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# Hypoglycaemic effect of Stigmast-4-en-3-one, from *Parkia speciosa* empty pods

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Oral administration of the chloroform extracts of *Parkia speciosa* (petai papan) empty pods to alloxan-induced diabetic rats produced a significant reduction in blood glucose levels. A hypoglycaemic assay guided extraction, isolation and structure elucidation gave stigmast-4-en-3-one. Stigmast-4-en-3-one produced 84% activity at 100 mg $kg^{-1}$ body weight (BW) compared to 111% activity of glibenclamide at 5 mg $kg^{-1}$ BW dosages. The minimum effective dose which produced statistically significant hypoglycaemic effect was 50 mg pericarp $kg^{-1}$ BW. Hypoglycaemic effect was not observed in healthy rats. Stigmast-4-en-3-one is therefore identified as a new oral hypoglycaemic agent occurring naturally in food.

## INTRODUCTION

Several plants contain hypoglycaemic phytosterin glycosides (Oliver-Bever, 1986). Hypoglycaemic β-sitosterol D-glycoside was isolated from the rootbark of *Ficus glomerata* and *F. religiosa* (Modak & Roa, 1966; Ambike & Rao, 1967; Vohora, 1970). Hypoglycaemic charantin or foetidin (1:1 β-sitosterol-βD-glucoside: $\Delta^{5,25}$-stigmastadiene-3β-ol) was identified in *Momordica charantia* L. (African cucumber, bitter gourd or balsam pear) (Sucrow, 1965; Vad, 1960) and *M. foetida* (Olaniji, 1975; Olaniji & Marquis, 1975). Charantin, which is more potent than tolbutamide, acts through a central pancreatic and slight extrapancreatic mechanism, but does not heal diabetic patients (Athar *et al.*, 1981). The synergistic hypoglycaemic actions of orally effective β-sitosterol and stigmasterol were reported by Jamaluddin *et al.* (1994).

Petai papan (*Parkia speciosa*, Hassk) is an edible legume believed by the locals to be able to control diabetes. Gmelin *et al.* (1981) found that *Parkia speciosa* seeds contain antibacterial cyclic polysulfides (hexathionane, tetrathiane, trithiolane, pentathiepane, pentathiocane and tetrathiepane) which are also responsible for their strong pungent, mushroom-like flavour; with Djenkolic acid (which can cause blockage of the ureter) and dichrostachinic acid, thought to be the precursors of these cyclic polysulphides (Susilo & Gmelin, 1982). Thiazolidine-4-carboxylic acid (Susilo & Gmelin, 1982; Pandeya, 1972) which has anticancer activity is also present in *Parkia speciosa*. The nutrient composition of *Parkia speciosa* has been reported by Mohamed *et al.* (1987). The green pericarp may be eaten together with the seeds and is also believed to have antidiabetic activity. This research attempts to investigate the hypoglycaemic activity of *Parkia speciosa* empty pods in normal and alloxan diabetic rats, and to elucidate the structures of the isolated active compounds.

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## MATERIALS AND METHODS

### Sample preparations

Fresh green pods of commercial *P. speciosa* were obtained from the evening market. The seeds were separated from the empty pods and the empty pods were air-dried and ground to a fine powder in a cyclone mill. Powdered samples were stored at room temperature. The dried powdered empty pods 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 pods overnight, the solution filtered and the solvent then evaporated. The extract was repeated three times, each using a fresh solvent. The combined extracts of each solvent were kept refrigerated for hypoglycaemic and chemical studies.

### Hypoglycaemic screening

Hypoglycaemic screening was carried out on Sprague–Dawley rats of mixed sexes from the same parents. Except for some healthy rats for control, all

other rats were made diabetic by injecting 60 mg alloxan monohydrate $kg^{-1}$ body weight (BW), intravenously at least 48 h before the oral administration of the extracts (Lundquist & Rerup, 1967). The rats were divided into 8 groups of 4 rats each, with group 1 as the healthy control. The rest of the groups (2–8) were used for the extract treatments (pet ether, chloroform, ethyl acetate, dichloromethane, ammoniacal chloroform, methanol and saline, respectively). The extracts and saline of 200 mg $kg^{-1}$ BW were co-administered orally with 1 g $kg^{-1}$ BW of glucose, on the 24 h-fasted rats. Blood samples were taken hourly by tail-end bleed using heparinized microhematocrit capillary-tubes (Riley, 1960), which were centrifuged at 30 000 rpm to obtain clear sera. The glucose in the serum was determined using the glucose oxidase method (Hill & Hessler, 1961) or Reflolux IIM reflectance photometer (Boehringer Mannheim GmbH).

### Chromatographic separation

The dark oily residue (43·5 g) resulting from the evaporation of chloroform extract of 7·97 kg air-dried pericarp showed hypoglycaemic activity and had at least seven unresolved components on TLC.

The residues were subjected to silica gel column chromatography ($h$ = 46 cm; i.d. = 6·5 cm) using 1000 ml pet ether/$CHCl_3$(1 : 1) followed by a gradual increase with 5% MeOH at each successive addition of solvent. Fractions of 100 ml were collected and analysed on TLC (silica gel). Fractions having similar TLC patterns were combined to give 12 combined fractions consisting of six major fractions: P-7, P-8, P-9, P-10, P-11 and P-12. When the six major fractions were screened, only fraction P-7 showed significant hypoglycaemic activity.

P-7 (1·7892 g) was further purified by preparative TLC using a pet-ether/$CHCl_3$ (3 : 7) solvent system to produce 4 bands. The first band, P-7·1, the major component was purified 4 times before a final single spot was obtained an analytical TLC using chloroform and detection under UV. P-7·1 was recrystallised with hot pet-ether and partially crystallised form upon cooling and evaporation of the solvent. The yellowish compound (200 mg) was dried under vacuum for 5 h and kept in the desiccator for hypoglycaemic assay.

The hypoglycaemic activity of P-7·1 (at 100 mg $kg^{-1}$ BW dose) was compared to that of glibenclamide (5 mg $kg^{-1}$ BW dose) given orally together with 1 g glucose $kg^{-1}$ BW. Glucose levels were monitored over a 24 h period using the Reflolux IIM.

### General spectroscopic analysis

Melting points were determined using a Kofler hot stage and are uncorrected. IR spectra were measured on Beckmann Model Acculab TM7 spectrophotometer, UV spectra on a Shimadzu UV-vis (UV-160) spectrophotometer, $^1$H-NMR spectra on Brucker WP-80 with TMS as internal standard. $^{13}$C-NMR with BBD and DEPT experiments were done on a Brucker CPX

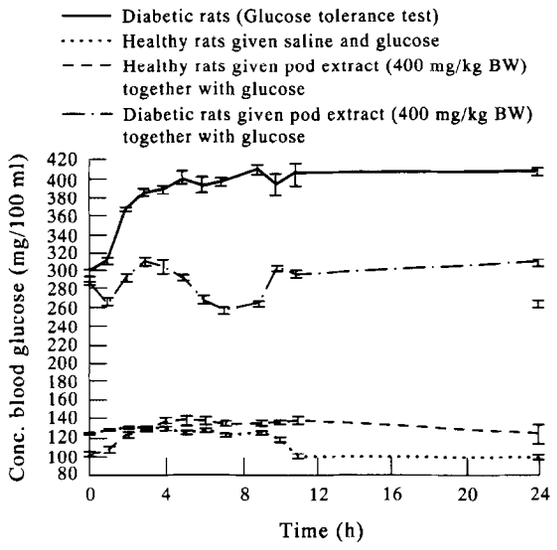


**Fig. 1.** Effect of chloroform extracts of *P. speciosa* on normal and diabetic rats. Data are means ± SE ($n$ = 4).

300 or a Brucker AM 500 spectrophotometer, respectively. MS were done on an AE1-MS12 spectrophotometer attached to a VG display data acquisition system (electron impact at 70 eV). Gas chromatography was carried out on a Shimadzu 9A using Alltech SE-30 capillary column and helium (flow rate at 30 ml/min) as the carrier gas. Oven temperature was set at 200–260°C at 5°C/min and injector port at 200°C. All solvents were distilled before used. Column and thin-layer chromatography utilized Merck Kieselgel 60 (230 mesh) and Merck Kieselgel 60 $PF_{254}$, respectively.

## RESULTS AND DISCUSSION

Preliminary hypoglycaemic tests showed that only the chloroform extract from *P. speciosa* pericarp had a significant activity. The initial rise in blood glucose level from 0 to 2 h suggested that glucose was absorbed

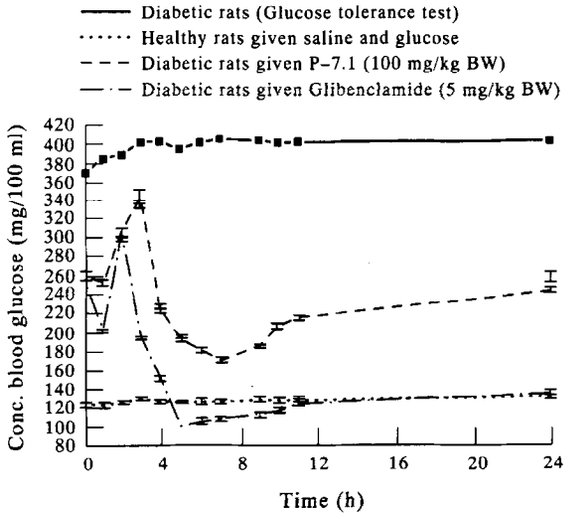


**Fig. 2.** Comparison study between the effects of glibenclamide (antidiabetic drug) and Stigmast-4-en-3-one P-7·1 on the blood glucose levels in alloxan-diabetic rats. Data are means ± SE ($n$ = 4).

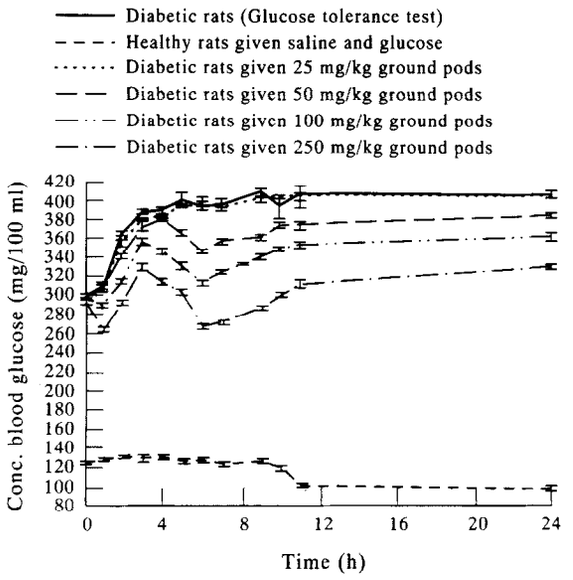


**Fig. 3.** Dose–response relationship of fresh and ground empty pods on blood glucose levels of alloxan-diabetic rats. Data are means ± SE ($n$ = 4).

from the alimentary canal and the rise in glucose level was effectively controlled only 2 h after ingestion of extract, and lasted for more than 24 h. The chloroform extract at 400 mg kg$^{-1}$ BW did not significantly change the blood glucose levels in normally fed or fasted rats but only affected diabetic rats (Fig. 1).

Glibenclamide (5 mg kg$^{-1}$ BW) lowered the blood glucose level by 111% (to 0·98 g litre$^{-1}$ blood), to below the glucose level of healthy rats (1·25 g glucose litre$^{-1}$ blood) (Fig. 2). Feeding of P-7·1 (100 mg kg$^{-1}$ BW) significantly ($P$ < 0·0001) reduced blood glucose level from 400 mg glucose (untreated diabetic rats) to 1·70 g glucose litre$^{-1}$ blood (84% reduction).

The empty pods of *P. speciosa* exerted the hypoglycaemic effects in a dose-dependent manner (Fig. 3) and the statistically significant ($P$ < 0·0001, SD 22·03) minimum effective dose was 50 mg empty pods kg$^{-1}$ BW. The dose–response relationship after 4 h is tabulated below and the % lowering of blood glucose is approximately proportional to the square root of the pod concentration given.

| | | | | | |
|---|---|---|---|---|---|
| Dose (mg pods kg$^{-1}$ BW) | [X] | 25 | 50 | 100 | 250 |
| % Decrease in blood glucose level | [Y] | 0 | 18 | 31 | 47 |

The % lowering of blood glucose [Y] = 1·4 $[X]^{0.59}$ [correlation coefficient $r^2$ = 0·94] where [X] = mg pods kg$^{-1}$ BW.

### Structure identification of P-7·1 Stigmast-4-en-3-one (Table 1) (200 mg)

P-7·1 melted at 87–88°C and showed significant hypoglycaemic activity in alloxan-induced diabetic rats. The $^1$H-NMR and $^{13}$C-NMR spectra (Table 2) indicate the compound to be steroidal, absorbing in the characteristically complex proton peaks from $\delta$ = 0·7–2·35. The higher-field signals in the $^1$H-NMR ($\delta$ < 1) spectrum were assigned to Me-18 ($\delta$ = 0·71, s, 3H), Me-19 ($\delta$ = 1·05, s, 3H), Me-21 ($\delta$ = 0·95, d, 3H). The broad multiplets for (-$CH_2$-CO-, 2H) appeared downfield in the range of about $\delta$ = 2·2–$\delta$2·35 and the vinylic proton signal at $\delta$ = 5·72 (br s, –CO–CH=C). The presence of an $\alpha,\beta$-unsaturated carbonyl group was well supported by the UV absorption band at 241 nm ($\epsilon_{max}$ 5590) and also the IR absorption band at 1640 cm$^{-1}$. The $^{13}$C-resonance peak at 123·66 ppm was consistent with an $\alpha$-unsaturated C4, whereas the peak at 171·57 ppm was consistent with the $\beta$ of C5 of the $\alpha,\beta$-unsaturated cyclohexanone. The carbonyl carbon at C3 appeared at 199·49 ppm. The assignments of these signals were in agreement with literature (Isabel *et al.*, 1983; Krishna *et al.*, 1973; Takayuki & Shigeharu, 1974) and shown in Table 2.

Some ambiguity, however, remains due to the appearance of $^1$H-resonance peaks at $\delta$3·5 which could be attributed to the 3-OH group and at $\delta$5·1 attributed to the vinylic proton of the side chain. These peak areas are, however, relatively too small to be considered as part of the molecule. The signal for the 3-OH group, however, was absent in the IR spectrum, i.e. the

**Table 1. Spectral data of P-7·1**

IR (cm$^{-1}$, KBr pellet)
2962, 2926, 2872, 2853, 1640, 1465, 1450, 1375 and 722
$^1$H-NMR (500 MHz, $CDCl_3$)
0·71 (s, 3H, H-18), 0·84 (d, 6H, H-27), 0·87 (t, 3H, H-29), 0·95 (d, 3H, H-21), 1·05 (s, 3H, H-19), 2·2-2·35 (br m, 2H) and 5·72 (br s, 1H, H-4).

**Table 2. Carbon chemical shifts (ppm) of P-7·1 Stigmast-4-en-3-one ($^{13}$C-NMR, 75·5 M Hz, $CDCl_3$)**

| Carbons | P-7·1 (Stigmast-4-en-3-one) |
|---|---|
| 1 | 37·2 |
| 2 | 31·7 |
| 3 | 199·5 |
| 4 | 123·7 |
| 5 | 171·6 |
| 6 | 32·7 |
| 7 | 31·7 |
| 8 | 31·6 |
| 9 | 50·1 |
| 10 | 36·5 |
| 11 | 21·1 |
| 12 | 39·7 |
| 13 | 42·3 |
| 14 | 56·6 |
| 15 | 24·3 |
| 16 | 28·9 |
| 17 | 56·1 |
| 18 | 11·6 |
| 19 | 19·0 |
| 20 | 36·1 |
| 21 | 18·8 |
| 22 | 33·9 |
| 23 | 26·1 |
| 24 | 45·6 |
| 25 | 29·1 |
| 26 | 21·2 |
| 27 | 19·0 |
| 28 | 23·0 |
| 29 | 12·2 |

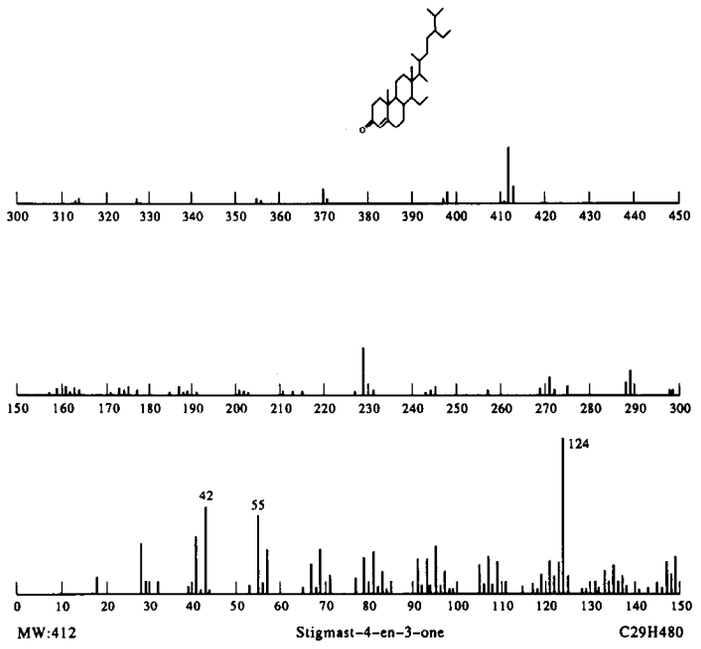


**Fig. 4.** The MS spectrum of Stigmast-4-en-3-one.

band at 3400 $cm^{-1}$. In addition, the $^{13}C$-resonance peak at around 71 ppm and the signal for the disubstituted double bond of the side chain (≈ 138 ppm and ≈ 123 ppm, respectively) were also absent in the $^{13}C$-NMR spectrum. On the other hand, peaks at 26·11 and 33·92 ppm, respectively, were present, suggesting the replacement of unsaturation with saturated carbons.

The compound was probably not a mixture because the HPLC showed a single peak and the GC–MS spectrum also confirmed that it was a single component. There were some contradictions between the $^{1}H$-resonance signals and other spectra. The appearance of the olefinic $^{1}H$-resonance signals, supposedly for C22–C23, and the hydroxyl group at C3, however, could not be confirmed by derivatization or other approaches due to the limited amount of the isolate. The appearance of these peaks may be due to some impurities in the NMR sample solution. The MS spectrum showed that this compound had a molecular ion peak at m/e 412, consistent with the molecular formula $C_{29}H_{48}O$ (Fig. 4). Other fragmentation ions were at m/e 395; 275; 135; 124; 107; 77; 67 and 42.

In conclusion, based on the comparison of the spectral data of P-7·1 with the literature, the structure of P-7·1 was suggested to be a stigmast-4-en-3-one or sitost-4-en-3-one (Fig. 5). This compound was reported to have a melting point of 89–90°C (Takayuki & Shigeharu, 1974).

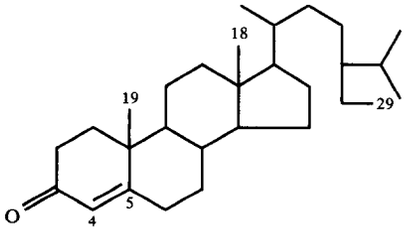


**Fig. 5.** Stigmast-4-en-3-one.

The occurrence of the stigmast-4-en-3-one is reported for the first time from *Parkia speciosa* and suggested as another orally hypoglycaemic agent. There has been no report on other biological activities of this compound, except that the metabolite stigmast-4-en-3-one may be a necessary intermediate in the metabolism of β-sitosterol. For example, the biosynthesis of spirostanols such as tigogenin and gitogenin from cholesterol must pass through stigmast-4-en-3-one (Stohs & El Olemy, 1971).

Phytosterols have been reported to have hypocholesterolaemic effects (Peterson *et al.*, 1953), cardiotonic activity (Loynes & Gowdey, 1952) growth promoting and sexual reproduction inducing activity (Vishniac & Watson, 1952; Nes *et al.*, 1982) in animals and humans. Hypoglycaemic charantin or foetidin (1:1 β-sitosterol-βD-glucoside: $\Delta^{5,25}$-stigmastadiene-3β-ol) from *Momordica charantia* L. (African cucumber, Bitter gourd or balsam pear) (Sucrow, 1965; Vad, 1960) and *M. foetida*, (Olaniji, 1975; Olaniji & Marquis, 1975) are more potent than tolbutamide and also have slight antispasmodic and anticholinergic effects (Athar *et al.*, 1981).

## CONCLUSION

This work reinforces the fact that plant sterols may act as micronutrients that play some role in regulation of physiological processes in the body.

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