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TRITERPENE AGLYCONES FROM VARIOUS *PHYTOLACCA DODECANDRA* POPULATIONS

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Key Word Index—*Phytolacca dodecandra*; Phytolaccaceae triterpene; saponin; microanalysis; molluscicide.

Abstract—Fifteen samples of *Phytolacca dodecandra* collected over a wide geographical range were evaluated. The triterpenoids were obtained from hydrolysis of the saponins from a single berry. The analyses were carried out by gas chromatography of the methyl derivatives. The results of the analysis of the total aglycone derivatives divided the population into the high oleanolic acid group that contained more than 80% (mean, 89 ± 6%) oleanolic acid and the low oleanolic acid group that contained less than 66% (mean, 53 ± 7%) oleanolic acid.

INTRODUCTION

The potent molluscicidal activity and high yield of crude triterpene saponins (over 25% dry weight) from *Phytolacca dodecandra* attracted the attention of Lemma [1] over 20 years ago. Early field studies in Ethiopia showed the dramatic effect of these molluscicidal saponins in controlling the vector of schistosomiasis [2]. The fruit (berries) of *P. dodecandra* (endod in Ethiopian) have been used in Africa for centuries as a soap for washing clothes; since then, the saponins have been found to be fungicidal [3] and emetic [3] larvicidal for the mosquito [4] and a potent spermicide [5]. Continued interest in the biology of this plant has uncovered female antifertility activity [6] and prompted a more careful look at the chemistry and botany [3, 7].

The proceedings of the 1983 workshop on *P. dodecandra* dealing with research on the chemical, toxicological, molluscicidal and agronomic aspects of this plant including field trials have raised even greater interest [3]. The endod bush has been identified as one of the most promising plants to be used for the local control of schistosomiasis on a self-help basis [8]. Work has continued on the chemistry of the active components [3, 7, 9], the toxicology [10], extraction [11], agriculture [7] botany [12] and additional field studies [13]. Many rural villages in endemic areas have already started or are anxious to start self help programs with endod [14]. This brings considerable pressure on the scientific community to have answers to most efficient use, and environmental impact of the wide spread use of endod.

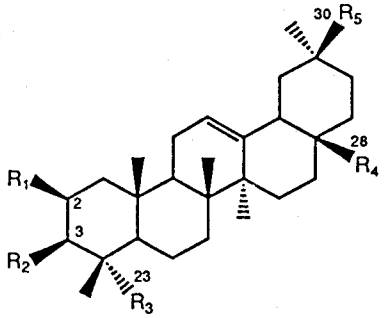
Early recognition [15] that there were different varieties of endod plants growing wild throughout Africa has led to the selection of three types: 3, 17, and 44 as standards—each with its own unique characteristics of high yield, high molluscicidal activity and resistance to drought and insects. *Phytolacca dodecandra* seems to separate itself from most other *Phytolacca* species in having mostly triterpene 28-monocarboxylic acid saponins (Fig. 1) rather than the 28,30-dicarboxylic acids [16–18]. Oleanolic acid, 2-hydroxyoleanolic acid, hederogenin and bayogenin have been identified in the aglycones [17] of a crude saponin extract obtained from berries purchased for use as a soap on the open market in Addis Ababa (1970). Because this determination was done on a crude saponin mixture obtained from *ca* 100 kg scale extraction of diverse wild plants, no information was obtained on the variations of aglycones within a plant type.

It was of interest to determine the triterpene skeleton and the patterns of oxidation at the various carbon positions, rather than attachments of sugars or the esterification of the carboxylic acid groups. It was felt that a rather simple straightforward method would be more useful for distinguishing plant types, and glycosidation and esterification would unnecessarily complicate the picture and be more subject to variation than skeletal changes. The complete separation and identification of all the triterpene products was also hoped to be unnecessary for distinguishing the various plant types.

The purpose of this study was to determine the variation in the aglycones from the several standard types as well as wild types of *P. dodecandra* using gas chromatography. If possible, this should give an objective chemotaxonomic method for distinguishing berries from these important types, even though the plant is not available for inspection and this would provide a method for monitoring accidental outbreeding of cultivated plants.

RESULTS AND DISCUSSION

The mean area of the oleanolic acid methyl ether methyl ester peak ($R_t = 2.40$ min) as a percentage of the total permethylated aglycones that were eluted from the 1.8 m packed column at 270° for each type of berry is shown in Table 1. The total area of methylated triterpene aglycones includes permethyl derivatives of 2-hydroxyoleanolic acid ($R_t = 2.93$ min), hederogenin ($R_t = 3.02$ min) and bayogenin ($R_t = 3.66$ min) as well as a



28-monocarboxylic acids $R_4 = CO_2H$; $R_5 = CH_3$
28,30-dicarboxylic acids $R_4 = CO_2H$; $R_5 = CO_2H$

oleanolic acid	$R_1 = H$	$R_2 = OH$	$R_3 = CH_3$	$R_4 = CO_2H$	$R_5 = CH_3$
2-hydroxyoleanolic acid	$R_1 = OH$	$R_2 = OH$	$R_3 = CH_3$	$R_4 = CO_2H$	$R_5 = CH_3$
hederogenin	$R_1 = H$	$R_2 = OH$	$R_3 = CH_2OH$	$R_4 = CO_2H$	$R_5 = CH_3$
bayogenin	$R_1 = OH$	$R_2 = OH$	$R_3 = CH_2OH$	$R_4 = CO_2H$	$R_5 = CH_3$

Fig. 1. Triterpene derivatives.

Table 1. Oleanolic acid as a per cent of total aglycones for types of berries by location

Type of berry	% Oleanolic derivative
P. heptandra	
South Africa	38.5 ± 0.5
P. dodecandra	(low oleanolic type)
Bale, Ethiopia	45 ± 11
Shoa, Ethiopia	45 ± 3
Zimbabwe	47 ± 4
Bale Facil, Ethiopia	47 ± 2
Nigeria	51 ± 2
Zimbabwe	53 ± 2
Type 17 (mature)	58 ± 7
Wollo, Ethiopia	61 ± 7
Type 17 (immature)	66 ± 5
mean =	53 ± 7
P. dodecandra	(high oleanolic type)
Type 3 (mature)	82 ± 4
Type 3 (immature)	83 ± 3
Type 44 (mature)	83 ± 3
Ilubabar, Ethiopia	88 ± 3
Type 44 (immature)	88 ± 3
Eritrea	89 ± 2
Begemdir, Ethiopia	91 ± 6
Kenya	98 ± 4
Gojam, Ethiopia	99.9 ± 0.1
mean =	89 ± 6

number of other minor products. The minor products are not completely resolved using the packed column, and peaks 1000 times smaller than the oleanolic derivative and peaks having retention times longer than 6 min were not measured. The short analysis time was desirable due to the large number of samples, about 10 for each type of berry collected.

It can be seen that all samples separate clearly into two groups; either the high oleanolic acid group, above 80% of the total triterpene aglycones, or the low oleanolic acid group, containing *ca* 66% or less oleanolic acid with the remaining triterpenes more highly oxygenated. There is a wide gap in the distribution of the two types of mature berries, with means of 89 ± 6% and 53 ± 7% respectively. The value for the sample of *P. heptandra* is not included in the calculation of the mean in Table 1 even though the sample could probably not be distinguished from *P. dodecandra* by observation of the berries alone. Standard types 3 and 44 are unfortunately not distinguishable from each other by this method but they are nicely separated from standard type 17 and many of the wild varieties.

The individual aglycone components are more clearly separated using temperature programming and the megabore column. The chromatograms for type 17 and 44 are shown in Fig. 2. Under these conditions the methyl derivatives of oleanolic acid, hederogenin, 2-hydroxyoleanolic acid and bayogenin have retention times of 6.6, 7.0, 7.7 and 7.9 min respectively.

The chromatogram of type 3 is not shown because of its close similarity to type 44. Peaks that were not identified by comparison with authentic samples were assigned numbers representing the M_r from the mass spectrum for

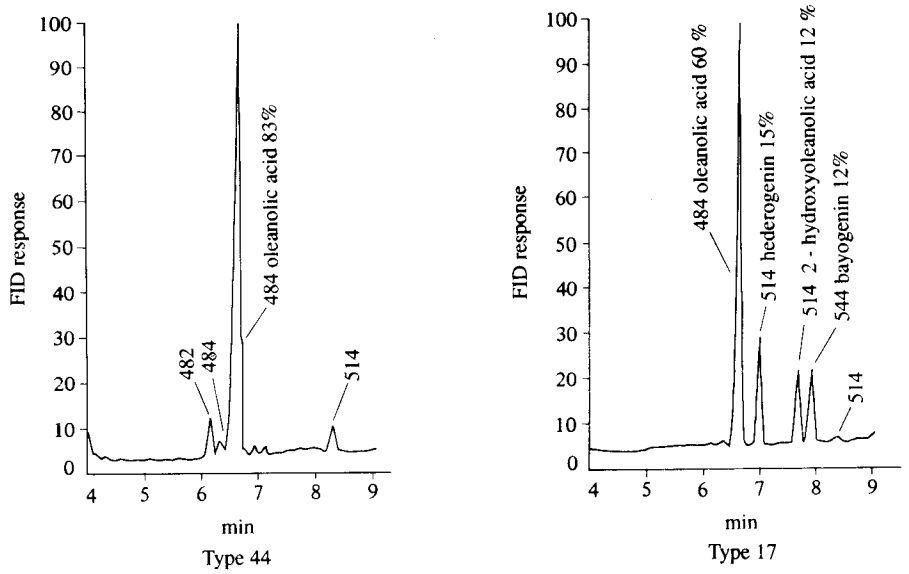


Fig. 2. Triterpene derivatives from type 17 and 44.

that peak. The percentage composition of the oleanolic acid derivative in the mixture was within the experimental error, since the oleanolic acid derivative was well separated even on the packed column and both sets of data used the flame ionization detector (FID).

The analyses of all berries, both standard types and wild types, were done on mature berries. Analyses of immature berries of the three standard types were also made to assess the effect of the maturation of the berry on the distribution of aglycones. In all three cases, the immature berries tended to be the same or slightly higher in oleanolic acid derivatives than the mature berries, although the differences were within experimental error or very small. If oleanolic acid is further elaborated via enzymes during maturation, one might expect immature berries to be higher in oleanolic acid [16]. The differences between the oleanolic acid content of mature and immature berries was certainly small when compared with the differences in the two types of berries. The mean values of oleanolic acid for the low group, 53 ± 7% and the high group, 89 ± 6% are clearly separated, as well as the highest of the low group, 66 ± 5%, and the lowest of the high group, 82 ± 4%, a span of 16 percentage points of no overlap.

Lemma [2] has pointed out that endod exists in two main varieties, 'arabe' with pinkish and 'ahiyo' with greyish berries. It is not always easy to make this distinction after berries have been dried and stored for some time. There seemed to be no correlation between colour and oleanolic acid content in the few cases we could assign a colour type.

A simple micromethod for chemically separating endod berries into two classes based on the oleanolic acid content of the triterpene aglycones has been presented. While it is known that the attached sugars as well as the aglycones have an effect on the molluscicidal activity of the end product, we hope that this analysis will become a useful tool in the overall process of molluscicide production. The standard types were grown under similar conditions in central Ethiopia and therefore most likely reflect a true genetic difference. The wild types were grown under many different conditions and it is not known what effect soil, climate and health of the plant may have had on the aglycone composition. The appearance of two distinct types would, however, favour a large genetic component.

EXPERIMENTAL

Berries (10 g) of each of the standard collections were weighed individually. The mean weights and standard deviations for types 3, 17 and 44 were 20 ± 10, 15 ± 10 and 19 ± 10 mg respectively. The berries taken for analysis were classified as mature if their wts were above the mean. Berries classified as immature were smaller than the mean wt by one standard deviation or more, yet were symmetrically developed. If insufficient sample of some of the wild type berries were available, berries over 20 mg were considered to be mature.

One mature berry (*ca* 15–50 mg) was placed in 2 ml H_2O in a small vial (teflon screw cap) and allowed to stand 24 hr. To the foamy soln obtained was added 2 ml of *n*-BuOH and the mixture shaken and allowed to separate. The *n*-BuOH was removed as the upper layer and placed in another small vial for evapn under a slow stream of inert gas. To the almost colourless solid saponin remaining was added 1 ml 0.5 N H_2SO_4 and the mixture capped and heated on the steam bath for 24 hr during which time the aglycone precipitates. The cooled mixture is extracted with 2 ml $CHCl_3$ and again evapd. To the aglycone was added *ca* 500 μl dry dimethylformamide (maintained over 3Å sieves), 10 mg NaH (washed free of oil with hexane) and 300 μl of MeI and the mixture allowed to stand at room temp. for several hr, diluted with 2 ml of H_2O (care!) and extracted with 2 ml of $CHCl_3$. After washing H_2O several times, the solvent was evapd in a slow stream of inert gas. The triterpenoid aglycone permethyl derivatives were taken up in 500 μl of $CHCl_3$. The GC was a Hewlett-Packard 5710-A with 6 ft × 2 mm glass column packed with 1% SE-30 on GR-Q; flame ionization detector (FID) at 350°; injector at 300° and column at 270° isothermal with He flow = 22 ml/min. An HP 3380 A integrator was used to collect data.

10 berries were used for each determination, with the exception of Bale and Gojam where 3 and 6 berries were used due to short supply.

Additional data were collected using a 15 m megabore capillary column coated with Durabond-23 (J&W Scientific, Folsom, California) film thickness 0.5 mm, i.d. 0.53 mm, programmed from 200 to 260° at 8°/min with He flow = 15 ml/min. Mass spectral data were collected with a Ribermag R10-10C mass spectrometer using negative ion chemical ionization with NH_3 reagent gas.

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