two being sitosterol and sitosterolin. Sitosterol was found to bind directly to muscarinic receptors with consequent inhibition of nerve signal transmission, supporting the finding that roots of \textit{Alangium} species showed significant muscle relaxant effects in dog. In addition, sitosterol and sitosterolin were able to interact with control compounds such as mesulergin and cause large increases in their binding activities to five types of receptors.

Another factor to consider is that tannins or polyphenols, which are major constituents of \textit{Rhoicissus tridentata} extracts, generally show non-specific binding to proteins, and may also affect receptor binding,\textsuperscript{44} so influencing the CNS.

It is clear therefore that there are several polar and non-polar compounds in \textit{Rhoicissus tridentata} extracts with the means to affect the CNS either directly or indirectly. There is likely to be a complex synergistic effect between the various plant ingredients, which could explain the reported CNS depression. Although cell-line studies\textsuperscript{41,42} have shown that medicinal decoctions of the plant are non-toxic, the seasonal variation in the concentrations of polymeric proanthocyanidins in plants should be taken into account to avoid both uterine hyperstimulation or other adverse medical effects such as CNS depression.

\subsection*{Conclusions}
The proanthocyanidin Fraction A and the hydrogel Fraction B, which both contain polyhydroxy polymeric material, were the principal spasmogens in \textit{Rhoicissus tridentata} extracts, and enhanced the effect of acetylcholine at all concentrations tested. Although these water-soluble fractions are non-toxic when taken orally in solution, they have the capacity to cause uterine hyperstimulation, with potential medical complications. Traditional healers need therefore to consider the higher concentration of proanthocyanidins in summer compared to winter when preparing medications. Branches are also a good source of proanthocyanidins, so could be an alternate choice to roots for healers. Sitosterol, sitosterolin and the methylene chloride extract showed oestrogenic effects, but only slightly stimulated uterine muscle tissue at low acetylcholine concentrations, inhibiting this response at higher concentrations.

Several compounds found in \textit{Rhoicissus tridentata} extracts with known health benefits support the traditional healers’ claims that \textit{isimuzi} promotes good health during pregnancy. Proanthocyanidins are powerful antioxidants, beneficial for the heart, cardiovascular and immune systems. Powerful antioxidant as well as antioxidant activity has been attributed to lupenone. Triacontanol, a plant growth hormone, has cholesterol-lowering properties, and triterpenoids such as oleanolic acid, lupenone and taraxastananol are known for their anti-inflammatory action.

A complex synergistic or interactive effect between bioactive plant ingredients, such as sterols, sterol glucosides and polyphenols, could explain isolated reports of CNS depression caused by crude extracts of \textit{Rhoicissus tridentata}.

The generous assistance of the following institutions and persons is gratefully acknowledged: the National Research Foundation and Technikon Mangosuthu for funding this research; K.H. Pegel (formerly of the University of Natal, Durban) for donating sitosterol, sitosterol glucoside and (+)-catechin standards; E.V. Brandt (University of the Free State) for providing 10 proanthocyanidin standards; and D. Ferreira of Mississippi University for assistance and advice on column chromatography of proanthocyanidins.

Received 16 May 2005. Accepted 16 March 2006.


