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FLAVONOIDS OF THE FRUITS AND LEAVES OF *TRIBULUS TERRESTRIS*: CONSTITUTION OF TRIBULOSIDE

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Abstract—Kaempferol, kaempferol-3-glucoside, kaempferol-3-rutinoside and tribuloside (kaempferol-3-β-D-(6″-*p*-coumaroyl) glucoside) have been isolated from *Tribulus terrestris*.

Tribulus is a cosmopolitan genus of fifteen species belonging to the family Zygophyllaceae. Three species, viz. *Tribulus cistoides*, *T. terrestris* and *T. alatus*, are of common occurrence in India. Among them *T. terrestris*, which is a trailing plant common in sandy soil, has been described to be of medicinal value. Since *Tribulus* species are known to cause photosensitivity in animals,[1] *T. terrestris* has been examined by a number of workers[2–4] who have recorded the presence of saponins yielding diosgenin, ruscogenin, gitogenin and 25-*D*-spirosta-3,5-diene as the aglycones. The present investigation is, therefore, restricted to the study of phenolic constituents.

The leaves and fruits of the plant *T. terrestris* were successively extracted with light petroleum, ether and alcohol. The first two extracts contained mostly chlorophyll and waxy material and were not examined further. The alcohol extract was the major fraction. On fractional crystallization, it deposited potassium nitrate and a mixture of saponins, which had been studied by the earlier workers.[2–4] The mother liquor was first extracted with light petroleum to remove chlorophyll and waxy material and then with ether and ethyl acetate.

The ether and ethyl acetate fractions were found to be identical and gave positive colour reactions for flavonoids. They were combined and chromatographed on silica gel. The different fractions furnished compounds *A*, *B*, *C* and *D*. From the extracted mother liquor a mixture of saponins was precipitated with ethanol. The ethanol-soluble portion, on column chromatography over silica gel, afforded compound *E*.

Compound *A* was identified as kaempferol. Compound *B* was found to be a triterpenoid by colour reactions. The i.r. spectrum of *B* showed the presence of hydroxyl groups and unsaturation. On acetylation, it furnished an acetyl derivative, m.p. 150–152°, but it could not be studied further due to lack of material. However, it was not identical with any of the sapogenins already known to occur in the plant.

Compound *C* was a new acylated flavonol glycoside and called tribuloside. The analytical data agreed with the molecular formula $C_{30}H_{26}O_{13}$. It gave an olive-green colour with alcoholic $FeCl_3$ and red colour with magnesium and hydrochloric acid, and zinc and hydrochloric acid. These observations suggested that tribuloside was either a dihydroflavonol or

[1] M. HENRICI, *Ondersetepoort J. Vet. Sci. Animal Ind.* **10**, 367 (1938).
[2] W. T. DE KOCK and P. R. ENSLIN, *J. S. African Chem. Inst.* **11**, 33 (1958).
[3] J. M. BROWN and W. T. DE KOCK, *S. African Ind. Chem.* **13**, 189 (1959).
[4] M. TOMOVA and D. PANOVA, *Farmatosiya (Sofia)* **15**, 210 (1965).

3-substituted flavonol.[5] The former possibility was ruled out by its u.v. spectrum with maxima at 270 and 315 nm (stronger), whereas dihydroflavonols have different absorption characteristics[6] (high max. at 278 to 292 nm and low maximum at about 325 nm). Therefore it appeared to be a 3-substituted flavonol. However, there was some doubt because the long wavelength band (340–380 nm), characteristic of flavonols, was missing.[7]

Tribuloside is highly soluble in sodium carbonate and to a lesser extent in sodium bicarbonate solution, suggesting the presence of a free 7-hydroxyl, which was confirmed by a bathochromic shift (8 nm) in the shorter wavelength band of the spectrum in sodium acetate. The aluminium chloride shift in the spectrum indicated the presence of a free 5-hydroxyl.

The i.r. spectrum had, in addition to the usual flavonoid carbonyl frequency at 1665 cm^{-1}, a strong peak at 1700 cm^{-1}, due to an ester carbonyl, strong absorption at 3400–3600 cm^{-1}, due to hydroxyl groups, and multiple absorption at 1600–1625 cm^{-1}, due to aromatic double bonds.

Tribuloside resembled a glycoside in solubility and behaviour on TLC; however, Molisch's test was not definite. It formed a heptaacetate as indicated by analytical data and NMR spectrum discussed below. On methylation with diazomethane it gave a tetra-methyl ether which did not give any reaction with $FeCl_3$ indicating that the chelated hydroxyl group had also been methylated. The i.r. spectrum of the methyl ether still had a strong band in the hydroxyl region showing the presence of free alcoholic hydroxyl groups.

That tribuloside was an *O*-glycoside was shown by acid hydrolysis which yielded glucose, kaempferol and *p*-coumaric acid. However, alkaline hydrolysis under mild conditions was more convenient to liberate *p*-coumaric acid, identified by spectral data and comparison with an authentic sample, and another compound which was found to be kaempferol-3-glucoside on the basis of hydrolytic and spectral studies.

All these observations indicate that tribuloside is composed of 1 mole each of kaempferol, glucose and *p*-coumaric acid, and that glucose is attached to the 3-hydroxyl of the kaempferol and the acid was esterifying either a phenolic hydroxyl or a sugar hydroxyl. The latter possibility was indicated by the low carbonyl frequency at 1700 cm^{-1} and was confirmed by the acid hydrolysis of the methyl ether of tribuloside when kaempferol-5,7,4′-tri-*O*-methyl ether was formed along with glucose and *p*-methoxycinnamic acid. As no hydroxy acid was produced in the above hydrolysate, the possibility of the sugar being linked to the phenolic hydroxyl of the acid moiety was eliminated.

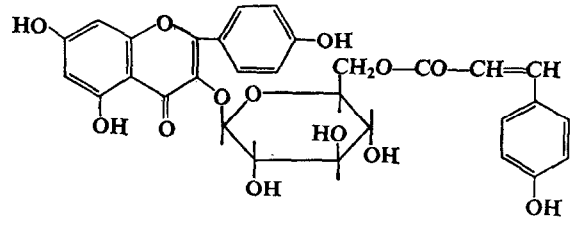
The above conclusions are supported by the NMR spectrum of tribuloside acetate which showed seven acetoxyl signals in the range δ 2·00–2·50 ppm integrating to twenty-one protons. Of four aromatic acetoxyls, δ 2·47 ppm (3H) could be assigned to the 5-acetoxyl, δ 2·35 ppm (9H) to 7 and 4′-acetoxyls and the acetoxyl of *p*-coumaric acid unit. The three alcoholic acetoxyls were at δ 2·04 ppm (6H) and 2·12 ppm (3H).

In order to locate the position of attachment of the acid group, periodate oxidation experiments were carried out using the methyl ether of tribuloside; 2·1 moles of the oxidant were consumed. Further, formic acid was formed as one of the oxidation products. These results could be explained only if the acid group were attached to the 6-hydroxyl of the glucose moiety and the sugar group was in the pyranose form.

That tribuloside has a β-glycosidic linkage was indicated by the NMR spectrum which

[5] J. C. PEW, *J. Am. Chem. Soc.* **70**, 3031 (1948); M. SHIMIZU, *J. Pharm. Soc. Japan* **71**, 1329 (1951).
[6] *Comparative Biochemistry of Flavonoids*, J. B. HARBORNE, p. 90, Academic Press (1967).
[7] L. JURD, *The Chemistry of Flavonoid Compounds* (edited by T. A. GEISSMAN), p. 110, Pergamon Press, Oxford (1962).

showed a doublet at δ 5·35 ppm (J=7 cps)[8] integrating for one proton. This was confirmed by the enzymatic hydrolysis experiments when the compound could be hydrolysed by emulsin and not with Taka-diastase. Hence, tribuloside is kaempferol-3-β-D-(6″-*p*-coumaroyl) glucoside (I)



Tribuloside (I)

A similar compound, tiliroside, has been reported to occur in *Tilea argentea*, having all the component parts as in tribuloside. However, considerable differences were observed in m.p. and specific rotation between them: tribuloside, m.p., 224–226°; $[\alpha]_D^{30}$ −81·3° (methanol): tiliroside,[9] m.p. 247–256°; $[\alpha]_D^{20}$ −62·5 (methanol). Further, the glucoside linkage of tiliroside was not β because it was not hydrolysed with β-glucosidase.[10] A direct comparison of the two could not be made due to non-availability of an authentic sample of tiliroside.

Compound *D* was a flavonoid glycoside from its colour reactions and chromatographic behaviour. U.v. and visible spectra showed it to be a kaempferol derivative. On acid hydrolysis it gave kaempferol and glucose and the latter was assigned to the 3 position of kaempferol on the basis of u.v. spectral data.[7] Thus the compound *D* was identified as kaempferol-3-glucoside (astragalin) and its properties agreed with those recorded in literature.[11]

Compound *E* was again a 3-substituted kaempferol derivative from its colour reactions and u.v. and visible spectral data and on acid hydrolysis it furnished kaempferol, glucose and rhamnose. Complete methylation with dimethyl sulphate and subsequent acid hydrolysis yielded 5,7,4′-trimethyl ether of kaempferol identified by comparison with an authentic sample. Compound *E* was thus identified to be kaempferol-3-rutinoside and its properties agreed with those recorded in literature.[11]

EXPERIMENTAL

R_f values were determined by circular paper chromatography on Whatman No. 1 filter paper using *n*-butanol:acetic acid:water (4:1:5) (a), 15% acetic acid (b), water saturated with phenol (c) and 25% acetic acid (d). TLC was carried out on silica gel, using toluene:ethyl formate:formic acid (5:4:1) (e) and benzene:methanol (4:1) (f). The flavonoid compounds were developed by exposure to ammonia vapour and TLC plates were also developed with 10% H_2SO_4 and heating at 120° for a few minutes. The u.v. spectra taken in ethanol are marked (i), with sodium acetate (ii), with $AlCl_3$ (iii), with boric acid and sodium acetate (iv) and with sodium ethoxide (v).

[8] T. J. MABRY, J. KAGAN and H. RÖSLER, *Nuclear Magnetic Resonance Analysis of Flavonoids*, The University of Texas Publication, Sept. 1964, No. 6418.

[9] L. HÖRHAMMER, L. STICH and H. WAGNER, *Naturwissenschaften* **46**, 358 (1959).

[10] J. B. HARBORNE, *Phytochem.* **3**, 151 (1964).

[11] S. HATTORI, *Chemistry of Flavonoid Compounds* (edited by T. A. GEISSMAN), p. 317, Pergamon Press, Oxford (1962).

Extraction

Tribulus terrestris was collected in November from Haridwar (India). Air-dried and coarsely powdered leaves and fruit (1 kg) of the plant were soxhleted successively with light petroleum, ether and alcohol. The alcohol extract (5 l.) deposited KNO_3 (2 g) on keeping overnight. The extract was concentrated to 500 ml when another solid (5 g) separated out; it gave positive test for saponins and was found to be a complex mixture. The mother liquor was further concentrated to a viscous liquid, diluted with water and extracted successively with light petroleum (to remove waxy material and chlorophyll), ether and ethyl acetate.

The ether and ethyl acetate fractions were identical on TLC (solvent e) and gave positive tests for flavonoids. The two were combined (4·5 g) and chromatographed on silica gel column (180 g). The column was first eluted with benzene and benzene:$CHCl_3$ (1:1) mixture, which removed most of the chlorophyll. Further, elution with benzene:ethyl acetate (1:1) afforded first, compound *A* (20 mg) admixed with chlorophyll, which was identified as kaempferol. Subsequent elution of the column with benzene:ethyl acetate (1:1) gave a mixture of two components (solvent e). It was rechromatographed over silica gel to remove chlorophyll. The eluate separated into acetone-insoluble (40 mg, compound *B*) and acetone-soluble fractions (300 mg, compound *C*). Benzene:ethyl acetate (1:3) eluate of the column gave compound *D* (80 mg). Finally acetone eluate contained resinous material and a mixture of saponins.

The mother liquor from the extraction on concentration gave a gummy mass (6 g) which was taken up in methanol and precipitated with absolute ethanol. The precipitate was a mixture of saponins. The mother liquor (2 g) was chromatographed over silica gel. Elution with methanol:ethyl acetate (1:19) furnished compound *E* (100 mg).

Compound B

Compound *B* crystallized from methanol, m.p. 305–310°, and gave positive Liebermann-Burchard test. ν_{max}^{KBr} 3600 (hydroxyl), 1635 cm^{-1} (unsaturation).

Compound C (*Tribuloside*)

The compound crystallized from acetone–ethyl acetate as pale yellow needles, m.p. 224–226°. (R_f 0·3 (e) and R_f 0·55 (f).)

Tribuloside was soluble in methanol and ethanol; sparingly soluble in acetone, ethyl acetate and insoluble in water and other organic solvents. (Found: C, 58·6; H, 5·1. $C_{30}H_{26}O_{13}.H_2O$ required: C, 58·8; H, 4·6 per cent.) U.v., (i) 270 ($\log\epsilon$ 4·35), 305 (inflex.), 315 ($\log\epsilon$ 4·48), 355 nm (inflex.); (ii) 278, 315, 360 (inflex.); (iii) 278, 305, 320 (inflex.), 399 nm; (iv) 270, 315, 360 nm (inflex.) and (v) 279, 315, 376 nm. I.r. ν_{max}^{KBr} 3600 (s), 3400 (s), 1700 (s), 1665 (m), 1625 (s), 1600 (m), 1555 (s), 1505 (w), 1420 (m), 1360 (s), 1300 (s), 1250 (s), 1210 (s), 1175 (s), 1140 (s), 1125 (s), 1065 (s), 1030 (s), 1015 (s), 990 (m), 978 (m), 825 (s) cm^{-1}.

Acid Hydrolysis

The compound (50 mg) was refluxed with 2 N HCl:ethanol (1:1, 20 ml) for 3 hr. Ethanol was removed and the solution, after extraction with ether, taken to dryness. The residue contained glucose. The acetone-soluble fraction of the ether extract was identified as kaempferol. The acetone-insoluble fraction was treated with aq. $NaHCO_3$ (2%) and from the soluble portion *p*-coumaric acid isolated.

Alkaline Hydrolysis

The compound (50 mg) was refluxed at 100° with 1 N KOH:ethanol (1:1, 10 ml) for 1 hr. The mixture was cooled, acidified (dil. HCl) and extracted with ether and ethyl acetate. The ether extract yielded *p*-coumaric acid, and the ethyl acetate extract yielded a glycoside (R_f values 0·75, 0·4 and 0·8 in solvent systems (a), (b), and (c) respectively), m.p. 175–176°. U.v., (i) 268, 345 nm; (ii) 276, 350 nm; (iii) 278, 340, 390 nm; (iv) 274, 325, 390 nm. On further hydrolysis with 7% H_2SO_4 it afforded kaempferol and glucose. The observed data agreed with that reported for kaempferol-3-glucoside.

Acetylation

Acetylated tribuloside (acetic anhydride and pyridine), crystallized from ethyl acetate–light petroleum (60–80°), had m.p. 120–122°; ν_{max}^{Nujol} 1760 (acetoxyl $\rangle$CO), 1710 (ester $\rangle$CO) and 1660 cm^{-1} (flavonoid $\rangle$CO). (Found: C, 59·5; H, 4·7. $C_{44}H_{40}O_{20}$ required: C, 59·5; H, 4·5 per cent.) NMR taken in $CDCl_3$ on a Varian A-60 instrument (TMS) gave (δ values ppm; no. of protons; assignment): 2·47 (S, 3H, 5-acetoxyl); 2·35 (S, 9H, 7,4′-acetoxyls and the phenolic acetoxyl of *p*-coumaric acid); 2·04 (S, 6H, sugar acetoxyls); 2·12 (S, 3H, sugar acetoxyl); 5·35 (broad, 1H, $J=7$ cps);. The remaining sugar protons were obtained between δ 3·80 to 4·40 ppm. The various aromatic protons, observed between δ 6·20 to 8·20 ppm, could not be fully interpreted due to mixing up with the $CHCl_3$ peak (δ 7·40 ppm) present as an impurity in $CDCl_3$.

Methyl Ether of Tribuloside

Methylation with diazomethane gave a compound, crystallized from methanol, m.p. 148–150° (decomp.); ν_{max}^{Nujol} 3550 (OH), 1710 (ester >CO), 1655 cm^{-1} (flavonoid >CO). (Found: C, 59·1; H, 5·9; OCH_3, 16·9. $C_{34}H_{34}O_{13}.2H_2O$ required: C, 59·5; H, 5·8; OCH_3, 18·1 per cent.)

The ether on hydrolysis (2 N HCl:ethanol (1:1)) and subsequent fractionation gave *p*-methoxy-cinnamic acid and kaempferol-5,7,4′-tri-*O*-methylether, m.p. 149–150°, u.v., (i) 260, 305 (inflex.), 355 nm.

Periodate Oxidation

(i) The methyl ether was treated with 0·05 N $NaIO_4$ for 24 hr at 0°, consumed 2·09 moles of the oxidant and distillation of the solution gave positive chromotropic acid test for formic acid.

Compound D (*Kaempferol-3-Glucoside*)

Compound *D*, m.p. 175–177°, gave an olive-green ferric reaction, test for flavonoids and Molisch's test. It had the same R_f values and u.v. spectra as the glycoside obtained from alkaline hydrolysis of tribuloside. On hydrolysis it gave kaempferol and glucose only.

Compound E (*Kaempferol-3-Rutinoside*)

The compound crystallized from methanol as pale-yellow granules, m.p. 220–222°. It gave similar colour reactions as *D*. On paper chromatography it had the R_f values 0·60, 0·55, 0·70 and 0·75 in solvent systems (a), (b), (c) and (d) respectively. U.v., (i) 266, 350 nm; (ii) 274, 355–360 nm; (iii) 275, 395 nm; (iv) 265, 350 nm and (v) 277, 410 nm.

On hydrolysis it gave kaempferol, and the aqueous hydrolysate, on chromatographic examination, was found to contain glucose and rhamnose.

On methylation (Me_2SO_4–K_2CO_3) and subsequent hydrolysis compound *E* furnished 5,7,4′-tri-*O*-methyl ether of kaempferol, identified in the usual manner.

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