Phytochemistry, 1971, Vol. 10, pp. 7.497 to 2503. Pergamon Press. Printed in England.

ISOLATION AND CHARACTERIZATION OF STEROIDS AND OTHER CONSTITUENTS FROM *TRIGONELLA FOENUM-GRAECUM*

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(Received 14 *October 1970)*

Abstract-Diosgenin and its 25β-epimer, yamogenin were separated and shown to be the major sapogenins of ***Trigonella foenum-graecum*** L. Trigonellagenin, found by other workers, is considered to be a mixture of these two epimers. They are obtained by acid hydrolysis, in different proportions from West Pakistani and Moroccan seed, with the total yield higher from the latter. The presence of 25α-spirosta-3,5-diene, an artefact, was confirmed, together with gitogenin, and evidence was obtained for the isolation of their 25β-epimers. Co-crystallization of 25α- and 25β-spirosta-epimers accounts for the variations in m.p. reported. A trace of tigogenin was detected by TLC from the Moroccan seed only. Petrol extraction of the powdered seeds yielded fixed oil (7 per cent) in which squalane-like hydrocarbons were tentatively identified. The sterols included β-sitosterol and cholesterol. Gitogenin was not found in the leaf, stem and root, which otherwise afforded the compounds derived from the seed.

INTRODUCTION

Trigonella foenum-gruecum L. (fenugreek) (Leguminosae) is an annual, herbaceous plant widely distributed in many parts of Asia, Africa and Europe. The seed is considered[1] to be a potential economic source of diosgenin and yamogenin for the steroid industry which is estimated to require some 1·5 million kg of plant steroid in 1973.[2] A mixture of diosgenin and yamogenin is as acceptable as diosgenin alone for the partial synthesis of pharmaceutical steroids. The genins of this seed have been the subject of somewhat contradictory reports.[3–8] Marker *et al.*[5] isolated from powdered seed diosgenin, gitogenin and a trace of tigogenin. Soliman and Mustafa[6] found diosgenin and gitogenin together with another sapogenin, trigonellagenin, but they could not find tigogenin. From the defatted powdered seed, Bedour *et al.*[7] isolated 25α-spirosta-3,5-diene besides the three sapogenins reported by Marker *et* al. but failed to find trigonellagenin. Varshney[8] reported only diosgenin and gitogenin.

Our preliminary studies[9] on 52 samples of fenugreek seed from 18 countries indicated the presence of variable amounts of total sapogenin by IR method.[10] From the root of

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[1] R. HARDMAN to National Research Development Corporation, London, (a) ***Brit. Put.*** 1,136,626 (1968), (b) 1,198,626 (1970), and (c) ***Brit. Put. Appl. 39765*** (1967).
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[9] F. R. Y. FAZLI and R. HARDMAN, ***Trop. Sci.*** 10, 66 (1968).
[10] K. R. BRAIN, F. R. Y. FAZLI, R. HARDMAN and A. B. WOOD, ***Phytochem. 7, 1815 (1968).***

Balanites aegyptiaca we have characterized yamogenin,' [1] a genin not reported in ***T. foenum-graecum.*** Serpukhova[12] classified fenugreek seed into three major groups with many varieties on the basis of their shape, size and colour. We also found this classification useful. [9] Assuming that there may be different chemical races of *T.* ***foenum-graecum*** affording various genins we selected two samples of seed which belonged to different groups in the above classification and were available commerically. The seed of one was small, approaching square in shape, yellowish brown in colour and was from West Pakistan (Serpukhova's Group: Indicae), while the other seed was comparatively larger in size, elongated, yellow-cinnamon in colour and was from Morocco (Group: Aethiopicae). The sterols of the seed and the steroids of the root, stem and leaf required investigation because of their possible economic value, the crop being an annual. The co-occurrence of saponins and squalane was of interest because of the effect of this hydrocarbon on sapogenin yield.[1a,1b,13]

RESULTS AND DISCUSSION

The total sapogenin content of the whole seed of ***T. foenum-graecum*** when obtained by the IR method [10] was 1·27% (25α-epimers, 62 % and 25β-epimers, 38 %) for the West Pakistani seed and 1·50% (both epimers equal) for the Moroccan seed.

West Pakistani Seed: Spirostans

The neutralized and dried acid insoluble matter from the whole seed was fractionally extracted with petrol: from the *first* ***extract*** a fixed oil was obtained which contained 25-spirosta-3,5-diene and sapogenin. The latter, afforded needles from acetone, m.p. 189–190°, which co-chromatographed with a natural mixture of diosgenin and yamogenin. From the IR spectrum equal absorption at 900 cm^{-1} and 917 cm-' indicated that a mixture of almost equal amounts of diosgenin and yamogenin had been obtained. By Soxhlet extraction a ***second extract*** also gave fixed oil and sapogenin and the latter gave needles from acetone, m.p. 203-204" undepressed on admixture with an authentic specimen of diosgenin. Its spectrum was identical with that of diosgenin from ***Dioscorea*** *sylvatica* tuber and the much stronger peak at 900 cm^{-1} than at 917 cm-' indicated that it was diosgenin with not more than 10 per cent of yamogenin. The mother liquor yielded a mixture of almost equal parts of diosgenin and yamogenin. A ***third extract*** gave 'gitogenin' with a trace of diosgenin. After column chromatography the 'gitogenin' gave needles from acetone, m.p. 260" (reported 268–270°,[7] 271°[14]) and a diacetate, m.p. 243". The spectrum of this 'gitogenin' showed all the characteristic peaks but pronounced absorption at both 900 cm^{-1} and 917 cm^{-1} suggested that it was a mixture of gitogenin with its 25β-epimer, neogitogenin. These could not be separated with the solvent systems used.

The m.ps of some sapogenins, as reported in the literature by various workers, frequently differ by as much as 20". Marker ***et al.*** also noticed such variations in their extensive work with plant sapogenins and suggested that polymorphic forms could occur.[15] The variations in the melting points in our work were due to the isolation of mixtures of the 25α- and 25β-epimers of a sapogenin or (see below) of a diene.

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[12] V. I. SERPUKHOVA, ***Trudy, Prikl. Bot., Genet i Selekcii Sen 7*** **(1),** (1934).
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[14] C. W. SHOPPEE, ***Chemistry of Steroids,*** Butterworths, London (1964).
[15] R. E. MARKER, R. B. WAGNER, P. R. ULSHAFER, E. L. WITTBECKER, D. P. J. GOLDSMITH and C. H. RUOF ***J. Am. Chem. Soc. 69, 2167 (1947).***

Mother liquors were combined, the solvent removed and the product after saponification and column chromatography yielded hydrocarbons, 25-spirosta-3,5-dienes and a mixture of equal parts of diosgenin and yamogenin. When submitted to preparative TLC this genin mixture yielded yamogenin as a lower band which was removed by chloroform and crystallized from alcohol: m.p. 190"; $[\alpha]_D^{24\cdot7}$ -125"; IR and mass spectral data was as required (m/e = 414). Elemental analysis of its acetate (m.p. 160-162") and its spectra further confirmed the identification. Diosgenin (upper band) from acetone gave m.p. 211° and its spectrum and acetate confirmed its purity.

Moroccan Seed: Spirostans

The Moroccan seed afforded by the same procedure a mixture containing almost equal parts of diosgenin and yamogenin from both the ***first*** and ***second extracts.*** The m.p. of the various crops obtained, varied between 190 and 194". In this case, diosgenin could not be separated from yamogenin from the ***second extract*** without a tedious preparative TLC procedure with continuous elution. Separation of the two epimers appears possible only from a seed which contains predominantly diosgenin, as in the West Pakistani seed.

'Trigonellagenin'

Soliman and Mustafa[5] isolated a compound with m.p. 189–190°, acetate m.p. 156–158°, and named it trogonellagenin. Bedour *et al.*[7] could not find this compound nor did they report yamogenin. We consider that trigonellagenin is not a new compound but a mixture of diosgenin and yamogenin. The reason that Bedour *et al.* could not find 'trigonellagenin' was probably because the seed they used contained predominantly diosgenin.

25-Spirosta-3,5-diene

The 25-spirosta-3,5-diene, an artefact from the acid hydrolysis, being readily soluble in petrol was present only in the above ***first extract*** and in the first fractions from the column chromatography. These, on removal of the solvent, afforded needles from ethanol, m.p. 147-148". The m.p. was about 15" lower than previously reported by Bedour *et al.*[7] and thus its identity was suspected. The spectrum showed pronounced absorption at 985, 917, 900 and 868 cm^{-1} revealing a steroidal sapogenin nucleus with an intact spiroketal side chain. Comparatively higher absorption at 900 cm^{-1} than at 917 cm^{-1} indicated 25α-configuration but this did not exclude the possibility of presence of its 25β-epimer. There was no apparent absorption for a free hydroxyl group at 3490 cm^{-1}. The absence of absorption in the region 1700-1800 cm^{-1} indicated that a OH group combined as an ester was also absent. The UV spectrum in ethanol exhibited triplet maximal absorption with peaks at 227·5, 234·5 and 243 nm and thus the presence of a hetero-annular conjugated diene system of the type present in cholesta-3,5-diene. It could not be acetylated, confirming its lack of hydroxyl groups. TLC examination with continuous development disclosed a mixture containing 25α-spirosta-3,5-diene and 25β-spirosta-3,5-diene. The latter has recently been isolated and characterized by Hardman and Wood[16] from the seed of ***Balanites orbicularis*** which also yields yamogenin. Their sample of dienes, 25α- of m.p. 165°, and 25β- of m.p. 181°, were used as references for the comparison. A mixture of these two dienes when crude had a m.p. of 141". It seems that the presence of the 25β-epimer of spirosta-3,5-diene is due to dehydration of yamogenin and crystallization of mixtures of the

[16] C. N. WOOD, personal communication.

two dienes will account for the variation in the m.ps previously reported: Wall *et al.*[17] obtained a product of m.p. 130-140" by refluxing yamogenin with concentrated hydrochloric acid in 95% ethanol for 96 hr and designated it 25α-spirosta-3,5-diene after comparison with a 'somewhat purer' sample prepared from diosgenin. The 25α-diene must have resulted from isomerization during the prolonged treatment. We boiled for only 2 hr with 2 N HCI and in the absence of ethanol in order to minimize contamination of the sapogenin by diene. Bedour *et al.*[7] in obtaining their diene, m.p. 162–163°, from fenugreek seed used 4 N HCI with a small quantity of butanol and boiled for 3 hr.

The total weights of spirostans obtained from the Pakistani and Moroccan seeds are given in Table 1. Seed from two different groups of Serpukhova's classification yielded various amounts of the same sapogenins. Only in the Moroccan sample was a trace of tigogenin-like substance detected on TLC examination of the diosgenin/yamogenin-containing fraction. Its amount was too small to be separated quantitatively for further investigation.

TABLE 1. GRAVIMETRIC YIELD OF SPIROSTAN FROM WEST PAKISTANI AND MOROCCAN FENUGREEK SEED

Spirostan	Per cent yield on moisture free base	
	West Pakistani	Moroccan
Diosgenin and yamogenin	0.83	0.92
Gitogenin and neogitogenin	0.02	0.03
25α- and 25β-spirosta-3,5-dienes	0·03	0.03
Tigogenin	absent	a trace
Total	0.88	0·98

Squalane-like Hydrocarbons (preliminary results) and Sterols

Powdered Pakistani seed was extracted with petrol and TLC examination of the oil obtained showed the absence of steroidal sapogenins. The unsaponifiable matter from the oil, when subjected to column chromatography, yielded hydrocarbons in the first fraction eluted with petrol. The compound identical with squalane in TLC, was separated by preparative TLC to afford a colourless oil. On standing, crystalline material separated out leaving behind an oil. This oil was partially miscible with acetone (like squalane). Its mol. wt. (MS) was 423 (mol. wt. squalane 422.83). Its IR spectrum showed the peaks 734 cm^{-1} for (CH,), where $n < 4$; 1175 cm^{-1} for C-C skeletal branched chain; 1380 cm^{-1} for H_3C \ C- and C-CH, ; 1470 cm^{-1} for CH, bend (scissors) and 2950 cm^{-1} for H_3C / C-H stretch (CH, and CH,) and indicated that the compound was a saturated branched, long chain hydrocarbon. The spectrum was also superimposable on the IR spectrum of authentic squalane. The crystalline material, after recrystallization from acetone gave needles, m.p. 57°–58°, and had a mol. wt. of 480 (m.s.). Its IR spectrum was similar to that of the oil indicating this to be also a saturated branched long chain hydrocarbon. The difference between the two may be in the length of the chain. These hydrocarbons and others of *T. foenum-graecum* are being further investigated,

[17] M. E. WALL, S. SEROTA and L. P. WITHAUER, *J. Am. Chem. Soc.* 77, 3086 (1955).

Squalene has been frequently isolated from plants where it is usually present in low concentration. So far there is little evidence of the natural occurrence in plants of squalane or squalane-like compounds, that is branched chain hydrocarbons with about 30 carbon atoms, fully or almost fully saturated. From our present investigation some evidence has been obtained for the co-occurrence of squalane-like hydrocarbons with saponins in the unsaponifiable matter of fenugreek leaf as well as in the seed. Hardman *et al.* have investigated and discussed the co-occurrence and acid release of hydrocarbons and diosgenin and yamogenin in the seed of species of *Balanites*[18] and in the tubers of *Dioscorea deltoidea.*[19] The effect of a variety of extraneous hydrocarbons, including squalane, on the yield of steroidal sapogenin from plant material[1a,1b], has been reported.[13]

The seed oil unsaponifiable matter yielded β-sitosterol via an alumina column. The mother liquor when combined with other sterol fractions from the column and submitted to GLC showed 4 major components. Two of these corresponded to β-sitosterol and cholesterol in their retention time and chromatographed with authentic specimens. Acetate and TMS derivatives confirmed their presence. The other two components were not identified. Reports of cholesterol in sapogenin-affording tissue are now common.

Leaf, Stem and Root from Moroccan Seed Sown in England

Because ***T. foenum-graecum*** is an annual, and with ripening of the fruits leaf fall may occur, the plants were harvested when they carried fully developed pods but contained unripe seeds. Oils obtained by separate extraction of the powdered dried leaf, stem and root yielded after saponification squalane-like hydrocarbons and β-sitosterol, but no free sapogenin or spirostadiene. Acid hydrolysis of the defatted powdered leaf yielded 25α- and 25β-spirosta-3,5-diene, a 1: 1 mixture of diosgenin and yamogenin, and β-sitosterol. The stem and root when similarly treated showed the same steroids in trace amounts by TLC examination. Gitogenin was not detected in the leaf, stem or root and was found in the seed only. The leaf, stem and root from plants grown in West Pakistan from the West Pakistani seed gave very similar results.

Sterols were present in all parts of the plant and occurred in both a combined and a free state. There was no evidence of sapogenins in the fixed oil or in the unsaponifiable matter from the oil from the seed or other morphological parts. Saponins have been isolated from fenugreek seed[5] and sapogenins were found only after acid hydrolysis of the seed and other parts. This suggests that the sapogenins occur in the plant only as their glycosides and as such are not directly in association with the stored fat, but rather with cell wall material and as free saponin in the circulatory system of the plant thus effecting easy transportation of the steroid and protecting the latter. Glycoside formation involving the cell wall[20,21] may well be a method of steroid storage in the plant and of controlling excess steroid, so preventing its interference in normal cellular mechanisms. The interrelationship of steroidal saponins and plant growth regulators have recently been discussed by Hardman *et al.* following studies using powdered tubers of ***Dioscorea deltoidea***[22] and powdered fruits of ***Balanites orhicularis.***[23] *No* doubt the steroidal sapogenins and their glycosides have several functions in a given plant.

[18] R. HARDMAN, C. N. WOOD and E. A. SOFOWORA, *Phytochem.* 9, 1087 (1970).
[19] R. HARDMAN and K. R. BRAIN, Phytochem. 10, 1115 (1971).
[20] G. BLUNDEN, R. HARDMAN and W. R. WENSLEY, *J. Pharm. Pharmac.* 17,274 (1965).
[21] R. HARDMAN and E. A. SOFOWORA, *Planta Medica* (in press).
[22] R. HARDMAN and K. R. BRAIN, *Phytochem.* 10, 519 (1971).
[23] R. HARDMAN and C. N. WOOD, *Phytochem.* 10, 757 (1971).

EXPERIMENTAL

Materials. T. foenum-graecum seed was cultivated in District Sialkot of West Pakistan, and that of Moroccan origin was purchased in London. All seed had a germination rate of at least 99 per cent. The Moroccan seed was also grown under field conditions in Nottingham. The whole plant was dried at 55" for 24hr in separate parts and finely powdered. Dried leaf, stem and roots were also received from West Pakistan and powdered.

Methods. Petrol is light petroleum (b.p. 40–60°) unless otherwise stated. Sapogenins and sterols were detected with 300% w/v $SbCl_3$ in conc. HCI on TLC. For prep. chromatography plates were coated with 1 mm silica gel PF 254 + 366 'for prep. TLC E. Merck' or silica gel rhodamine 6G. Developing solvents: hexane; hexane-acetone (4:1); hexane-di-iso-propyl ether (96:4) or CH_2Cl_2–Et_2O (98:2). M.ps are corrected; $[\alpha]_D$ in $CHCl_3$ in 1 cm tubes; UV using 96 % ethanol; IR using a KBr disc and a thin film between rock salt discs; GLC columns 6 ft x $\frac{1}{4}$ in. o.d. (glass), silicone gum rubber SE 30 coated on acid-washed-HMDS Chromosorb G. 80-100 mesh, temp. 225°, injector temp. 275" and detector temp. 250°, carrier (NJ 30 ml/min. Reference sterols, 1 μl 2·0% w/v in anal. $CHCl_3$ injected.

Extraction of spirostans from Pakistani seed. Whole seed (2 kg) was boiled under reflux with 10 1.2 N HCI for 2 hr and the mixture cooled and filtered. The insoluble matter was washed with water, ammonia soln. (20% w/v) and again with water. The alkaline residue was dried at 75" in a hot air stream for 24 hr, finely powdered and placed in a Soxhlet thimble. The thimble was covered with petrol for 2 hr without heat giving the ***first extract. The second extract*** was obtained after refluxing for 4 hr, and refluxing for 7 days gave the ***third extract.*** **The** ***first extract,*** after concentrationgave an oil, which on dilution with petrol afforded a solid (2.78 g) after 2 days. This gave a mixture of diosgenin and yamogenin, m.p. 189-190". The second ***extract*** freed of solvent. when fluxed with petrol (b.p. 60–80°) gave a solid. 6.88 g after 2 days which yielded needles from acetone, m.p. 203–204°, of a mixture of diosgenin (about 90%) and yamogenin, $C_{27}H_{42}O_3$; acetate, from acetone, m.p. 193–194°, $C_{29}H_{44}O_4$. (The ***Moroccan seed*** similarly gave a 1 :1 mixture of diosgenin and yamogenin, m.p. 190–192°). The ***third extract,*** freed of petrol, gave a yellowish solid, (TLC, mainly gitogenin with a trace of diosgenin) which on elution from a column of activated alumina (Type H 100/200 mesh, Peter Spence & Sons Ltd., England) with a mixture of benzene–$CHCl_3$ (7:3) afforded gitogenin from acetone, m.p. 260°, $C_{27}H_{44}O_4$; diacetate, m.p. 243°, $C_{31}H_{48}O_6$.

The combined mother liquors were concentrated and the residue saponified by boiling under reflux for 1 hr with 3 1. N/2 alcoholic KOH. Alcohol was removed and the residue in 500 ml ether was added to a separator containing 500 ml water. The unsaponifiable matter was extracted with ether and the solvent removed. The paste obtained was dissolved in petrol and separated on activated alumina (50 g, in column 512 x 30 mm) with petrol. Elution was monitored with UV. Fractions (50 ml) showing the same spot or spots by TLC were bulked. The solvents were petrol, benzene, $CHCl_3$, ethanol in sequence, and the proportion of each new solvent was gradually increased through 1, 2.5, 5, 10, 25, 50 and 100%. Fraction 1 gave hydrocarbons; fractions 7-11, from petrol and benzene, gave 25-spirosta-3,5-diene, needles from ethanol, m.p. 147–148°, $[\alpha]_D^{24\cdot7}$ −166°, $C_{27}H_{41}O_2$, and when subjected to TLC with continuous development in hexane: *iso*-propylether (96:4) for 2 hr gave the two epimers (25α and 25β). Fractions 27-35, of benzene-$CHCl_3$ showing a greenish-yellow band in UV gave a mixture of diosgenin and yamogenin of m.p. 188-190 after recrystallization. By prep. TLC on continuous elution for 18 hr using dichloromethane–ether (97:3) the two epimers were separated: Yamogenin, needles from ethanol, m.p. 190°, IR, $C_{27}H_{42}O_3$; m.p. 160–162°, $C_{29}H_{44}O_4$. Diosgenin, needles from acetone, m.p. 211°, IR; acetate, m.p. 194–195°.

Isolation of squalane-like hydrocarbons and sterols. Pakistani seed powdered to pass B.S. sieve No. 40, was Soxhlet extracted with dried ($MgSO_4$) petrol for 48 hr. Solvent was removed to give an oil, 29.41 g (6·5% m.f.b.). This was saponified (1 hr with 600 ml of N/2 alcoholic KOH) and the unsaponifiable matter, 1.685 g, 5·6% of the oil, 0·37% of the seed, m.f.b. was separated on activated alumina (Spence, Type H) (20 g, 400 x 18 mm). Petrol elution in 25 ml fractions. was monitored with UV. Fractions 1-3. were bulked and subjected to prep. TLC on silica gel rhodamine 6G. The spot identical in R_f value and colour in daylight (pale pink) and fluorescence in UV (pink) with squalane was separated and the oil obtained on removal of the solvent was set aside in darkness to yield a white crystalline solid (31 mg) and a colourless oil. Their physical properties are given in the Results and Discussion. Fractions 23–27 on removal of the solvent gave β-sitosterol, $C_{29}H_{50}O$, 0.378 g (0·08% of the seed, m.f.b.). m.p. mixed m.p., IR, GLC, mol. wt. 414 (m.s.); acetate, $C_{31}H_{52}O_2$, m.p., mixed m.p. Fraction 22, combined with the mother liquor from the crystallization of β-sitosterol, and the solvent removed gave a solid which when submitted to GLC showed β-sitosterol and cholesterol. Acetate and TMS derivatives of the mixture were prepared and GLC confirmed their identity.

Investigation ofpowdered leaf, stem and root. Leaf (480 g) was defatted with petrol in a Soxhlet for 7 days to give an oil, 14.3 g (3·5%, m.f.b.). On standing for 3 days, an amorphous substance, 0·315 g, separated from it, which on repeated deposition from acetone solution gave a white solid, m.p. 61-62". Its IR spectrum suggested it was an ester of a long chain fatty acid. The oily-mother liquor was saponified and the unsaponifiable matter was chromatographed on alumina as before. Fractions 1 and 2 by prep. TLC afforded the squalane-like material as a crystalline solid and an oil, as from the seed. Fractions 7 and

8 via prep. TLC on silica gel PF 254 + 366 plates in hexane: acetone system gave β-sitosterol, $C_{29}H_{50}O$, m.p., mixed m.p., IR; acetate, m.p.

The defatted leaf was refluxed with 2 N HCl(4 1.) for 2 hr and the acid insoluble matter treated as in the case of seed afforded, after extraction with petrol for 5 days, a solid 9.75 g (239 % m.f.b.). This was chromatographed on neutral activated alumina (60 g, Brockman Type H) eluting with increasing proportions of $CHCl_3$, in benzene while monitored with UV. Fractions 2-5 contained 25-spirosta-3,5-dienes (0.245 g, 0·06% m.f.b.), needles from ethanol, m.p. 147-148". Fractions 14–18, gave a greenish solid (O-98 g) which by TLC on silica gel PF 254 + 366 plates, and when recrystallized from acetone gave a mixture of diosgenin with yamogenin, (O-218 g, 0·05% m.f.b.) IR, m.p. 190–191°, $C_{27}H_{42}O_3$.

Stem (341 g) extracted with petrol by Soxhlet for 7 days gave 3·40 g of an oil, TLC of which showed hydrocarbons and β-sitosterol. The defatted stem when hydrolysed with 4 1.2 N HCl and in the usual way afforded an oil, 1.579 g. TLC of this revealed diosgenin/yamogenin and 25-spirosta-3,5-diene. Root (60 g) when subjected to similar extraction procedures gave the same compounds as from the stem but in smaller amounts.

Acknowledgements-We thank Rhalifa Muhammad Saeed of Alomahar, District Sialkot, West Pakistan for having the crop grown and Professor T. Reichstein for sending samples of gitogenin. We are grateful ta the National Research Development Corporation for financial assistance. One of us (F.R.Y.F.) also thanks the Government of Pakistan and the Commonwealth Foundation for their research grants.