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Hypoglycaemic effect of *Parkia speciosa* seeds due to the synergistic action of β-sitosterol and stigmasterol

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Oral administration of the chloroform extracts of *Parkia speciosa* seeds (petai papan) to alloxan-induced diabetic rats produced a significant depression in blood glucose levels. Extraction, isolation and structure elucidation of the hypoglycaemic fraction (S-9·4) gave a mixture of β-sitosterol (66%) and stigmasterol (34%). Fraction S-9·4 produced 83% hypoglycaemic activity at 100 mg/kg body weight (BW) compared with 111% activity of glibenclamide at 5 mg/kg BW dosages. The minimum effective dose which produced statistically significant hypoglycaemic effect was 25 mg seeds/kg BW. When tested individually β-sitosterol and stigmasterol showed no hypoglycaemic effects, indicating that synergism between β-sitosterol and stigmasterol was necessary to effect the hypoglycaemic activity. The hypoglycaemic effect was not observed in healthy rats. The synergism of β-sitosterol and stigmasterol to produce hypoglycaemic activity and their occurrence in *Parkia speciosa* Hassk has never been reported.

INTRODUCTION

It was reported in 1976 that 5% of the population in the United States have diabetes and the number is increasing by 6% per year (the National Commission on Diabetes). In Malaysia the incidence of diabetes reported annually is only about 0·1% of the population, but many cases and deaths due to diabetes probably go unreported. Petai papan (*Parkia speciosa*, Hassk), a legume which is consumed as a condiment in food, for its flavour and texture, is believed by the locals to be able to control diabetes. This research attempts to investigate the alleged hypoglycaemic activity of *P. speciosa* in normal and alloxan-diabetic rats, and to elucidate the structures of the isolated active compounds.

Parkia speciosa has been used in folk medicine for its antibacterial activity on kidney, ureter and urinary bladder infections; its effect is due to the presence of several cyclic polysulphides (Gmelin *et al.*, 1981). The cyclic polysulphides (hexathionane, tetrathiane, trithiolane, pentathiepane, pentathiocane and tetrathiepane) are also responsible for the strong pungent, mushroom-like flavour of petai. Djenkolic acid and Dichrostachinic acid, which are found in the seeds, were thought to be the precursors of these cyclic polysulphides (Susilo & Gmelin, 1982). Petai may also have anticancer activity, resulting from the presence of thiazolidine-4-carboxylic acid (Pandeya, 1972; Susilo Gmelin, 1982). The seeds contain the minerals Ca, P, Na, K, Mg, Fe, Mn, Zn and Cu and 31 mg/100 g vitamin C, 11 mg/100 g niacin, 6–10% protein and 1·6–1·8% fat (Mohamed *et al.*, 1987).

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MATERIALS AND METHODS

Sample preparation

Fresh green pods of commercial petai papan (*P. speciosa*) were obtained from the evening market. The seeds were separated from the pods and both portions were air-dried and ground to a fine powder in a cyclone mill. Powdered samples were stored at ambient temperature. The dried, powdered seeds were successively extracted with petroleum ether, chloroform, dichloromethane, ethyl acetate, 25% ammoniacal chloroform and methanol. A general extraction procedure was followed for each solvent by soaking the powdered seeds overnight, filtering the solution and then evaporating the solvent. The extraction was repeated three times, using a fresh solvent each time. The combined extracts of each solvent were kept refrigerated for hypoglycaemic and chemical studies.

Hypoglycaemic screening

Hypoglycaemic screening was carried out on 200–450 g *Sprague drawley* rats of mixed sexes from the same parents. The rats were made diabetic by injecting 60 mg alloxan monohydrate/kg body weight (BW), intravenously at least 48 h before the oral administration of the extracts (Lundquist & Rerrup, 1967). The rats were divided into eight groups of four rats each, with group I as the control. The rest of the groups were used for the extract treatment. The extracts of 200 mg/kg BW were co-administered orally with 1 g/kg BW of glucose, on the 24 h-fasted rats. Blood samples were taken hourly from tail-end bleed using heparinized microhematocrit capillary-tubes (Riley, 1960), which were centrifuged at 30 000 rev/min to obtain a clear sera. The glucose in the serum was determined using the glucose oxidase method (Hill & Hessler, 1961) or a Reflolux IIM reflectance photometer (Boehringer Mannheim GmbH).

Chromatographic separation

The dark oily residue (23·8 g) resulting from the evaporation of the chloroform extract showed hypoglycaemic activity and had at least seven unresolved components on thin-layer chromatography (TLC). The residue was subjected to silica gel column chromatography ($h = 46$ cm; i.d. = 6·5 cm) using 1000 ml petroleum ether/$CHCl_3$ (1:1) followed by $CHCl_3$ as eluents. Elution was then continued with a $CHCl_3$/MeOH mixture at increasing polarity. Fractions of 100 ml were collected and analysed on TLC (silica gel). Fractions having similar TLC patterns were combined to give seven combined fractions. Each fraction was screened for hypoglycaemic activity using eight groups of four rats each. Only the fraction designated as S-9 showed significant activity. The fraction (16·3 g) was further purified by column chromatography on silica gel using a $CHCl_3$/MeOH gradient mixture to give nine fractions. the major fraction, designated as S-9·4 (6·5 g) gave white needles upon repeated recrystallization from petroleum ether, m.p. 135–136°C. Thin-layer chromatographic analysis gave a single spot but the ^{1}H-NMR spectrum of the crystals suggested the fraction was a mixture of two sterols.

The hypoglycaemic activity of S-9·4 at 100 mg/kg BW dose was compared with that of glibenclamide at 5 mg/kg BW given orally together with 1 g glucose/kg BW. Glucose levels were monitored over a 24-h period using the Reflolux IIM reflectance photometer.

General

Melting points were determined using a Kofler hot stage and are uncorrected. Mass spectra were recorded on Varian MAT CH7 and Finigan MAT GC-MS SSQ 710 spectrometers. Gas chromatography was carried out on a Shimadzu 9A instrument using an Alltech SE-30 capillary column and helium (flow rate 30 ml/min) as the carrier gas. The oven temperature was set at 200–260°C at 5°C/min and the injector port temperature at 200°C. IR spectra were recorded with a FTIR 1650 Perkin-Elmer spectrophotometer. ^{1}H-and ^{13}C-NMR

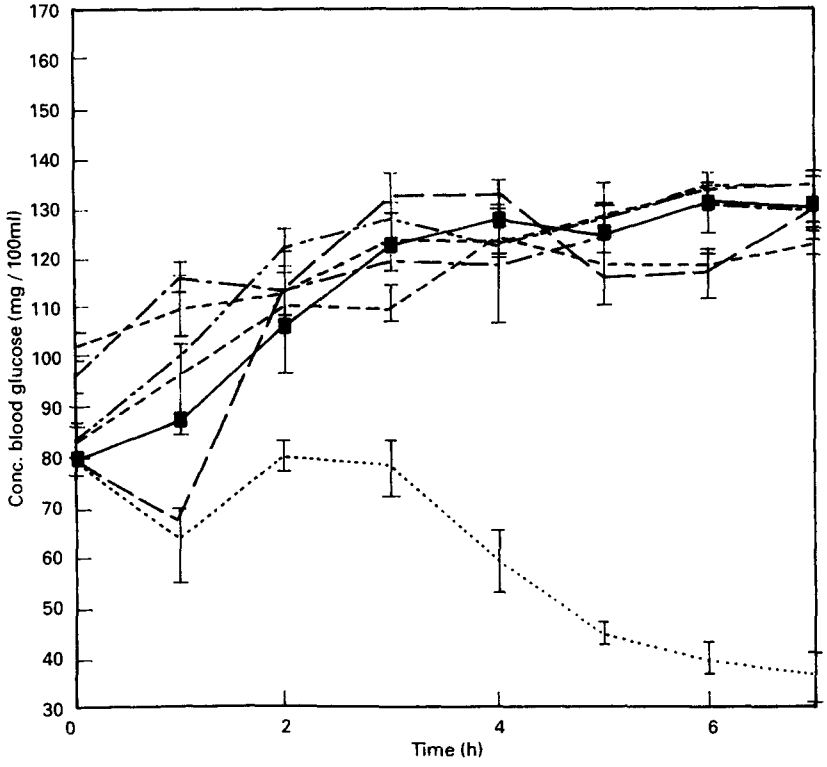

Fig. 1. Effect of different chemical solvent extracts of *P. speciosa* on blood glucose levels in alloxan-diabetic rats. Data are means ± SE (n = 4). -■- Diabetic (untreated). Treated with extract (1 g/kg) of --- petroleum ether, ···· chloroform, – – – ethyl acetate, —·—·— dichloromethane, —·—·— ammoniacal chloroform and —— methanol.

were obtained with a Bruker CPX 300 spectrometer. Authentic stigmasterol and β-sitosterol were obtained commercially from the Sigma Chemical Co. All solvents were distilled before use. Column and thin-layer chromatography utilized Merck Kieselgel 60 (230 mesh) and Merck Kieselgel 60 PF_{254}, respectively.

RESULTS AND DISCUSSION

Preliminary tests showed that only chloroform extract from *P. speciosa* gave a significant hypoglycaemic activity (Fig. 1). The increase in glucose level was effectively controlled only 3–4 h after ingestion of extract, and lasted for more than 24 h. The chloroform extracts at 400 mg/kg BW did not change the blood glucose levels in normally fed or fasted rats but only affected diabetic rats (Fig. 2). Only fraction S-9·4 had antidiabetic activity, as shown in Fig. 3.

Lowering of the blood glucose level by 111%, (to 98 mg/100 ml blood), was observed in alloxan-induced diabetic rats, to below the glucose level of healthy rats (125 mg glucose/100 ml blood) when the rats ingested 5 mg glibenclamide/kg BW (Fig. 4). In comparison, 100 mg S-9·4/kg BW reduced the blood glucose level by only 83% (to 173 mg glucose/100 ml blood), which is significantly ($p < 0.0001$) lower than that in untreated diabetic rats (400 mg glucose/100 ml blood). Time-course studies showed glibenclamide elicited the fall in blood glucose level 3 h after oral administration as compared with 4 h for S-9·4, which may indicate a different mechanism of action.

The seeds of *P. speciosa* exerted hypoglycaemic effects in a dose-dependent manner and the statistically significant ($p < 0.0001$, SD 22·03) minimum effective dose was 25 mg seeds/kg BW. The dose–response relationship is set out in Table 1 and the per cent lowering of blood glucose is approximately related to four times the square root of the dose of seed given.

Table 1. Dose–response hypoglycaemic activity of *P. speciosa* seeds

Dose (mg seeds/kg BW) (x)	25	100	500	3 000
Per cent decrease in blood glucose level (y)	25	48	77	116

The per cent lowering of blood glucose (y) = 4·31 $x^{0.43}$ (correlation coefficient r^2 = 0·95) where x = mg seeds/kg BW.

Structural identification of S-9·4 (a mixture of β-sitosterol and stigmasterol in the ratio 66 : 34)

Compound S-9·4 was suspected of being a mixture of two related sterols, most probably β-sitosterol and stigmasterol in the ratio of 2 : 1, based on the uneven integrals of olefinic signals in the nuclear magnetic resonance (NMR) spectrum and two molecular ion peaks in the mass spectrum (MS).

The steroidal-type compounds absorbed in the characteristically complex proton peaks from δ = 0·6–2·4. There were two angular methyl groups at C18 and C19, each showing a singlet peak at δ = 0·68 (s, 3H) and δ = 1·00 (s, 3H), respectively. The other two geminal methyl groups at C26 and C27 signals occurred as a doublet at δ = 0·82 (d, 6H). The methyl group at the C21 peak also appeared as a doublet at δ = 0·93 (d, 3H) and, finally, the methyl group at the C29 signal occurred at δ = 0·84 (t, 3H). Direct comparison of these signals with NMR data from the literature showed a close match of the corresponding peaks (Thompson & Dukty, 1972). The mixture also showed

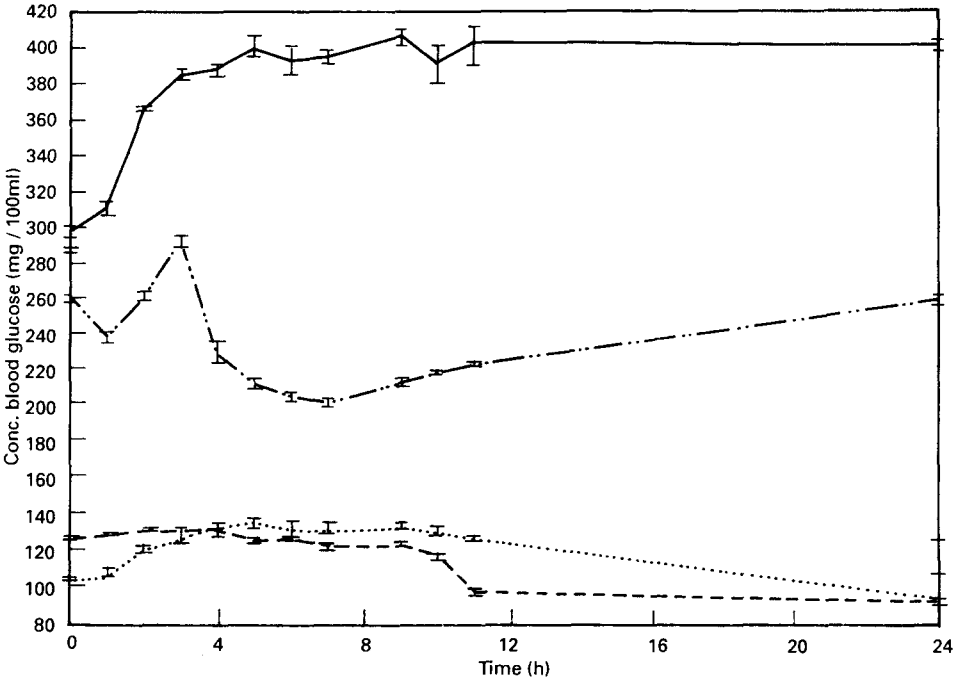

Fig. 2. Effect of chloroform extracts of *P. speciosa* on normal and diabetic rats. Data are means ± SE (n = 4). —— Diabetic (untreated), – – – saline, ····· normal (seed extracts 400 mg/kg) and –·–·– treated (seed extracts 400 mg/kg).

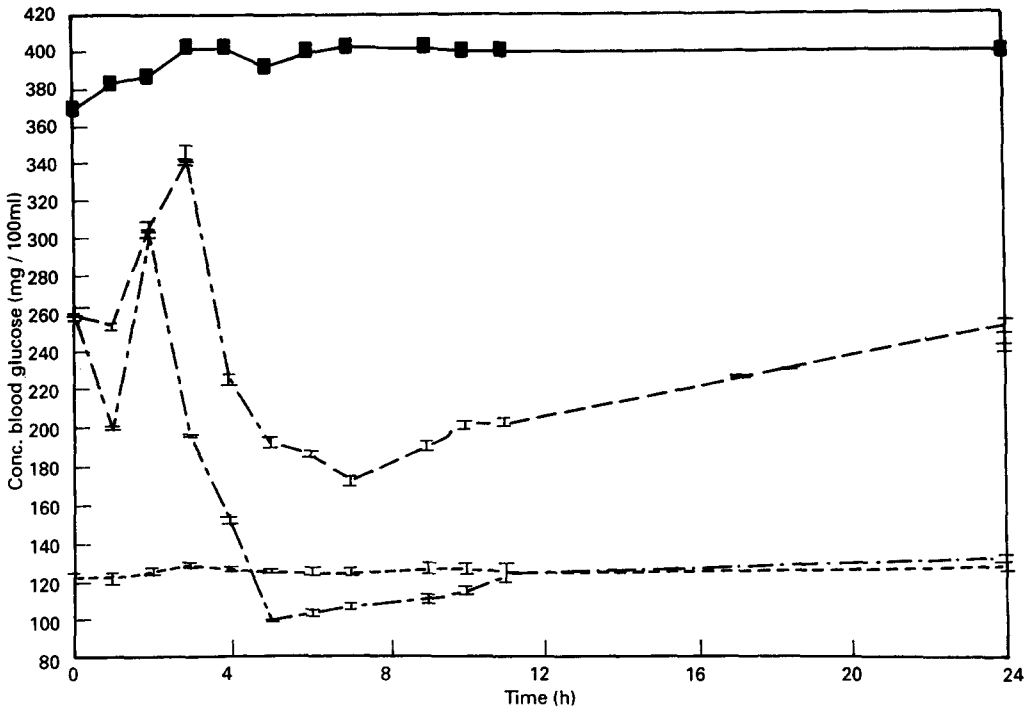

Fig. 3. Comparison study between the effects of glibenclamide (antidiabetic drug) and S-9·4 on the blood glucose levels in alloxan-diabetic rats. Data are means ± SE (n = 4). —— Diabetic (untreated), ---- saline, – – – compound S-9·4 and ·····– glibenclamide.

the resonance signal of oxymethine at δ = 3·5 (m, 1H) (lit. δ = 3·4, m, 1H), characteristic of 3-OH sterols with four neighbouring protons. The vinylic proton signal was at δ = 5·35 (t, 1H) (lit. δ = 3·25, t, 1H) (Chow & Quon, 1968). Finally, the vinylic protons at δ = 5·1 (dd, 2H) was assigned for the AB system of a disubstituted double bond at C22–C23 of stigmasterol with each vinylic proton (J geminal 16 Hz) coupled to a single neighbouring proton.

The mass spectrum of S-9·4 had a parent ion at m/e = 414 with other fragmentation peaks at m/e = 399 (M-CH_3), 396 (M-HOH), 381 (M-CH_3-HOH), 329, 303, 275, 273 (M-side chain), 255 (M-side chain-HOH), 246, 231, 229 and 213 suitable for β-sitosterol. The presence of stigmasterol was also supported by the existence of a parent ion at m/e = 412 with other fragmentation peaks at m/e = 397 (M-CH_3), 394 (M-HOH, 369, 351, 300, 271 (M-side chain -2H), 255 (M-side chain -HOH), 231, 229 and 213. These MS values are in agreement with that reported by Knapp and Nicholas (1969). The ^{13}C-NMR spectra of S-9·4 were similar to the literature signals (see Table 2). It is interesting to note that all the carbon chemical shifts in both β-sitosterol and stigmasterol were indicated at almost the same signals, except at *C22–C23 (Table 2).

The steroids, β-sitosterol and stigmasterol, which have five isolated unsaturation sites were shown to possess a characteristic IR band pattern, having significant absorption centres at or near 3425 cm^{-1} (free OH-stretch), 2962, 2872, 2853, 1465, 1450, 1380 and 1370 cm^{-1} (C-H bending for isopropyl group with strong doublet: geminal dimethyl groups), 1061, 1023, 956, 882, 840 and 803 cm^{-1} (trisubstituted olefin—the most characteristic bands for β-sitosterol and stigmasterol).

Table 2. Carbon chemical shifts (ppm) of β-sitosterol and stigmasterol

Carbons	β-sitosterol		Stigmasterol	
	a	b(lit)	a	b(lit)
1	37·23	37·31	37·23	37·31
2	31·52	31·57	31·67	31·69
3	71·60	71·69	71·60	71·81
4	42·21	42·25	42·28	42·35
5	140·73	140·76	140·73	140·80
6	121·70	121·59	121·70	121·69
7	31·63	31·92	31·67	31·94
8	31·63	31·92	31·67	31·94
9	50·11	50·17	50·11	50·20
10	36·14	36·51	36·45	36·56
11	21·06	21·11	21·06	21·11
12	39·75	39·81	39·67	39·74
13	42·21	42·33	42·28	42·35
14	56·64	56·79	56·74	56·91
15	24·28	24·32	24·34	24·39
16	28·23	28·26	28·91	28·96
17	56·07	56·11	56·03	56·06
18	11·64	11·87	11·96	12·07
19	19·01	19·40	19·39	19·42
20	36·13	36·17	40·49	40·54
21	18·76	18·82	21·06	21·11
*22	33·92	33·95	138·30	138·28
*23	26·03	26·13	129·25	129·32
24	45·60	45·85	51·22	51.29
25	29·12	29·18	31·63	31.87
26	19·80	19·84	21·19	21.26
27	18·97	19·07	18·87	19.02
28	23·04	23·09	25·40	25.44
29	12·24	12·32	12·03	12.27

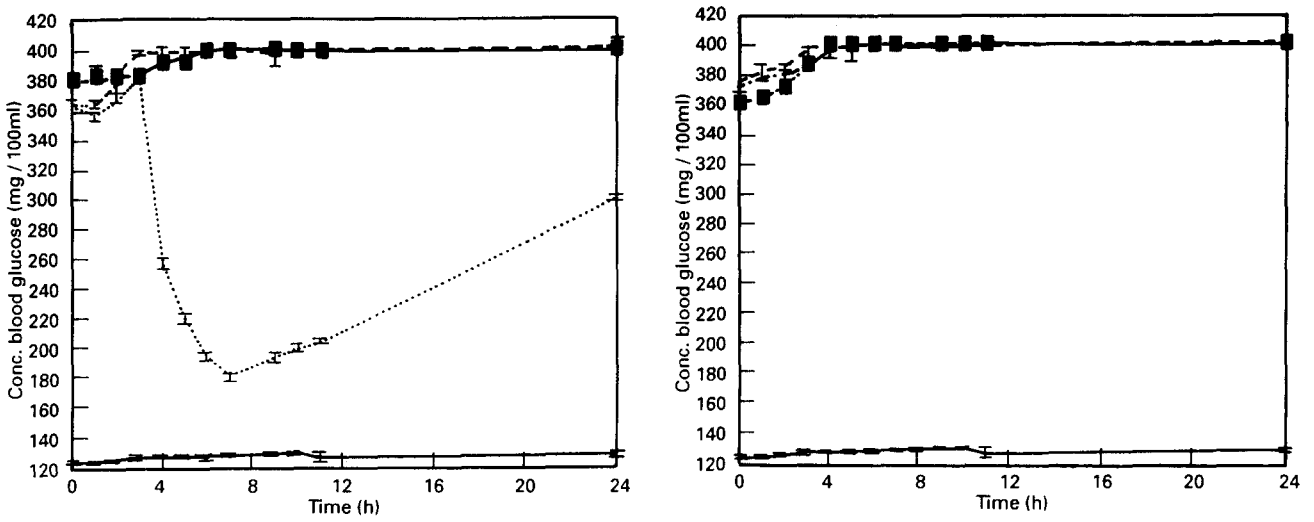

Fig. 4. Determination of seven seed fractions for hypoglycaemic activity. Data are means ± SE (n = 4). (a) —— Saline, ----, Diabetic (Untreated), ····· Treated with Fraction 9 (200 mg/kg)–Active, – – – Treated with Fraction 13 (200 mg/kg)–Non Active, – · · · – Treated with Fraction 14 (200 mg/kg)–Non Active, – · – · – Treated with Fraction 16 (200 mg/kg)–Non Active, (b) —— Saline, ---- Diabetic (Untreated), ····· Treated with Fraction 17 (200 mg/kg)–Non Active, – – – Treated with Fraction 18 (200 mg/kg)–Non Active, – · · · – Treated with Fraction 20 (200 mg/kg)–Non Active.

These data are in good agreement with those reported in the literature (Idler, *et al.*, 1953; Abu-Mustafa *et al.*, 1960).

The UV spectrum gave absorption at 206 nm (ϵ_{max} = 3040) (lit. 204 nm, 3200) where this value was supported for the C=C chromophore of the sterols (Abu-Mustafa *et al.*, 1960). The melting point of β-sitosterol is 136–138°C and stigmasterol melts at 168–169°C (Ott & Ball, 1944). In addition, β-sitosterol gave a pink to blue to green colour in the Lieberman–Burchard reaction (Mitsuhashi & Shimizu, 1960).

Since S-9·4 consisted of a mixture of two related sterols (the minor component with unsaturation in the side chain, i.e. stigmasterol), attempts were made to assess the hypoglycaemic activity of authentic β-sitosterol (100 mg/kg BW) and stigmasterol (100 mg/kg BW) individually and also in a mixture of these two compounds (66 : 34 mg/kg BW), based on the ratio of the two constituents of sample S-9·4 (in a 2 : 1 ratio according to the relative integrals of olefinic signals in NMR and two peaks in MS). Interestingly, the individual authentic β-sitosterol and stigmasterol did not produce any significant decrease in blood sugar levels in alloxan-treated diabetic rats (Fig. 5). Never-

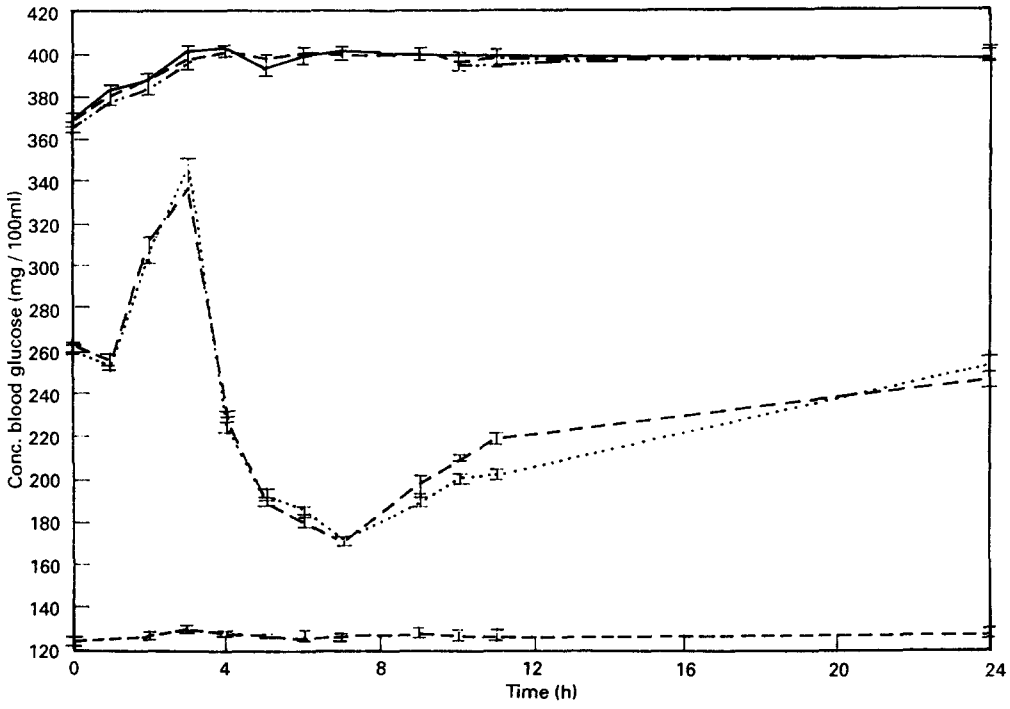

Fig. 5. Hypoglycaemic activity of authentic β-sitosterol, stigmasterol and a mixture of the two (66 mg : 34 mg) compared with the isolated S-9·4. Data are means ± SE (n = 4). —— Diabetic (untreated), – – – saline, ····· compound S-9·4 (100 mg/kg), – – – mixture (66 mg : 34 mg), – · · · – β-sitosterol (100 mg/kg) and – · – · – stigmasterol (100 mg/kg).

theless the mixture of the above authentic compounds in the ratio 66:34 did show similar hypoglycaemic activity to S-9·4, with a similar percentage activity (84%). This hypoglycaemic synergism has not been reported before in work related to these sterols. Admixtures of β-sitosterol and stigmasterol may therefore be used as a new orally effective hypoglycaemic agent, besides their hypocholesterolaemic property in animals and humans (Peterson *et al.*, 1953), cardiotonic activity (Loynes & Gowdey, 1952) and the growth-promoting and sexual reproduction-inducing activity of β-sitosterol (Vishniac & Watson, 1952; Nes *et al.*, 1982).

Several plants have been shown to contain hypoglycaemic phytosterin glycosides (Oliver-Bever, 1986). The active constituent from the rootbark of hypoglycaemic Ficus species (*Ficus glomerata* and *F. religiosa*) was found in India to be β-sitosterol D-glycoside, which had a peroral hypoglycaemic effect in fasting and alloxan-diabetic rabbits and in pituitary-diabetic rats comparable to the effect of tolbutamide (Modak & Rajarama Roa, 1966; Ambike & Rajarama Rao, 1967; Vohora, 1970). The hypoglycaemic principle isolated from *Momordica charantia* L. (African cucumber, bitter gourd or balsam pear) called charantin, was found to be a mixture of equal parts β-sitosterol-β-D-glucoside and $\Delta^{5,25}$-stigmastadiene-3β-ol (Sucrow, 1965). The hypoglycaemic principle in *M. foetida*, called foetidin, has been isolated and characterised as a chromatographically homogeneous product of equal parts of β-sitosterol-β-D-glucoside and $\Delta^{5,25}$-stigmastadien 3β-ol-glucoside and was thus shown to be identical with charantin (Olaniji, 1975; Olaniji & Marquis, 1975). Extensive investigations have shown that an extract of the dried fruits of *M. charantia* has marked hypoglycaemic properties, giving good results in clinical trials (Vad, 1960; Athar *et al.*, 1981). Charantin has a more potent hypoglycaemic action than tolbutamide in equivalent doses. A dose of 50 mg/kg of charantin reduces hyperglycaemia by 42% in rabbits. It has slight antispasmodic and anticholinergic effects but does not heal diabetic patients (160 cases). The action is present, although less pronounced, in depancreatised cats, indicating the existence of a slight extrapancreatic as well as pancreatic action. Doses of 400 mg/kg given intraperitoneally are not lethal in mice. Foetidin has been shown to lower the blood glucose level in normal rats but, contrary to results published earlier, Marquis *et al.* (1977) noticed no significant effect, such as the control of blood glucose, blood cholesterol, cardiotonic activity, growth and sexual reproduction, in diabetic albino rats.

CONCLUSION

This work reinforces the observation that plant sterols, in particular β-sitosterol and stigmasterol, may act as micronutrients that play some role in the regulation of physiological processes in the body.

REFERENCES

Abu-Mustafa, E. A., Fayez, M. B. E., Gad, A. M. & Osman, F. (1960). Isolation of β-sitosterol from Chufa Tubers. *J. Org. Chem.*, **25**, 1269.

Ambike, S. H. & Rajarama Rao, M. R. (1967). Studies on phytosterolin from the bark of Ficus religiosa. *Indian J. Pharmacy*, **29**, 91–2.

Athar, M. S., Athar, M. A. & Yaqub, M. (1981). Effect of Momordica charantia on the blood glucose level of normal and alloxan diabetic rats. *Planta Medica*, **42**, 205–12.

Chow, Y. L. & Quon, H. H. (1968). Chemical constituents of the Heartwood of Mesua ferrea. *Phytochemistry*, **7**, 1871–4.

Gmelin, R., Susilo, R. & Fenwick, G. R. (1981). Cyclic polysulphides from Parkia speciosa. *Phytochemistry*, **20**(11), 2521–3.

Hill, J. B. & Hessler, G. (1961). Automated determination of glucose utilising a glucose oxidase-peroxide system. *J. Lab. Clin. Med.*, **57**, 970–80.

Idler, D. R., Nicksic, S. W., Johnson, D. R. Merloche, V. W., Schuette, H. A. & Baumann, C. A. (1953). Seperation and tentative identification of two new sterols from Oats. *J. Am. Chem. Soc.*, **75**, 1712–5.

Knapp, F. F. & Nicholas, H. J. (1969). The sterols and triterpenes of banana peel. Phytochemistry, **8**, 207–14.

Loynes, J. S. & Gowdey, C. W. (1952). Cardiotonic activity of certain steriods and bile salts. *Can. J. Med. Sci.*, **30**, 191–201.

Lundquist, I. & Rerrup, C. (1967). Blood glucose level in mice III corticotropin-induced hypoglycaemia. *Acta Endocrinol*, **56**(4), 713–25.

Marquis, V. O., Adanlawo, T. A. & Olaniyi, A. A. (1977). The effect of foetidin from Momordica foetida on blood glucose level of albino rats. *Planta Medica*, **31**, 367–74.

Mitsuhashi, H. & Shimizu, Y. (1960). Constituents of Asclepiadacae plants I. The components of cynachum caudatum Chem. *Pharm. Bull.*, **8**, 313–17.

Modak, A. T. & Rajarama Roa, M. R. (1966). Phytosterolin from Ficus religiosa. *Indian J. Pharmacy*, **28**, 105.

Mohamed, S., Mohamed Shamsuddin, A. R., Sabturiah, S. & Fauziah, A. (1987). Some nutritional and anti-nutritional components in jering, keredas and petai. *Pertanika*, **10**(1), 61–8.

National diabetes Data Group (1979). Classification and diagnosis of Diabetes Mellitus and other catogories of glucose intolerance. *Diabetes*, **28**, 1039–57.

Nes, W. D., Hanners, P. K., Bean, G. A. & Patterson, G. W. (1982). Inhibition of growth and sitosterol-induced sexual reproduction in Phytophthora cactorum by steroidal alkaloids. *Phytopathology*, **72**(4), 447–50.

Olaniji, A. A. (1975). A neutral constituent of Momordica foetida. *Lloydia*, **38**, 361–2.

Olaniji, A. A. & Marquis, V. O. (1975). Phytochemical and preliminary pharmacological investigation of the alkaloid contained in Momordica foetida. *J. Pharmacy (Nigeria)*, **6**, 117–19.

Oliver-Bever, B. (1986). Medicinal plants in tropical West Africa. Cambridge University Press, Cambridge, UK.

Ott, A. C. & Ball, C. D. (1944). The isolation of stigmasterol and α-sitosterol from Phaseolus vulgaris. *J. Am. Chem. Soc.*, **66**, 489–91.

Pandeya, S. N. (1972). Role of sulphides (Thioethers) in biological systems. *J. Sci. Ind. Res.*, **31**, 320–31.

Peterson, D. W., Shneour, E. A., Peck, N. E. & Gaffey, H. W. (1953). Dietary constituents affecting plasma and liver cholesterol in cholesterol-fed chicks. *J. Nutrition*, **50**, 191–201.

Riley, V. (1960). Adaption of orbital bleeding technic to rapid serial blood studied. *Proc. Soc. Exp. Biol. Med.*, **104**, 751–4.

Sucrow, W. (1965). Uber steringlucoside und ein neues

Stigmastadienol aus Momordica charantia. *Tetrahedron letters*, **26**, 2217.

Susilo, R. & Gmelin, R. (1982). Precursor of cyclic polysulphides in seeds of Parkia speciosa. *Z. Naturforsch*, **37c**, 584–6.

Thompson, M. J. & Dukty, S. R. (1972). NMR Spectra of C-24 isomeric sterols. *Phytochemistry*, **11**, 1781.

Vad, B. G. (1960) Place of M. charantia in the treatment of diabetes *Maharasthra Med. J.*, **6**, 773.

Vishniac, H. S. & Watson, S. W. (1952). steroid requirements of Labyrinthula vitellina var. pacifera. *J. Gen. Microbiol.*, **8**, 248–55.

Vohora, S. B. (1970). Antidiabetic studies on *F. bengalensis L. Indian J. Pharmacy*, **32**, 68–70.