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Accumulation of Steroidal Sapogenins and Fixed Oil in Developing Fruits of *Balanites aegyptiaca* Del.

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Abstract. The fruits of *Balanites aegyptiaca*, a wild savannah tree, constitute a promising source of steroidal sapogenins, chemicals in demand by the pharmaceutical industry, in addition to an untapped source of an edible fixed oil . Large guantity of the fruit (*ca.* 400 000 tons per annum) are traditionally collected and marketed in Sudan due to their edible mesocarp. This study is concerned with accumulation of the fixed oil as well as of steroidal sapogenins in 11 fruit developmental stages, from the young immature to the fully ripe, dry stages. The fruits were separated into 'kernels' and combined 'epicarp+ mesocarp' tissues. An infra-red spectrophotometric method was used for sapogenin quantification. Total sapogenin contents expressed per tissue was 0.5 mg in kernels and 2.5 mg in 'epicarp=mesocarp' in the first stage (S1). Thereafter, these values rose sharply in later fruit developmental stages so that by stage S7 (a mature but still green stage), they contained maximum values of 22.0 and 60.0 mg /tissue, respectively. Similarly, balanites kernel oil (BKO) dramatically increased in amount with fruit development; from < 5.0% in the first two very young stages to over 45.0% of kernel dry weight by stages 7 and 8. Thus, harvesting fruits at the still green 7th stage would minimize losses of the dry, sapogenin-rich epicarp encountered during traditional harvesting and handling of dry fruits. 'Epiarp+mesocarp' tissue contained both 25α and 25β sapogenins, the proportion of 25β increased with fruit maturation. On the other hand, kernel tissue contained only 25α (diosgenin type) sapogenin till the very late fruit stage, when plastid degradation occurred. Linoleic acid was the major fatty acid of balanites kernel oil in all fruit developmental stages. Linolenic and palmitoleic acids were present in the kernel oil of very young fruits but disappeared with the degradation of plastid- membrane lipids on further fruit maturation and desication.

Keywords: *Balanites aegyptiaca,* steroidal sapogenin, fruit development, kernel oil, fatty acids

Introduction

Balanites aegyptiaca Del. (Zygophyllaceae), commonly known as desert date tree, is a drought-resistant wild plant native to the dry land areas of Africa and South Asia. The saponin rich tree thrives well in the wild in Sudan, where it is known as hijleej and is valued for several local uses. The mature dry fruits (laloab) are offered in local Sudanese markets for their edible sweetish mesocarp. The oil rich fruit kernels are traditionally used in certain parts of Sudan as a source of an edible oil. Large guantity of the edible fruit, estimated to amount to 400 000 tons (Abu-el-Futuh, 1983) are traditionally collected annually for sale in local markets.

Saponins, glycosides of triterpene (C_{30}) or steroid (C_{27}) aglycones are natural compounds of important biological and economic importance. Many saponins were shown to possess interesting biological activities which are of potential medical applications, such as their anti-tumor (Thakur *et al.*, 2011; Podolak *et al.*, 2010; Fuchs *et al.*, 2009), anti-diabetic (Al-Thobaiti and Abu Zeid, 2019; Hassanin *et al.*, 2018; Ezzat *et al.*, 2017; Elekofehinti , 2015), antifungal (Hussanin *et al.*, 2019), and anthelmintic activities (Shalaby *et al*., 2018). Asrade *et al.* (2017) reported antiplasmodial activity for the leaves of another species of Balanites, *B. rotundifolia*. Isolated diosgenin was reported to play a potential role, as a phytoestrogen in animal production (Sirotlein *et al*., 2019). Sapogenins, aglycones of steroidal saponins, are regarded as important starting materials for the partial synthesis of many steroidal drugs e.g (Chen and Wu, 1994).The role of saponins in plant physiology and in plant defenses against pathogens, pests and herbivores have been reviewed (Moses *et al*., 2014).

Terpenoid biosynthesis involves condensations of an active 5-carbon isoprenoid unit which is generated in plants *via* two biosynthetic pathways, *viz*., the conventional acetate-mevalonate and the more recently discovered non-mevalonate pathway (Rohdich *et al.*, 2002). The product of enzymatic condensation of three

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such C5 units eventually results in the formation of farnesyl diphosphate (C15), two units of which dimerize to produce the linear 30-carbon squalene, the parent precursor of triterpenes and steroids. Spirostanol and furostanl steroidal sapogenins are derived from cyclized parent precursors eventually generated from squalene by structural modifications through further cyclization, oxidation, reduction, isomerization and hydration etc. (Moses *et al*., 2014). Glycosylation of the aglycone formed, to give the respective saponin is believed to be the final step in steroidal saponin biosynthesis and it is thought to be involved in regulating the biological activities of the whole saponin molecule (Kohara *et al*., 2005). It should be noted that the detailed biosynthetic pathways of steroidal saponins and enzymes involved are yet to be elucidated (Augustin *et al*., 2011). Despite many research efforts undertaken using traditional radiotracer techniques reported by (Brenac and Sauvaire, 1996; Stohs *et al*., 1974; Bennett *et al*., 1970) or currently much used transcriptome analysis and gene expression studies (Kwon *et al*., 2019; Ciura *et al*., 2018; Zhu *et al*., 2018; Abdelrahman *et al*., 2017; Upadhyay *et al*., 2014; Kalra *et al*., 2013; Augustin *et al*., 2011; Kim *et al*., 2010; Suzuki *et al*., 2002).

Balanites fruits have long been recognized as a promising economic source of steroidal sapogenins, suitable for industrial use as raw materials for commercial steroid drug production (Hardman and Sofowora, 1972;1970). The two major steroidal aglycones of saponins of *B. Aegyptiaca* (the balanitins) are diosgenin and yamogenin, which, as epimers have the same stereoisomerism but differ only in the orientation of one methyl group attached to one chiral carbon, C-25 (Fig. 1).

In a previous study (Osman-Bashir and Elhussein, 2017a), we reported on the quantitative distribution of sapogenins within morphological parts of the mature (market-stage) fruit of balanites and among several Sudanese fruit-kernel accessions. We also reported on the two other sapogenin by-products of balanites fruits, namely the fixed edible oil (and its contained lecithin) and crude protein enriching the kernels (Osman-Bashir and Elhussein, 2017b). We also showed that available fruit material traditionally collected in Sudan from wild tree stands could sustain its industrial exploitation as a commercial source of sapogenins and edible oil. In this report we evaluate accumulation of sapogenins and fixed oil as affected by fruit developmental stage. The infrared spectroscopic method originally developed by the Bath group (Brain *et al*., 1968) was used to determine

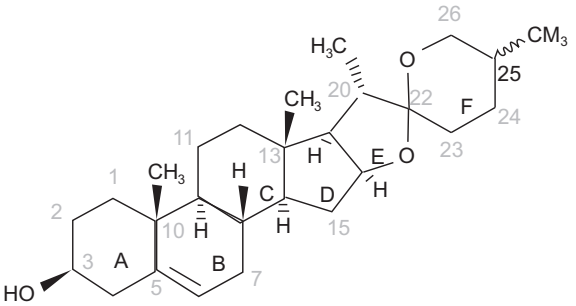

Fig. 1. Structures of diosgenin and yamogenin. The two compounds belong to the spirostanes, the major sub group of steroidal sapogenins, which are characterized by the two spiro-connected heterocyclic rings (E and F). The two compounds share the same configuration about all chiral carbons but differ only in the orientation of the methyl group attached to C-25 (wavy bond in the figure). With the F ring having a chair conformation, the methyl group at C-25 is equatorial and alpha in diosgenin (25α-spirost-5- en-3β-ol). and is axial and beta in yamogenin (25β-spirost-5- en-3β-ol).

α- , β- and total sapogenins also desribed earlier by (Osman *et al*, 2014; Osman-Bashir and Elhussein, 2017a).

Materials and Methods

Balanites fruit material at different stages of development were collected from a single tagged tree growing wild in central Sudan (Umbaroana Public Park, Wad-Medani City area). The stages, designated S1 to S11 covered all phases of fruit development from the very young immature green stage (S1) to the fully ripe brownskinned, market stage (S11). Figure 2 is a photograph of these stages. The collection period extended for a total of about three months with a variable time lapse between successive stages, generally taking intervals of 4-8 days.

For chemical analysis of sapogenin and fixed oil contents, the fruit was dissected into three morphological parts, 'kernels' 'mesocarp+epicarp' and 'endocarp'. It was too difficult to separate mesocrp from epicarp tissue, especially in young fruit stages. Photographs of these fruit parts were included in our previous report (Osman-Bashir and Elhussein, 2017a).

Extraction of sapogenins, Accurately weighed amounts of dried and powdered 'epicarp +mesocarp' combined

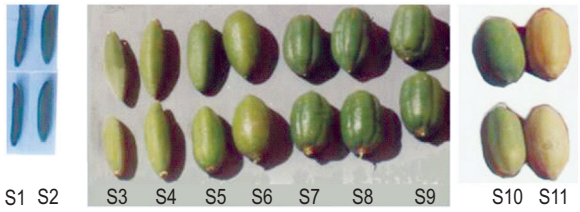

Fig. 2. Photos of balanites fruit developmental stages. The stages are referred to in the text by the codes shown, from the very young immature stage (S1) to the fully ripe brownish, market stage (S11). The colour of S1 and S2, not clearly reproduced in the photo which is green. Actual length of the mature fruit is about 3 cm.

tissues were hydrolyzed, under reflux with 2N HCl (30 mL/gm plant material) for 3 h, after cooling, the aqueous acid was filtered off and the residue (containing the sapogenins) was briefly washed with distilled water before it was neutralized with 10% NH_4OH, washed again with distilled water and dried over-night at 50 °C, steroidal sapogenins, thus prepared by *in situ* hydrolysis of their saponins, were then extracted with petroleum ether (b.r. 40°-60°) in a Soxhlet extractor for 24 h. The Soxhlet extracts were carefully dried down in a rotary evapourator and the semi-solid residue (the sapogenins) was dissolved in a known volume of spectrosol-grade $CHCl_3$ and taken for infrared spectrophotometric analysis. For optimum recovery of sapogenins from oil rich kernel tissue. It was found necessary to de-fat the tissue prior to acid hydrolysis. Usually the fixed oil was first Soxhlet extracted from kernels before the Marc was subjected to acid hydrolysis of the saponins.

Infrared spectroscopic assays of sapogenins. The sapogenin extract, finally taken up in spectrosol grade chloroform which was used for infrared spectroscopic quntification (usig a Shimadzu Infrared Spectrophotometer Model IR 435). The infrared spectrosopic assay of sapogenins was based on the fact that 25α- and 25β–sapogenins absorb most intensely at either wave numbers 900 cm^{-1} or 915 cm^{-1}, respectively. However, when present together in appreciable amounts, the two epimers mutually absorb at both wavenumbers. Thus the influence of the presence of one epimer on the absorption of the other was routinely corrected by using a graph prepared according to the 'ratio method' of Brain *et al*. (1968). Details of the preparation of calibration graphs for the absorption of diosgenin (α-) and yamogenin (β-sapogenin) as well as the ratio graph were as we previously reported (Osman-Bashir and Elhussein, 2017a; Osman *et al*., 2014). Thus the final chloroform solutions of crude sapogenins prepared were routinely assayed by scanning in the IR region 1000-800 cm^{-1}. Total 25α-sapogenins and total 25β-sapogenins present in extracts were calculated as diosgenin or yamogenin. Total sapogenin values were obtained by summing up values for total 25α- and 25β-sapogenins.

Fixed oil extraction. Oven-dried, powdered tissue (kernel or endocarp) was Soxhlet extacted for 6 h, using n-hexane.

Thin layer chromatography of polar lipids. Glass plates (20 x 20 Cm) pre-coated with silica gel G 60, 0.2 mm thickness (Merck Co., Germany) were normally used for Thin Layer Chromatography (TLC). Polar lipid extraction solvent, TLC development solvents and detection reagents are included in the legends to Fig. 7 and 8.

Gas liquid chromatography of fatty acids. For the preparation of fatty acid methyl esters, aliquots of balanite kernel oil were dissolved in the methylation mixture methanol benzene conc. sulfuric acid (20: 10: 1), in glass ampoules that were sealed and heated at 75 °C– 80 °C for 90 min. After allowing to cool, an equal volume of sodium bicarbonate solution (5%) was added and the mixture extracted twice with hexane. Anhydrous sodium sulfate granules were added to the combined hexane layers (containing the methyl esters), before filtration and subsequent GLC analysis. A Varian Instrument Group Series 00-997140-01 gas chromatograph equipped with a computing integrator was used for the analysis of fatty acid methyl esters. Analysis was carried out isothermally at column oven temperature of 170 °C, column inlet and detector oven temperatures of 180 °C and the carrier gas flow rate was 50 mL/ min.

Results and Discussion

As mentioned above, all fruit stages of balanites fruits analysed in this study were collected from a single tagged wild tree growing in central Sudan..

Accumulation of 'total sapogenin' during balanites fruit development. The amount of total sapogenin was calculated by summation of the values obtained for alpha- and beta- epimers as determined by the infra-

red spectroscopic method. The total sapogenin value per morphological fruit part increased progressively with fruit development, from as low as 0.5 mg and 2.4 mg in S1 to reach maxima of over 20 mg and 60 mg per tissue in 'kernel' and 'mesocarp+epicarp', respectively, in stage S7 (Fig 3). 'Epicarp + mesocarp' combined tissue always had a total sapogenin content higher than kernels all through fruit developmental stages. In both fruit tissues analyzed, a plateau was reached by stage S7, a still-green but mature stage. The rest of the fruit represented by the woody endocarp, analyzed in all fruit stages, was devoid of sapogenins, in agreement with our previous report (Osman-Bashir and Elhussein, 2017a).

The results of total sapogenin content per whole fruit (i.e., values for 'kernel' plus 'mesocarp+epicarp'), showed a similar pattern of increase with balanites fruit developmental stage, maximum total sapogenin accumulation in the whole fruit occurring at stage S7 (Fig 4). Our previous study on the distribution of sapogenins within the morphological parts of balanites fruit (Osman-Bashir and Elhussein, 2017a) showed that most of the fruit total steroidal sapogenin resided in the mesocarp (64%), followed by the kernel (ca.25%).The inedible fragile epicarp (fruit skin) contained about 10%. The finding that maximum sapogenin content of balanites fruits is reached well before the dry market stage may be exploited to avoid losses of fragile epicarp tissue during harvesting and transport. Thus economic harvesting could be considered to be done before the epicarp is completely dry. Stage S8 is particularly suitable for harvesting since at this stage kernal oil content is also maximum, as discussed below.

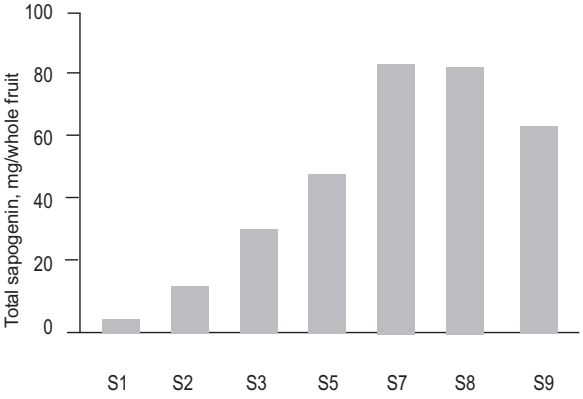

Fig. 4. Total sapogenin content of developing balanites fruits (mg/whole fruit).

It is noteworthy that expression of the results for sapogenin accumulation on the basis of oven dry weight of the two fruit tissues analyzed (results not given) showed a much less sharp increase with fruit development and maturation. The rise in sapogenin level was partly obscured by the large decline in tissue moisture content with fruit development, as recorded for kernels (Fig 5). Such loss of moisture content (desiccation) is a normal physiological process of seed maturation (Srivastava, 2002).

Accumulation of alpha and beta sapogenin epimers during fruit development. Table 1 shows that kernel tissue synthesized and accumulated sapogenin

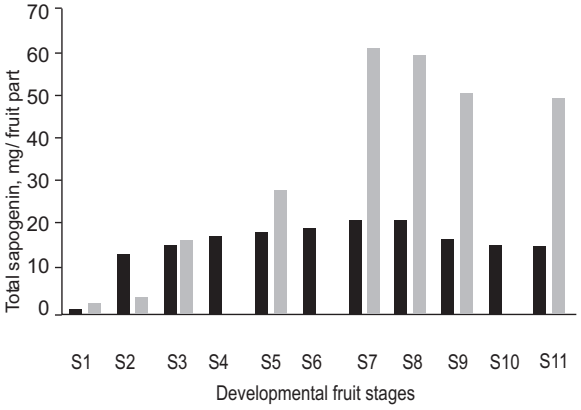

Fig. 3. Accumulation of total sapogenins (mg/ fruit part) in 'kernel' and 'epicarp+ mesocarp' tissues during balanites fruit development expressed as mg total sapogenin/ morphological part.

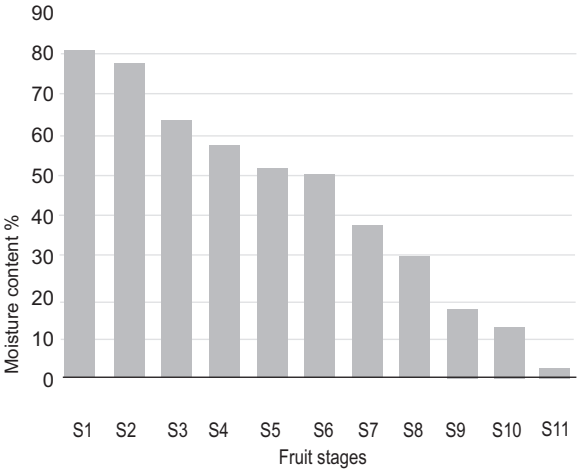

Fig. 5. Moisture content of the kernels of balanites fruits at different stages of maturity.

exclusively of the diosgenin- type (25α). This was true for kernels of all fruit developmental stages, except for the very late mature and dry stage (S10) in which a considerable amount of 25β sapogenin (about one third that of 25α) accumulated. In 'epicarp+mesocarp' combined tissues 25α sapogenins were also predominant. The ratio of 25α to 25β was close to 3:1 in 'epicarp +mesocarp' tissue from very young fruits of stage S1 (Table 1). However, in later fruit stages the proportion of yamogenin- type sapogenin progressively increased, so that by stage S10 the two epimeric sapogenins were present in equal proportions (Table 1).

This dominance of 25α-sapogenin in kernels is in agreement with our previous report involving kernel tissue of 15 acessions of mature ripe fruits (Osman-Bashir and Elhussein, 2017a). The Hardman research group at Bath (Brain *et al*., 1968), working on the sapogenins of *Balanites aegyptiaca*, had earlier hinted that the ratio of 25α to 25β sapogenins could be of biochemical significance. Despite their close structural similarity the two epimers diosgenin and yamogenin are not biosynthetically interconvertible in plants (Bennet *et al*., 1970). Rather, they biogenetically arise divergently from a single stereospecific reaction in their biosynthetic route, such as the postulated step involving a cis or a trans addition to an unsaturated intermediate (Gonzalez *et al*., 1974). The biosynthesis of 25α and 25β sapogenins in balanites fruits may be related to cellular compartmentalization of biochemical pathways. It may reflect the relative contribution of the mevalonate and non-mevalonate pathways of terpenoid biosynthesis. The more recently discovered non-mevalonate pathway (the deoxyxylulose phosphate pathway; reviwed by Dubey *et al*. (2003) which operates in the plastids (Rohdich *et al*., 2002) may be responsible for the preponderance of 25α sapogenins (diosgenin) in the kernels. Relative loss of this epimer in the later stages of fruit development may be related to plastid degradation (loss of plastid lipids).

Table 1. The ratio of diosgenin type (25α-) to yamogenin-type (25β-) epimers in 'kernel' and 'epicarp+mesocarp' tissues as affected by fruit developmental stage

Fruit stage	Fruit morphological part (α:β ratio)	
	'Kernel'	'epicarp+mesocarp'
S1	Pure 25α- epimer	2.8:1
S2	Pure 25α- epimer	2.4:1
S3	Pure 25α- epimer	2.1:1
S5	Pure 25α- epimer	2.1:1
S7	Pure 25α- epimer	2.4:1
S9	Pure 25α- epimer	1.9:1
S10	2.7:1	1.0:1

In this study we measured the amount of sapogenin aglycones obtained by acid hydrolysis of the parent saponin molecules. No free aglycones were detected in any part of the balanites fruit. sapogenins are functionally active in plants in their amphipathic glycoside form. Generally, following biosynthesis of the aglycone, the sugar moiety is added as the final step in saponin formation (Kohara *et al*., 2005). Not only the aglycone, but the sugar moiety (sugar type and mode of attachment) is important for physiological activity of saponin molecules (Thakur *et al*., 2011). Saponins of *Balanites aegyptiaca*, the so called balanitins, have notably been reported to occur as glycosides, mostly of the aglycone yamogenin (Yadav and Panghal, 2010; Chothani and Vaghasiya, 2011). Our results show that sapogenins of the 25α- type, such as diosgenin, are more dominant in the balanites fruit than their β-epimers. Moreover, saponins of the fruit kernels of balanites are exclusively based on diosgenin as the a glycone moiety. Further work involving intact saponins of balanites fruit is justified.

Accumulation of fixed oil during balanites fruit development. As previously reported (Osman-Bashir and Elhussein, 2017b) the oil of balanites fruit is present in the kernels with small amounts encountered in the endocarp (woody part). The edible kernel oil is traditionally prepared in south-western Sudan by stirring the crushed kernels in boiling water, recovering the floating oil layer free of the water-soluble bitter saponins. Balanites kernel oil (BKO), rapidly accumulated with progressive stages of fruit development. The value increased from about 2% of kernel dry weight in the very young stage to as high as 45% by the onset of stage S8, showing little increase thereafter (Fig 6). Thus maximum oil content was reached in the kernels, as the case for fruit sapogenin, before the onset of the mature dry market stage. Again, this is of advantage if fruit harvest is undertaken before the late dry stges. The results expressed on a unit fruit basis (g oil/100 fruit kernels; not shown) gave a somewhat similar pattern.

The unexpected occurrence of fixed oil in balanites endocarp (the woody fruit part) was previously reported (Osman-Bashir and Elhussein, 2017b). Table 2 shows

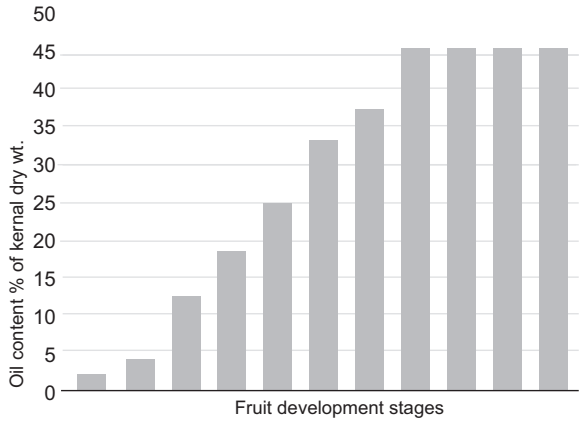

Fig. 6. Oil content (% of dry weight) of kernels of balanites at different stages of fruit maturity.

that the percentage of this oil amouted to as high as 8-9 % of the dry weight of the tissue, in the very early stages of fruit development. However, the content subsequently decreased with fruit maturation to about 5%, i.e., close to the values previously reported for kernels of mature fruits (Osman-Bashir and Elhussein, 2017b).

Changes in fatty acid composition of balanites kernel oil with fruit development. The fatty acid composition of balanites kernel oil (BKO) showed slight changes with fruit development (Table 3). Linoleic acid was the major fatty acid of BKO in all stages of fruit development. It amounted to more than 50% of BKO in the late mature fruit stages (Table 3). Oleic acid, the second most abundant fatty acid, initially increased to as high as 40% of total kernel oil fatty acids by stage S7, but later decreased to below 30% in kernels of later fruit stages. There was an overall slight decrease in the relative content of palmitic acid with a concomitant increase in stearic acid. The total unsaturation of fatty acids, as measured by the unsaturation index (Table 3), did not show much change. The presence oil in of palmitoleic was (16:1) and linolenic acids in the oil of kernels from very young fruit stages (Table 3). Palmitoleic acid disappeared in kernel tissue of stage S5 (and subsequent stages). This mono-unsaturated fatty acid was reported to be characteristically present in phosphatidyl glycerol (Pineau *et al.*, 2004; Weenink and Shorland, 1964), a phospholipid associated with plastidic membranes. Linolenic acid, which disappeared at a later stage (beyond stage S7) is particularly abundant in the plastidic lipids, mono- and di-galactosyl diglyceride (Kates, 1970). Loss of the two fatty acids was due to damage of plastid membranes of balanites kernels in maturing seeds. Figures 7 and 8 shows that, in the later stages of balanites fruit development, some phospholipids as well as the glycolipids are lost from the kernels This is consistent with literature reports that point out that during the course of seed filling and maturation of oilseeds, embryogenic plastids of oilseeds eventually lose their membrane structure, as reported for arabidopsis (Allorent *et al*., 2013; Mansfield and Briarty, 1992) as well as for soybean (Monma *et al*., 1994).

Thus it could be assumed that practically fruit developmental stage had little effect on fatty acid quality of BKO, a high linoleate-type of vegetable oil.

Table 2. Oil content of the woody part (endocarp) of the balanites fruit of different developmental stages. In very young stages (S1 and S2), the woody part was physically inseparable from the rest of the fruit

Fruit stage	Oil content, % of dry weight
S3	8.4
S5	9.2
S7	6.5
S9	6.2
S11	5.4

Table 3. Fatty acid composition of balanites kernel oil at different fruit maturity stages. The unsaturation index was calculated by multiplying the number of double bonds in an unsaturated fatty acid by its percentage composition (e.g 2x23.4, for oleic acid at stage 2) and summing up the total for each fruit stage

Fatty acid/ Unsat.	Fruit maturity stage					
Index	(S2)	(S4)	(S5)	(S7)	(S9)	(S11)
Palmitic acid (16:0)	15.7	18.1	12.7	10.4	10.0	8.4
Stearic acid (18:0)	4.3	4.3	4.8	3.4	8.2	10.0
Palmitoleic acid (16:1)	2.4	2.0	-*	-*	-*	-*
Oleic acid (18:1)	23.4	32.8	31.6	40.6	29.1	25.4
Linoleic acid (18:2)	50.8	40.2	49.2	44.2	52.5	56.0
Linolenic acid (18:3)	3.4	2.6	1.8	1.2	-*	-*
Unsaturation Index	142.4	127.0	134.5	133.5	134.0	137.0

* = trace or absent.

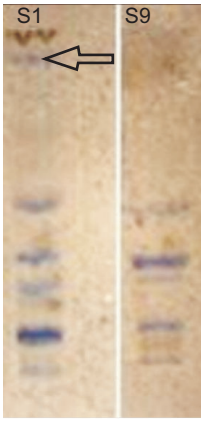

Fig. 7. TLC separation of phospholipids from kernels of immature (stage S1) and mature (S9) fruits of balanites. Polar lipids were extracted using chloroform/methanol (2:1). TLC solvent: chloroform/ methanol/ 7N ammonium hydroxide (130:60:8). Phospholipid detection: Dittmer's reagent (Dittmer and Lester, 1964).

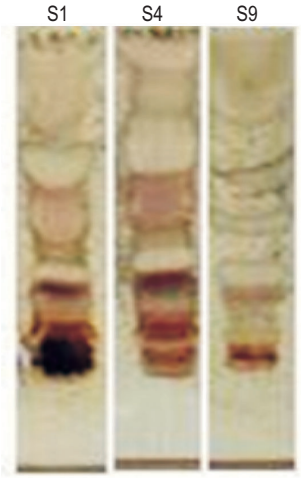

Fig. 8. TLC separation of individual glycolipids of balanites kernels at stages S1, S4 and S9. Extraction and TLC solvent as in Fig 7. Glycolipid detection: orcinol reagent (Skipski and Barclay, 1969).

Conclusions

Balanites fruits could be harvested at stages S7 or S8, still green but mature stages. At these optimum stages total fruit sapogenin in 'kernels' and in 'epicarp+ mesocarp' as well as kernel oil content are maximal. Thus loss of the sapogenin bearing, fragile epicarp, encountered during harvesting and handling of dry fruits is avoided. Moreover, the mechanically separated kernels would afford sapogenin purely of the 25α- type.

The finding that kernel tissue accumulated sapogenins predominantly of the 25α- type may reflect the operation of plastid-specific biosynthetic pathways of sapogenins.

The woody endocarp contained considerable amounts of fixed oil, particularly in young stages, confirming our previous observations.

The fatty acid composition of balanites kernel oil, a high linoleate type, did not show marked changes all through developmental stages. However, oil of kernels of very young fruit stages contained palmitoleic and linolenic acids, the two being associated with glycolipids and phospholipids of plastidic membranes which are lost during fruit maturation.

Conflict of Interest. The authors declare no conflict of interest.

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