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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF MOLLUSCICIDAL SAPONINS FROM *PHYTOLACCA DODECANDRA* (PHYTOLACCACEAE)

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SUMMARY

A high-performance liquid chromatographic method is described for the determination of oleanane saponins in *Phytolacca dodecandra* (Phytolaccaceae), a plant with potent molluscicidal properties. The molluscicidal monodesmosidic saponins of the berries were determined at 254 nm as their 4-bromophenacyl derivatives, whereas the non-derivatized bidesmosidic saponins, lacking a free carboxyl group, were determined at 206 nm. A comparison of different extraction procedures showed that with cold water predominantly monodesmosidic saponins were obtained, whereas hot water gave mainly bidesmosidic (non-molluscicidal) saponins.

INTRODUCTION

Molluscicides of plant origin are currently of great interest for the potential focal control of schistosomiasis in endemic countries[1]. This widespread tropical disease, affecting over 200 million people, is caused by parasites which require aquatic snails as intermediate hosts for their transmission. Some saponin-containing plants are particularly toxic to these snails and fulfil the majority of criteria for an effective plant molluscicide. One of the most promising and intensively studied of these plants is *Phytolacca dodecandra* l'Hérit (Phytolaccaceae), from Ethiopia[2]. A number of molluscicidal monodesmosidic and non-molluscicidal bidesmosidic saponins have been isolated from the berries of *P. dodecandra*[3–5] and a high-performance liquid chromatographic (HPLC) method has been developed for their determination[6]. The aim of this work was to determine quantitatively the content of saponins (Table I) in *P. dodecandra*. This is necessary for several reasons, the most important being the investigation of plant material from different strains and geographical locations, as it is advisable to maximize the content of molluscicidal saponins for the most effective treatment of infected sites. Quantitative analysis is also required in order to evaluate

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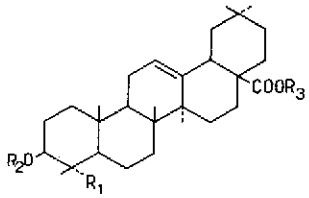
TABLE I

STRUCTURES OF SAPONINS



Compound	R_1	R_2*	R_3
1	CH_3–	Rha–(2)Glc–(2)Glc– (4)Glc	H–
1a	CH_3–	Rha–Glc–Glc– | Glc	Br–C$_6$H$_4$–$COCH_2$–
1b	CH_3–	Rha–Glc–Glc– | Glc	Glc–
2	CH_3–	Glc–(2)Glc– (4)Glc	H–
2a	CH_3–	Glc–Glc– | Glc	Br–C$_6$H$_4$–$COCH_2$–
2b	CH_3–	Glc–Glc– | Glc	Glc–
3	CH_3–	Gal–(3)Glc– (4)Glc	H–
3a	CH_3–	Gal–Glc– | Glc	Br–C$_6$H$_4$–$COCH_2$–
3b	CH_3–	Gal–Glc– | Glc	Glc–
4	$HOCH_2$–	Glc–(2)Glc– (4)Glc	H–
4a	$HOCH_2$–	Glc–Glc– | Glc	Br–C$_6$H$_4$–$COCH_2$–

* Rha = α-L-rhamnopyranosyl; Glc = β-D-glucopyranosyl; Gal = β-D-galactopyranosyl.

the efficiency of different extraction methods, involving changes of solvent, temperature and time. Information on the content of active saponins is essential for biodegradation and toxicological studies.

EXPERIMENTAL

Materials

Acetonitrile and methanol were of LiChrosolv grade (Merck, Darmstadt, F.R.G.). Pure saponins were previously isolated from *P. dodecandra*[4,5].

Preparation of saponin derivatives

Saponin (*ca.* 5 mg) was dissolved in water (2 ml) containing 1 mg of potassium hydrogencarbonate. The solution was lyophilized and treated with 1 ml of a mixture of 4-bromophenacyl bromide (Fluka, Buchs, Switzerland) (3.5 g) and 18-crown-6 (Fluka) (680 mg) in acetonitrile (100 ml). After refluxing at 100°C for 90 min and subsequent cooling, by-products were removed by passing the reaction mixture through a short column (2 cm × 1 cm I.D.) of Kieselgel 60 (15–40 μm) (Merck, Darmstadt, F.R.G.) and eluting first with dichloromethane (10 ml), followed by chloroform–methanol (1:1) (10 ml)[7]. Final purification was achieved by low-pressure liquid chromatography on a Lobar LiChroprep Si 60 (40–63 μm) column (13 cm × 1 cm I.D.) (Merck) with chloroform–methanol (9:1) as eluent.

For plant material, the lyophilized extract (20 mg) was treated in a similar fashion to the pure saponins, without the final column chromatographic step.

Apparatus

HPLC–UV analyses were carried out on a system consisting of a Spectra-Physics (San Jose, CA, U.S.A.) 8700 pump, a Rheodyne injector, a Hewlett-Packard (Palo Alto, CA, U.S.A.) 1040A photodiode array detector, an HP-85 computer and an HP 7470A plotter. Quantitative analyses were performed with Waters Assoc. (Milford, MA, U.S.A.) 6000A pumps, a Waters Assoc. 480 UV spectrophotometer, a Waters Assoc. 720 system controller and a Waters Assoc. 730 data module. Separations were performed on 10-μm μBondapak C_{18} (30 cm × 3.9 mm I.D.), 4-μm NovaPak C_{18} (15 cm × 3.9 mm I.D.) and 5-μm RP-8 (25 cm × 4.6 mm I.D.) (Knauer, Bad Homburg, F.R.G.) columns.

Chromatographic conditions

For non-derivatized saponins and extracts, a Knauer 5-μm RP-8 column was used, with a linear gradient from 30% to 50% of acetonitrile in water over 60 min, an elution rate of 1.5 ml/min and detection at 206 nm. Saponins were dissolved in methanol at a concentration of 1 mg/ml and extracts at 2 mg/ml. Samples of 10 μl were injected.

For derivatized saponins and extracts, RP-18 NovaPak and μBondapak columns were used, with a linear gradient from 40% to 70% of acetonitrile in water, an elution rate of 1 ml/min and detection at 254 nm. For injection 10-μl samples of 1 mg/ml saponin solutions or 5 mg/ml extract solutions were employed.

Extractions

In each instance, 1 g of dried berries was treated with 100 ml of water. The extract (50 ml) was filtered, lyophilized and weighed.

Method 1. Ground berries were extracted at 20°C for 24 h.

Method 2. Whole berries were heated at 90°C for 24 h in a stoppered flask.

Method 3. Ground berries were extracted under reflux for 2 h.

Method 4. Ground berries were extracted under reflux for 12 h.

Quantitative determination

For quantification purposes, derivatized saponins 1a, 2a and 3a were used as standards, together with the bidesmosidic saponins 1b, 2b and 3b. After the establishment of calibration graphs (obtained from the respective peak areas at different injected concentrations; least-squares analysis gave correlation coefficients of 0.998 for 1a, 0.999 for 2a and 0.999 for 3a), the amounts of the individual saponins in the extracts were determined as percentages of the total extracts. The amounts of the other saponins were calculated by summing the areas of the remaining minor saponin peaks in the chromatogram, using 2a and 2b as standards. HPLC runs were carried out in triplicate to obtain average results.

RESULTS AND DISCUSSION

Separations of saponins on reversed-phase columns have previously been carried out with detection at 206 nm owing to their poor absorption at higher wavelengths[6]. Consequently, there are limitations concerning the solvents and gradients that can be used. HPLC of an aqueous *P. dodecandra* extract (90°C, 24 h) with an acetonitrile–water gradient demonstrates the problems of baseline drift at 206 nm (Fig. 1). The bidesmosidic saponins elute between 10 and 20 min but the monodesmosidic saponins elute later and are consequently much more difficult to quantify. A three-dimensional representation of this chromatogram (Fig. 2) shows the rapid decrease in UV absorption of the saponins when moving to higher wavelengths.

Refractive index detection is not practicable under these conditions, so an

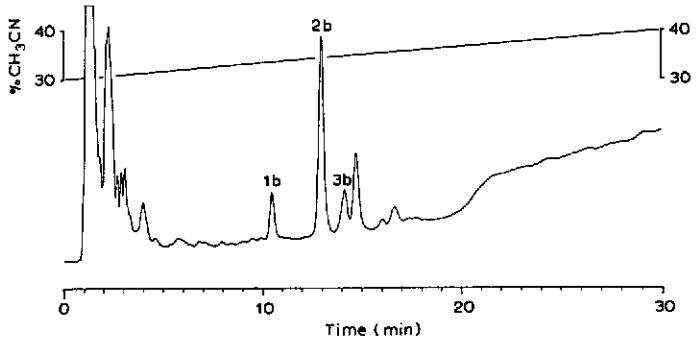


Fig. 1. HPLC analysis of an aqueous extract (method 2) of *Phytolacca dodecandra* berries. Column, Knauer RP-8, 5 μm (25 cm × 4.6 mm I.D.); 30–40% acetonitrile over 30 min; flow-rate, 1.5 ml/min; detection, 206 nm.

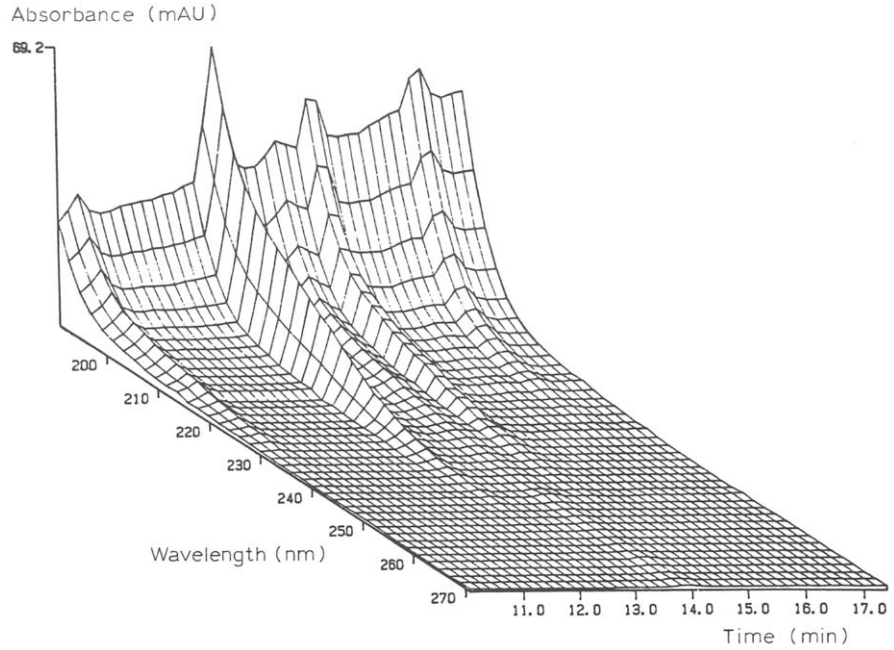


Fig. 2. Three-dimensional representation of a part of the chromatogram shown in Fig. 1.

alternative is to derivatize the saponins with a chromophore which facilitates UV detection at 254 nm. As the monodesmosidic saponins from *P. dodecandra* (which are responsible for the molluscicidal activity) possess a free carboxyl group at the C-28 position, derivatization can be carried out at this function. The approach used here is to introduce a 4-bromophenacyl chromophore by a reaction involving 4-bromophenacyl bromide and a crown ether (see Experimental). This method has previously been employed for the analysis of fatty acids[8] and prostaglandins[9]. The structures of four chosen derivatized pure saponins (1a, 2a, 3a, 4a) from *P. dodecandra* are shown in

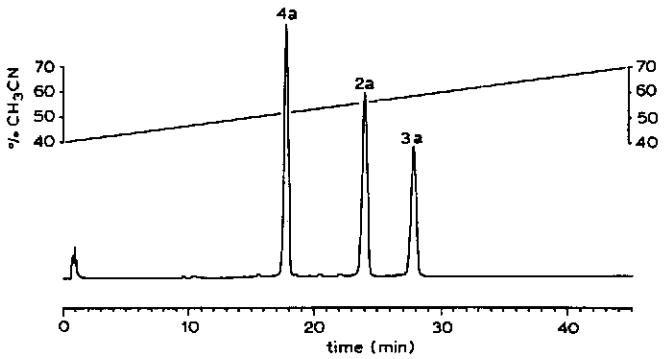


Fig. 3. Separation of derivatized saponins 2a, 3a and 4a. Column, NovaPak C_{18}, 4 μm (15 cm × 3.9 mm I.D.); 40–70% acetonitrile over 45 min; flow-rate, 1 ml/min; detection, 254 nm.

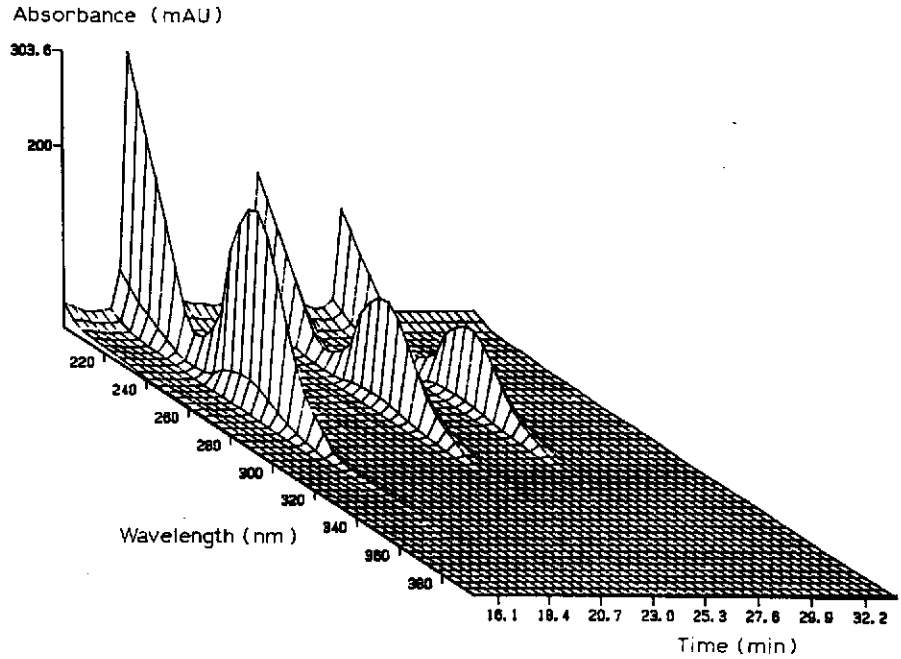


Fig. 4. Three-dimensional representation of the separation of derivatized saponins 2a, 3a and 4a shown in Fig. 3.

Table I. The four saponins selected for derivatization are found in appreciable amounts in *P. dodecandra* extracts and can be used for standardization and identification purposes. A mixture of three of these saponins was separated on an RP-18 column with detection at 254 nm, using a gradient from 40% to 70% of acetonitrile in water over 45 min without any baseline drift (Fig. 3). The three-dimensional chromatogram (Fig. 4) shows the excellent UV absorption of the derivatized saponins at 254 nm.

For quantification of the saponins in *P. dodecandra* extracts, two methods were considered: (a) use of the derivatives of the three isolated saponins as external standards; (b) use of naphthalene as internal standard. Comparison of the results showed virtually identical values for the two methods. Consequently, method (a) was employed throughout. Mixtures of the three derivatized saponins 1a, 2a and 3a were injected at different concentrations onto the HPLC column and the surface area under each peak was plotted against concentration to obtain the calibration graphs illustrated in Fig. 5.

Calculation of the percentages of saponins 1, 2 and 3 in any extract is therefore a relatively straightforward matter, after derivatizing the extract and performing a preliminary purification step before HPLC analysis.

The bidesmosidic saponins lack a free carboxyl functionality and therefore cannot be derivatized by the method employed for the monodesmosidic saponins. In order to quantify extracts containing bidesmosidic saponins, HPLC analyses were performed at 206 nm. By constructing calibration graphs with the pure glycosides 1b, 2b and 3b, the percentages of 1b, 2b and 3b in the extracts were ascertained.

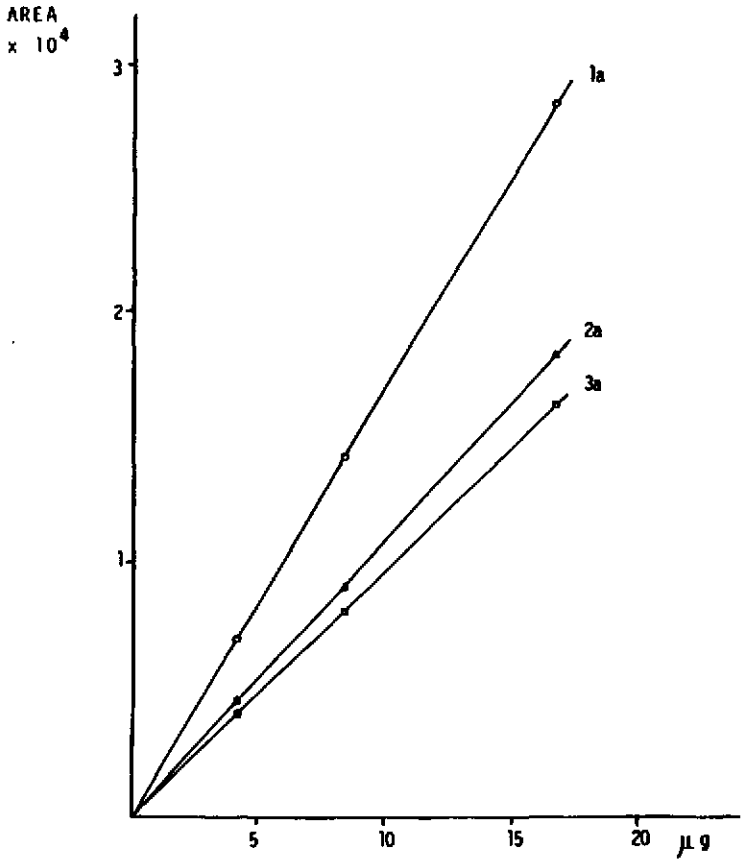


Fig. 5. Calibration graphs for derivatized saponins 1a, 2a and 3a. For conditions, see Fig. 3.

An aqueous extract of *P. dodecandra* berries (strain no. 17) was derivatized and subjected to preliminary purification (see Experimental) before analysis on a NovaPak C_{18} column (Fig. 6). The individual derivatized monodesmosidic saponins 1a–4a were clearly separated with a gradient of acetonitrile and water. Chromatography on a µBondapak C_{18} column gave a lower resolution of the peaks and a less satisfactory result. Analysis of the aqueous extract of another strain (no. 44) of *P. dodecandra* berries by the derivatization method is shown in Fig. 7. Although the modified saponins 1a, 2a and 3a are present, some of the other saponins found in strain 17 are missing, most notably the hederagenin glycoside 4a. Hence this analytical method is useful for both qualitative and quantitative purposes.

Different methods of extraction of *P. dodecandra* berries with water were investigated (it has already been established that extraction of the berries with methanol provides mainly bidesmosidic saponins[4,6]) in order to find both the weights of the extracts and the saponin compositions. The results are summarized in Table II.

The largest amount of extract was obtained by method 4, which involved refluxing ground berries with water for 12 h at 100°C (see Experimental). Extraction by method 1 gave both mono- and bidesmosidic saponins, whereas methods 2–4 gave

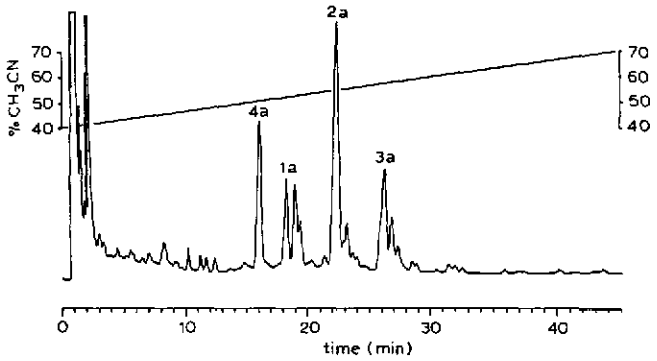


Fig. 6. HPLC analysis of the derivatized aqueous extract (method 1) from *Phytolacca dodecandra* berries (strain 17). Column, NovaPak C_{18}, 4 μm (15 cm × 3.9 mm I.D.); 40–70% acetonitrile over 45 min; flow-rate, 1 ml/min; detection, 254 nm.

only bidesmosidic saponins. Thus elevated temperatures increase the yield of extract but produce only bidesmosidic saponins. Kinetic experiments showed that the temperature limit for obtaining monodesmosidic saponins appeared to be *ca.* 60°C. A possible explanation for this phenomenon is that enzymes present in the berries cleave the glycosidic chain at position C-28 during extraction with cold water but that on heating the water the enzymes are deactivated and hydrolysis of the ester-linked sugars does not occur. Extraction of ground berries with cold water gave approximately 30% of total saponins by weight of extract. Saponin 2 constituted nearly 19% of the extract. All the hot water extraction methods gave a total of *ca.* 50% saponins as a percentage of the water extract. Bidesmosidic saponin 2b was the most important constituent, in amounts varying from 27 to 33% of the total extract. Extraction of non-ground berries (not shown) at 20°C gave 21% less extract than at 90°C. Thin-layer chromatography of the extracts obtained by method 1, 2 or 3 confirmed the presence or absence of saponins 1–3, with monodesmosidic saponins only detectable after method 1.

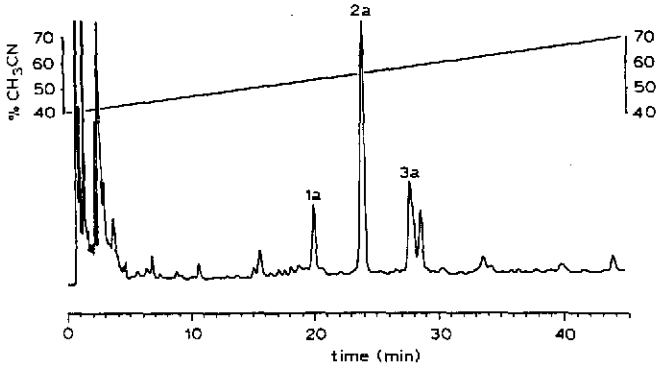


Fig. 7. HPLC analysis of the derivatized aqueous extract (method 1) from *Phytolacca dodecandra* berries (strain 44). Conditions as in Fig. 6.

TABLE II

PERCENTAGES OF SAPONINS IN *PHYTOLACCA DODECANDRA* AQUEOUS EXTRACTS

Method	*Total weight of extract (g)*	*Saponin in total extract (%, w/w)*						
		1a	*2a*	*3a*	*1b*	*2b*	*3b*	*Remaining saponins*
(1) 20°C, 24 h	0.413	3.5	18.9	3.6	*	*	*	5.4
(2) 90°C, 24 h	0.533	–	–	–	6.4	28.8	4.3	10.8
(3) 100°C, 2 h	0.500	–	–	–	8.3	32.9	4.5	9.9
(4) 100°C, 12 h	0.573	–	–	–	6.2	27.2	4.	9.8

* Bidesmosidic saponins not calculated by this procedure.

CONCLUSIONS

An HPLC method has been developed that permits the quantitative determination on reversed-phase supports of saponins in the molluscicidal plant *Phytolacca dodecandra* l'Hérit (Phytolaccaceae). The monodesmosidic saponins, which are also responsible for the molluscicidal activity of *P. dodecandra* berries, are quantified by means of their 4-bromophenacyl derivatives, permitting HPLC detection at 254 nm. Thus the concentrations of the most active saponins 2 and 3, with molluscicidal activities of 6 and 3 mg/ml, respectively[4], can be determined in crude plant extracts. The content of bidesmosidic saponins is calculated by HPLC analysis at 206 nm, without derivatization but using standard pure saponins.

This HPLC procedure involving derivatization with 4-bromophenacyl bromide can be extended to any saponins, provided either the aglycone or the sugar moiety contains a free carboxyl group.

A comparison of different aqueous extraction procedures for *P. dodecandra* berries showed that measureable amounts of monodesmosidic saponins were obtained uniquely at ambient temperatures. Extractions with hot water produced only bidesmosidic saponins, presumably owing to inactivation of the enzymes responsible for cleaving the glycosidic chain in position C-28 of the triterpene moiety. This important observation is relevant to the problem of schistosomiasis, as obviously only cold water extracts which contain monodesmosidic saponins will have any application as plant molluscicides. Thus, pounding the berries with cold water, the most practicable method of obtaining a vegetable molluscicide in endemic regions, conveniently provides the greatest concentration of saponins from *P. dodecandra* for application to sites of infestation by transmitter snails.

For toxicological and other investigations, it is therefore easy to reproduce field extraction conditions and then rapidly quantitate standard extracts for their respective active saponins by HPLC before submitting them to the tests required.

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