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ISOLATION AND CHARACTERIZATION OF SEED HYDROCARBONS FROM *BALANITES AEGYPTIACA* (*B. ROXBURGHII*) AND *B. PEDICELLARIS*

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Abstract—Solvent extraction, before and after acid treatment of the steroidal saponin-holding seeds of species of *Balanites* (Zygophyllaceae), was used to afford their hydrocarbons. The result of the GLC analysis of these is discussed with reference to the oil content and to the taxonomic classification of *Balanites aegyptiaca* and *B. roxburghii*. Both of these species, but not *B. pedicellaris*, afforded additional long-chain hydrocarbons after acid treatment. The qualitative variation in the hydrocarbon content of *B. pedicellaris* seed, as the fruit ripens, was also examined.

INTRODUCTION

LITTLE is known about the nature and the role of hydrocarbons *in* steroidal sapogenin-affording tissue. Their role may be of commercial importance in increasing the yield of sapogenin for the steroid industry[1] which is estimated[2] to require 1,000,000 kg of diosgenin by 1973. Consequently, the hydrocarbons of various plant sources of steroid are being currently investigated and some results for the diosgenin-yielding seeds of *Balanites* are now reported.

The taxonomic position of the genus *Balanites* has been in dispute for some time. Bentham and Hooker[3] as well as Dale and Greenway[4] placed the genus in the family Simarubaceae, a treatment now considered unsatisfactory. Hutchinson and Dalziel,[5] Engler[6] and Melchior[7] have placed it in the Zygophyllaceae, of which it is usually considered to form a distinct sub-family, Balanitoideae. Record[8] stated that the wood structure of *Balanites* has no resemblance to that of other members of the Zygophyllaceae. Heimisch[9] considered that apart from the medullary rays, the wood anatomy of *Balanites* suggests affinity with the Zygophyllaceae rather than with the Simarubaceae. In view of these conflicting opinions, Nair and Jain[10]

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[2] R. HARDMAN, *Phytochem*, **8**, 1319 (1969).
[3] G. BENTHAM and J. D. HOOKER, *Genera Plantarum*, Vol. 1, London (1862).
[4] I. R. DALE and P. J. GREENWAY, *Kenya Trees and Shrubs*, p. 531, Buchanan Kenya Estates in association with Hatchards, London (1961).
[5] J. HUTCHINSON and J. M. DALZIEL, *Flora of West Tropical Africa*, 2nd edition, Vol. 1, Part 2, p. 361, Crown Agents for Overseas Governments and Administrations, London (1958).
[6] A. ENGLER and K. PRANTL, *Die Natürlichen Pflanzenfamilien*, Vol. 19a, Verlag von Wilhelm Englemann, Leipzig (1931).
[7] H. MELCHIOR (ed), A. ANGLER, *Syllabus der Pflanzenfamilien*, 12th edition, Vol. 2, Gebrüder Borntraeger, Berlin-Nikolassee (1964).
[8] S. J. RECORD, *Bull. Yale. Sch. For.* **6**, 48 (1921).
[9] C. HEIMISCH, *Lilloa* **8**, 83 (1942).
[10] N. C. NAIR and R. K. JAIN, *Lloydia* **19**, 269 (1956).

studied the floral morphology and embryology of *B. roxburghii* Planch. and found that the genus *Balanites* has several embryological features characteristic of it and different from those of the Simarubaceae and the Zygophyllaceae. As a result of this work, Keay, Onochie and Stanfield[11] placed the genus in a separate family, Balanitaceae a treatment accepted by Hutchinson,[12] the most recent reviewer of the group.

Within the genus *Balanites* the Indian species, *B. roxburghii* Planch., is now regarded as identical with the African species, *B. aegyptiaca* Del.,[13] even though the Indian fruits are often larger than the African ones. For ease of reference, in comparing these two "species", the Indian source is still termed *B. roxburghii* in this report.

The use of hydrocarbons in chemotaxonomy has been reviewed by Eglinton and Hamilton.[14] Much of the work to date has centred on the hydrocarbon content of the plant cuticular waxes.[15,16] Evidence of possible family and generic taxonomic value is now presented, on the initial findings of the hydrocarbon content of the seeds of some species of *Balanites*.

RESULTS AND DISCUSSION

An unusual procedure for obtaining hydrocarbon was followed: The seed was defatted and its oil afforded hydrocarbon in the normal way. The defatted material was then treated by a method which we normally use to afford the sapogenin, namely by refluxing with 2 N acid.[17] After such treatment, in the case of *Balanites pedicellaris* seed which has a low oil content (4 per cent, w/w), no further hydrocarbon was obtained (Table 1 and Fig. 1). However, in the case of the oil-rich seeds (40–50 per cent, w/w) of *B. aegyptiaca* and *B. roxburghii*, the acid and subsequent treatment resulted in a significant further amount of hydrocarbon (Table 1). Such released hydrocarbon was of a long-chain saturated nature and excluded the shorter-chain members. Perhaps the released longer-chain hydrocarbon was bound because it was not yet required by the plant, considering that shorter-chain hydrocarbons can be metabolized by tuber tissue from sapogenin-affording species of *Dioscorea*.[18]

TLC of the crude hydrocarbon extract obtained before acid treatment indicated the presence of small amounts of unsaturated hydrocarbon in *B. aegyptiaca* and *B. roxburghii* but this class was not found in the samples of *B. pedicellaris* examined. Alumina column chromatography enabled the isolation of a single unsaturated compound which was shown by isothermal GLC and i.r. spectroscopy to be squalene or a close isomer. No unsaturated hydrocarbon was found after acid treatment of the seeds of any of the species studied. Although the presence of squalene in many seed oils has long been known,[19] previous attempts to detect squalene in *B. aegyptiaca* seed from ripe fruits had failed;[20] it was detectable only after

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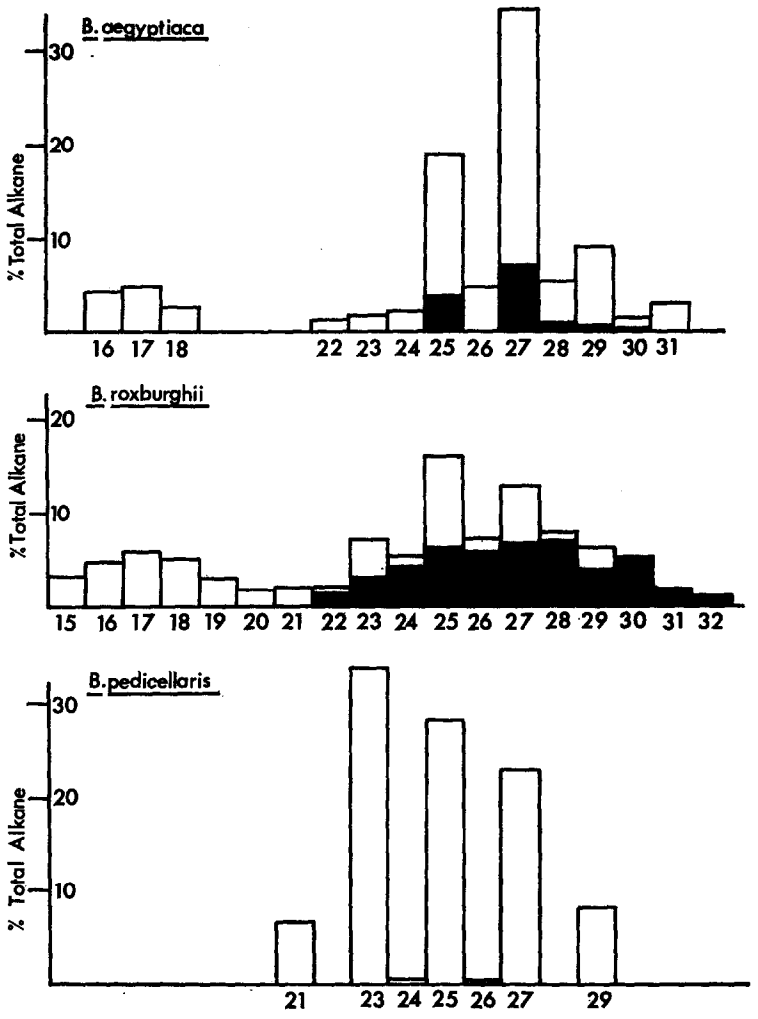

FIG. 1. DISTRIBUTION OF *n*-ALKANE OBTAINED BEFORE AND AFTER ACID TREATMENT OF *Balanites* SPECIES.

GLC analysis using 5% SE30 columns, temperature programmed at 2°/min.
■ Percentage of total alkane obtained after acid treatment.

TABLE 1. THE OIL CONTENT AND THE HYDROCARBON CONTENT OF THE SEEDS OF *Balanites* SPECIES FROM RIPE FRUITS

	B. roxburghii	*B. aegyptiaca*	*B. pedicellaris*
Oil (% w/w of seed, m.f.b.)	48·7	42·4	3·6
Total alkane (% w/w of seed, m.f.b.)	0·20 (46*)	0·010 (13*)	0·039 (0*)
Squalene (% w/w of total hydrocarbon)	11·9	2·3	0·0

* Percentage proportion obtained after acid treatment.

the seed had been incubated with sodium mevalonate-2-^{14}C. This variation in unsaturated hydrocarbon might well be accounted for by the different sources of the samples of *B. aegyptiaca* fruits.

After removal of any unsaturated hydrocarbon, the alkane fraction obtained from each of the three species was analysed by low-increment (2°/min) temperature programmed GLC following the procedure of Kuksis.[21] It was found that the alkanes consisted of chain lengths varying from C_{15} to C_{33} (Table 2) with *n*-alkane accounting for about 90 per cent of the total

TABLE 2. COMPOSITION OF THE SEED ALKANE IN RIPE FRUITS OF *Balanites* SPECIES

Carbon No.	*B. roxburghii*		*B. aegyptiaca*		*B. pedicellaris*
	*b	*a	*b	*a	*b
*n*15	6·0				
**i*16	T				
*n*16	8·7		5·3		T
*i*17	T		T		
*n*17	10·6		5·6		T
*i*18	T		T		
*n*18	9·0		3·0		T
*i*19	T				
*n*19	5·6		T		T
*i*20	T				
*n*20	3·1		T		T
*n*21	3·8	T	T		6·6
*i*22	T				
*n*22	1·5	2·9	1·5		T
*n*23	7·7	6·6	1·9	T	33·6
*n*24	2·3	9·2	2·4	T	0·4
*n*25	18·5	13·2	17·1	31·0	28·1
*n*26	2·9	12·6	5·5	T	0·2
*i*27	2·9	T	7·3	T	
*n*27	11·3	14·6	31·5	51·7	22·8
*i*28		T			
*n*28	1·9	15·2	5·1	7·2	T
*i*29		T			
*n*29	4·2	8·6	9·5	5·7	8·1
*i*30		T			
*n*30	T	11·1	1·3	3·5	T
*n*31	T	3·7	3·2	0·8	T
*n*32	T	2·2			T
*n*33	T	T			T

GLC analysis using 5% SE30 column, temperature programmed at 2°/min.
*b = Percentage of total hydrocarbon obtained before acid treatment.
*a = Percentage of total hydrocarbon obtained after acid treatment.
**i* = Mixed *iso* and ante-*iso* chains.
T = Trace (less than 0·2%).

hydrocarbon. Treatment of the alkane fractions with warm conc. H_2SO_4 caused no change in the relative proportion of each alkane, indicating that oxygenated compounds were absent.

[21] A. KUKSIS, *Biochem.* 3, 1086 (1964).

Samples of *B. pedicellaris* seed, representing three stages in the ripening of the fruit, were analysed to observe any effect of ripening on the seed alkane distribution. Changes had been previously observed in the total sapogenin content of *B. aegyptiaca* during fruit ripening.[20] As the fruit of *B. pedicellaris* ripened the qualitative alkane pattern remained the same, but the proportion of C_{23} *n*-alkane rose from 11 per cent to 21 per cent, w/w of the total alkane, while the proportion of C_{25}, C_{27} and C_{29} fell slightly (Table 3). GLC analysis of a separate batch of

TABLE 3. PERCENTAGE COMPOSITION OF THE SEED ALKANE IN *B. pedicellaris* AT THREE STAGES IN THE RIPENING OF THE FRUITS

Carbon No.	Unripe	Partially ripe	Ripe
*n*16	T	T	T
*n*17	T	T	T
*n*18	T	T	T
*n*19	1·8	2·6	4·1
*n*20	1·8	2·3	2·4
*n*21	1·8	3·2	4·5
*n*22	1·8	1·9	2·4
*n*23	10·5	18·8	21·3
*n*24	5·4	4·5	4·1
*n*25	28·2	23·6	21·3
*n*26	4·7	3·9	5·3
**i*27	T	T	T
*n*27	24·7	20·7	16·5
*n*28	3·6	4·9	4·5
*n*29	13·8	9·7	8·3
*n*30	1·8	2·3	2·8
*n*31	T	T	2·4
*n*32	T	1·7	T
*n*33		T	T

GLC analysis using 5% SE30 column, temperature programmed at 2°/min.
T = Trace (less than 0·2%).
**i* = Mixed *iso* and ante-*iso* chains.

B. pedicellaris, comprising almost entirely of seeds from ripe fruits, confirmed that C_{23} *n*-alkane predominated in such seeds (Table 2). From the elongation-decarboxylation pathway for the biosynthesis of hydrocarbons propsed by Kolattukudy,[22] it would appear that, as the fruit ripens, its ability to add C_2 units to the fatty acid chain is reduced, resulting in a preponderance of shorter-chain hydrocarbon. Alternatively, the existing longer chains might have been shortened.

The proportion of the *n*-alkane with odd carbon numbers predominated in the total alkane in all three species (Table 2 and Fig. 1). The *n*-alkane pattern of *B. pedicellaris* contrasted with that of the other two species in that shorter-chain alkanes (below *n*19) were only present in trace quantities. The alkane obtained before acid treatment from *B. aegyptiaca* and *B. roxburghii* resembled that of other seed oils.[21]

The similarity in the pattern of their hydrocarbons, both saturated and unsaturated, supports the proposals that *B. aegyptiaca* and *B. roxburghii* are taxonomically identical. Sofowora[20] had also noted a similarity in their steroidal sapogenin contents.

[22] P. E. KOLATTUKUDY, *Phytochem.* 6, 963 (1967).

EXPERIMENTAL

Plant Samples

Fruits of *Balanites roxburghii* were obtained from Poona-3 district, India; the fruits of *B. aegyptiaca* from the North West State of Nigeria and those of *B. pedicellaris* were obtained from Uganda. The macroscopic characters of the specimens agreed with the descriptions given in the literature. Unless otherwise stated, ripe fruits were analysed. A representative selection of healthy seeds was used from each sample.

Isolation of the Hydrocarbon

Seed, 175 g, obtained by cracking open the ripe fruits, was crushed in a mortar and defatted by soxhlet extraction for 24 hr with light petroleum (b.p. 40–60°). The oil fraction, obtained by evaporation of the light petroleum extract *in vacuo*, was saponified by refluxing with 1 l. of N/2 alc. KOH. The unsaponifiable matter was recovered by shaking with ether and fractionated on an 8 cm × 1·5 cm column of alumina (Peter Spence type H alumina 100/200 mesh packed in *n*-hexane). The column was eluted with *n*-hexane and the hydrocarbon collected in the first 20 ml fraction.

The solvent-free defatted seed was refluxed with 2 N HCl (2 l.) for 2 hr. The acid-insoluble residue was filtered, washed and neutralized with 10%, v/v NH_4OH. The residue was dried overnight at 50° before extraction with light petroleum (b.p. 40–60°) for 24 hr to afford a crude sapogenin fraction upon removal of the solvent *in vacuo*. The residue was recrystallized from acetone to remove most of the sapogenin. The mother liquor was concentrated to a small volume and chromatographed on alumina as above, to isolate its hydrocarbon.

The presence of unsaturated hydrocarbon was checked by TLC on 0·25 mm silica gel plates developed with *n*-hexane. Spots were located by spraying with a 0·1% aqueous solution of rhodamine 6G. Partial fractionation of the saturated and unsaturated hydrocarbons, for the isolation of squalene, was attained by chromatography on a 20 × 1·5 cm alumina (Spence type H) column. The column was developed with *n*-hexane and the eluate collected in 5-ml fractions. Fractions 3, 4 and 5 contained saturated alkane. The squalene-like hydrocarbon was located in fractions 9–13. It was recovered from the solvent *in vacuo*, and stored under refrigeration.

Three 30 g samples of *B. pedicellaris* seeds, from a further supply of fruits from the same geographical source, were sorted into green soft seeds (from unripe fruits), green hard seeds (from partially ripe fruits) and hard brown seeds (from ripe fruits). The hydrocarbon content of each of these groups of seeds was investigated as described above to provide the results in Table 3.

Further purification of all the alkane fractions was effected by treatment with 10 ml warm conc. H_2SO_4. After dilution with water, the hydrocarbons were recovered with *n*-hexane.

Identification of the Hydrocarbons

Hydrocarbons were analysed at all stages by GLC. 1 μl of an approximately 2·5%, w/v solution of hydrocarbon in *n*-hexane was resolved on a Varian Aerograph series 1520C Chromatograph equipped with dual columns and dual flame ionization detectors. Columns were ⅛ in. × 6 ft stainless steel coated with 5% w/w SE30 on acid-washed D.C.M.S. Chromosorb W. The initial column temperature was 100° and it was programmed at 2°/min to 260°. Injector temp.: 270°, detector temp.: 280°, carrier (N_2): 40 ml/min. Identification of the *n*-alkanes was achieved by chromatography with known standards (a range from C_{14} to C_{35}) which had a purity of at least 90%. Where standards were not available identification was based on the relative retention time of peaks. Confirmation of identity was achieved by isothermal GLC when a linear plot was obtained between the logarithm of the retention time and the carbon number of the *n*-alkane. It was assumed that branched alkanes had slightly shorter retention times than the corresponding *n*-alkane and these were tentatively identified on the basis of the relative retention time. Areas beneath peaks were estimated by the product of peak height and width at half peak height.

The isolated squalene was compared by isothermal GLC at 190° with an authentic reference sample. I.r. spectra were run on a 0·2%, w/w solution of authentic and suspected squalene in CCl_4 using a Hilger H800 Spectrophotometer with 1 mm fixed path length NaCl cells. Scan speed 15 min/rev from 5000 cm^{-1} to 2000 cm^{-1} and from 2000 cm^{-1} to 650 cm^{-1}. At all stages Analar grade solvents were used and contact with foreign sources of hydrocarbon, e.g. stopcock grease, was avoided.

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