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Hypoglycaemic and amylase inhibitory activities of leaves of *spondias mombin* linn

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Summary

Suppressing the production of glucose by inhibiting á-amylase / á-glucosidase activity is one of the therapeutic approaches for decreasing postprandial hyperglycaemia and a strategy for evaluating anti-diabetic activity. We investigated leaves of *Spondias mombin* because our previous ethnobotanical survey showed that it is used by traditional healers to manage diabetes in South West Nigeria. We report a bioactivity-guided study of *S. mombin* using glucose loading (1g/kg) alloxan-induced diabetic rats and inhibition of á-amylase as basis for isolation of active constituents. Hyperglycaemia was induced in albino rats and blood glucose levels monitored for 180 mins using a glucometer. Powdered leaves were macerated with 80% Methanol. The active extract was fractionated on column chromatography packed with silica gel G6OA eluting with gradient mixtures of *pet. ether and ethylacetate.* The most active á-amylase inhibiting fraction was purified on thin layer chromatography (TLC) and pure compound identified by spectroscopy. Peak decrease in blood glucose of 41.4% ($p< 0.05$) was recorded after 60 mins. This activity-guided study produced an active TLC band (69.8% amylase inhibition, $p < 0.05$) from which â-sitosterol was characterized as the main inhibitor. This is first report of hypoglycaemic and amylase inhibitory activities of *S. mombin*. The role of phytosterols in control of diabetes mellitus is discussed. This study justifies the ethnopharmacological use of this species in recipes for management of diabetes mellitus.

Keywords: *Spondias mombin, á-amylase inhibition, alloxan-induced diabetes, hypoglycaemic activity, hyperglycaemia*

Résumé

La production du glucose en inhibant l'activité de l'enzyme á amylase / á glucosidase est l un des approche thérapeutique pour réduire l'hyperglycémie post prandiale et une stratégie pour évaluer l'activité antidiabétique. Nous investiguons les feuilles de spondias mombin parce que notre étude ethnobotanique antérieure montrait que il était utilise par les guérisseurs traditionnels pour ménager le diabète au Sud-ouest du Nigeria. Nous rapportons l'étude de la bio activité guidée du S. mombin utilisant la concentration du glucose (1g/kg) d'alloxan induit diabète aux rats et inhibition de l'amylase comme base de l'isolation des ingrédients actif. L'hyperglycémie était induite aux rats et l'évaluation du taux du glucose pour 180 mins avec un glucomètre. Les poudres des feuilles étaient macérés avec le méthanol de 80%. L'extrait actif était fractionné a l'aide de la chromatographie a colonne rempli du gel de silicaG60A avec un gradient de mélange au pet éther et l'acide ethylacetique. La fraction du & amylase la plus inhibée était purifié la chromatographie a couche légère et le compose pur identifier par la spectroscopie. La réduction du taux du glucose était a 41.4% ($P<0.05\%$) enregistre après 60 mins dont le beta sitostérol était le inhibiteur principal. C'est le premier rapport d'un hypoglycémiant et des activités inhibitrices de l'amylase du S. mombin. Le rôle des phytosteroides dans le contrôle du diabète mellite est discute. Cette étude justifie l'usage de l'ethnopharmacologie de ces recipes pour les soins du diabète mellite.

Introduction

Diabetes mellitus is an endocrine and metabolic disorder characterised by chronic hyperglycaemia associated with risk of cardiovascular diseases [1]. The prevalence is increasing worldwide and two-third of adult sufferers live in developing countries [2]. The ability of a drug or diet to suppress the production/ absorption of glucose by inhibiting alpha-amylase or alpha-glucosidase activity is one of the therapeutic approaches for decreasing postprandial hyperglycaemia and a strategy for evaluating anti-diabetic activity [3].

Spondias mombin Linn. (Anarcadiaceae) commonly called "iyeye" (Yoruba) "iyawe" (Hausa) is a medium-sized deciduous tree found on farmland, around towns and villages and widely cultivated for

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his yellow pleasantly acid plum-like fruits [4]. It is used by herbalists in South West Nigeria to treat intestinal disorders particularly those associated with typhoid, diarrhea and dysentery [5] and it is also a component of traditional antituberculosis recipes [6]. Our interest in this plant arose because our previous ethnobotanical survey showed that it is used by herbalist in South West Nigeria to treat diabetes [7]. The antimicrobial potential of *S. mombin* has been published [5], there is, however, no report on the hypoglycaemic or alpha-amylase inhibitory activities of this plant. We report a bioactivity-guided study of *S. mombin* using glucose-loading, alloxan diabetic rats and inhibition of á-amylase as basis for isolation of active constituents of this species.

Materials and methods

Plant material

The plant was collected in Shagamu and authenticated at the Forest Herbarium, FRIN, Ibadan where an herbarium specimen had been deposited. The leaves were air-dried and powdered ready for analysis.

Extraction and solvent separation

1kg of dried powdered leaves of *S. mombin* was macerated with 80% methanol (MeOH) for five days and the combined filtrate was evaporated *en vacuo* in a rotary evaporator at 40°C and weighed. This crude aqueous MeOH extract was tested on glucose-loaded (1g/kg) and alloxan diabetic rats at concentration of 1g/kg as described below. The MeOH extract was suspended in methanol/water (1:4) mixture in a separatory funnel and partitioned into aliquots of chloroform ($CHCl_3$; 2 x 100mls) (Fraction A). The pH of the aqueous phase was adjusted to 2 with 2M sulphuric acid and partitioned into $CHCl_3$ (2 x 100mls) (Fraction B). The pH of the aqueous phase was raised to 9 with 20% sodium carbonate and partitioned into $CHCl_3$ (2 x 100mls) to give fraction C. Finally, the mother liquor, after neutralization, was then partitioned into aliquots of diethylether (2 x 100mls) (fraction D). The appropriate combined $CHCl_3$ and diethylether extracts (A – D) were dried over anhydrous sodium sulphate, filtered and solvent evaporated under reduced pressure to give fractions A – D which were separately tested on the á-amylase inhibition assay at concentration of 0.1g/ml.

Column fractionation and purification

The bioactive fraction D (1g) was fractionated on column chromatography packed with silica gel G60A, eluting with gradient mixtures of pet ether (40°-60°C) and $CHCl_3$. Fractions were monitored on analytical thin layer chromatography (TLC) on silica gel GF_{254}, developing in hexane /diethylether (1:1). Five fractions (D1-D5) were obtained and tested separately on the á-amylase assay at concentration of 2µg/ml.

The most active column fraction D1 (67% inhibition) was cleaned up on prep. TLC (silica gel GF_{254}, 0.5mm) developing in hexane/diethylether/ acetic acid (70:30:2). It produced three major active triterpene-rich TLC bands AJP, AJY and AJG. The most active TLC band AJP was finally purified on silica gel GF_{254} developing in hexane/diethyl ether (1:1) as mobile phase. This potent á-amylase inhibitor was subjected to spectroscopic analysis (UV, IR ^{1}HNMR, ^{13}CNMR)

Biological assays

Experimental animals

Albino Wister rats of both sexes weighing 80-200mg were obtained from the Central Animal House, University of Ibadan. They were kept in metabolic cages in a well-ventilated room fed on standard feed (Ladokun feed, Ltd, Ibadan) and water *ad libitum.*

Glucose-induced hyperglycaemia

The rats were divided into four groups (cages 1-4) of five rats each. The animals were fasted overnight (18hours) but allowed free access to water. Initial blood glucose (zero time) was determined before assay. Rats in groups 1-3 were made hyperglycaemic by oral administration of 1g/kg glucose solution. Rats in group 4 were neither glucose-loaded nor treated with extract. Blood samples were collected by snipping the tail and the glucose level determined using a glucometer as previously reported [8,9]. Details of this glucose-tolerance test have been previously described [8-10].

Alloxan-induced diabetes

The rats were divided into four groups of five rats each. Rats in cages 1-3 were fasted overnight, given a single intraperitoneal injection of 80mg/kg of alloxan monohydrate in isotonic saline and allowed to rest for three days to stabilize blood glucose level. Rats in cage 1 are diabetic but treated with the aqueous methanolic extract (1g/kg). Cage 2 consisted of untreated (control) diabetic rats. Cage 3 contained diabetic rats treated with glibenclamide (5 mg/ml) while cage 4 consisted of untreated normal rats (table 2). Basal blood glucose levels at zero time (fasting) were determined prior to oral treatment. The aqueous methanolic extract and glibenclamide were administered immediately to rats in appropriate cages

and blood sugar levels determined at 30 min interval for 180 min using a glucometer as previously published [9, 10].

Alpha-amylase inhibition assay

1ml of the plant extracts or â-sitosterol or acarbose (5mg/ml) were mixed with 1ml of 1% á-amylase [EC .3.2.11, 10units/ml, Sigma, Dorset) and incubated for 10 mins. 1ml of 1% soluble starch was added to the mixture and further incubated for 10 mins at 25°C. The reaction was stopped by adding 1ml of 1% dinitrosalicylic acid and boiled at 90°C in a water bath for 15 mins [11,12]. The cooled reaction mixture was diluted with 1ml de-ionised water and the absorbance of the test and control (without plant extract) mixtures were measured at 540nm in a 1cm cuvette using a UV-VIS-Spectrophotometer (Shimadzu, UV-VIS 1201) at 20°C.

Biochemical assays

The aqueous MeOH extract and glibenclamide were screened for their effects on some biochemical parameters in groups of five alloxan diabetic rats [13]. The blood cholesterol [14], protein [15] and albumin [16] levels were determined daily for fifteen days.

Spectroscopy

UV spectrum was recorded on a Shimadzu 1210 scanning spectrophotometer using MeOH as solvent; IR spectrum was recorded on Perkin-Elmer 1725 spectrophotometer on KBr discs; NMR spectra were on bruker Avance 300MHz spectrophotometer with fourier transform facility. TMS was internal standard while MS was recorded using Varian 1200 Triple Quarupole instrument at 70eV, inlet temp. of 230-300°C. Data acquisition, was monitored by a Varian Dell GX 150 computer.

Statistical analysis

Data are presented as mean ± SEM. The significance of the differences between the means of test and control animals were established by student's t-test.

Results

The aqueous MeOH extract from leaves of *S. mombin* at concentration of 1g/kg exhibited hypoglycaemic activity in the glucose-loaded hyperglycaemic rats (Table 1). Significant peak decrease of blood glucose level of 31.9% ($p<0.05$) was observed at 60 mins. Significant reduction of blood glucose was also recorded for the glibenclamide-treated (5mg/ml) rats producing peak decrease of 42.4% ($p < 0.05$) at 180 mins.

Table 2 shows effect of the crude aqueous MeOH extract on blood glucose level of the alloxan diabetic rats. Significant sustained anti-diabetic activity was observed from 30 mins of administration of plant extract. Peak reduction of blood glucose of 41.4% and 40.9% ($p<0.05$) were recorded at 60 mins and 120mins respectively.

Table 3 shows that only the partitioned diethylether soluble fraction D exhibited significant

Table 1: Effect of aqueous methanolic exctract of *S. Mombin* leaves on blood glucose level of glucose-loaded rats.

Groups	Mean blood glucose level (mg/dl ±SEM) 0	30min	60min	90min	120min	180min
S. mombin leaf extract (1g/kg, hyperglycaemic, cage 1)	82.3±4.291	161.2±0.158	106.5±1.155 (31.9%)*	120.2±0.310 (21.9%)*	124± 0.670 (22.2%)*	124.3±1.190 (18.7%)*
Hyperglycaemic untreated control (cage 2)	89.1±0.057	151.3±0.031	156.3±0.074	154.0±0.037	159.7±0.093	152. ± 0.375
Hyperglycaemic treated with glibenclamide (5mg/ml) (cage 3)	81.5+0.006	132.5±0.014 (12.4)%*	98.9±0.006 (36.7%)*	92.4±0.008 (40%)*	99.8±0.003 (37.5%)*	88.1±0.005 (42.4%)*
Normal rats (cage 4)	85±0.085	80.1±0.437	82.1±0.05	84.8±0.150	85.0±0.044	85.3±0.075

*Figures in parenthesis indicate % decrease in blood glucose level; n = 5, * Significantly different from control at $p < 0.05$*

Table 2: Effect of *S. Mombin* leaf extract on blood glucose level of alloxan induced diabetic rats.

Groups	Mean blood glucose level (mg/dl ±SEM) 0	30min	60min	90min	120min	180min
S. mombin leaf extract (1g/kg. diabetic) (cage 1)	182.2±1.856	116.3±0.792 (39.7%)*	116.6±1.112 41.4%)*	130.6±1.552 (36.7%)*	124.2±3.352 (40.9%)*	163.2±5.984 (26.6%)*
Diabetic untreated control. (cage 2)	172.3±0.314	193.0±0.208	199.1±0.259	206.4±0.094	210.1±0.089	222.4±0.073
Diabetic, treated with glibenclamide (5mg/kg) (cage 3)	191.8±0.249	177.4±0.184 (8.1%)	176.9±0.093 (11.2%)*	176.3±0.211 (14.6%)*	165.5±0.160 (21.2%)*	161.8±0.030 (27.3%)*
(Cage 4) Normal rats)	92.4±0.495	93.1±0.576	94.5±0.142	94.0±0.193	91.4±0.080	93.4±0.701

*Figures in parenthesis indicate % decrease in blood glucose level; n = 5, ** significantly different from control at p < 0.05*

Table 3: Alpha-amylase inhibitory activities of partitioned fractions A-D from *S. Mombin* leaf extract

Fractions (0.1g/ml)	Absorbance* 1	2	3	Mean +	% inhibition
A	0.789	0.929	0.712	0.810±0.110	13.0
B	0.766	0.741	0.899	0.802±0.085	14.0
C	0.831	0.836	0.784	0.817±0.028	12.0
D	0.239	0.234	0.268	0.247±0.018	73.0*
Acarbose (5mg/ml)	0.406	0.396	0.404	0.402±0.005	57.0**
Control (without inhibitor)	0.894	1.059	0.840	0.931+0.114	0.0

+ Values are mean ± SEM; n = 3 ëmax = 540 nm; 1% á-amylase (10 units/ml); ** significantly different from control at p < 0.05.*

Table 4: Alpha-amylase inhibitory activities (*in vitro*) of column fractions D1-D5 from leaf extract of *S. Mombin*

Column fractions (2µg/ml)	Absorbance* 1	2	3	Mean +	% inhibition
D1	0.307	0.331	0.299	0.312±0.091	67.0**
D2	0.568	0.484	0.550	0.534±0.014	43.6**
D3	0.663	0.635	0.701	0.666±0.033	29.0**
D4	0.786	0.722	0.855	0.787±0.082	16.0
D5	0.805	0.834	0.991	0.877±0.16	5.3
Acarbose (5mg/ml)	0.406	0.396	0.404	0.402±0.058	57.0**
Control (without inhibitor)	0.894	1.059	0.840	0.931+0.157	0.0

*+ Values are mean ± SEM; n = 3; *ëmax = 540 nm; 1% á-amylase (10 units/ml); ** significantly different from control at p < 0.05*

á-amylase inhibitory activity at 0.1mg/ml. 73% inhibition of á-amylase ($p<0.05$) was recorded for fraction D compared to 57% inhibition by the reference compound, acarbose at concentration of 5mg/ml. (Table 3). Further separation of fraction D on column chromatography produced six sub-fraction D1-D5.

Result shown in table 4 shows that column fraction D1 replicated the á-amylase inhibitory activity of the crude extract; showing 67% inhibition

($p<0.05$) at concentration of 2µg/ml. Acarbose exhibited 57% inhibition which was significant at $p<0.05$. Column fractions D2 and D3 also exhibited significant inhibition in same assay but were not further investigated.

Further purification of the most active column fraction D1 on preparative TLC gave three main bands (AJP, AJY and AJG) which inhibited á-amylase by 77%, 57% and 61% respectively at 2µg/ml. Spectral analysis showed that the three bands were triterpenoid-rich mixtures. TLC band AJP was further cleaned up on silica gel GF_{254} (0.5mm thickness) developing in hexane/diethylether/acetic acid (70:30:2). Final purification was achieved on preparative TLC plates coated with silica gel GF_{254}, developing in hexane/diethylether (1:1) as mobile phase. This potent inhibitor reacted violet to anisaldehyde-sulphuric; blue to phosphomolybdic acid and green to the Lieberman-burchard spray reagents indicative of a steroid.

Spot AJP exhibited the following spectral characteristics: **UV**: ëmax 240nm (MeOH); **IR**: Vmax (KBr)cm^{-1}: 2925 (- CH_3 stretch), 2850, 1652 (C=C stretch), 1457 (C-H bending, angular methyl group), 1376, 1082, 832.

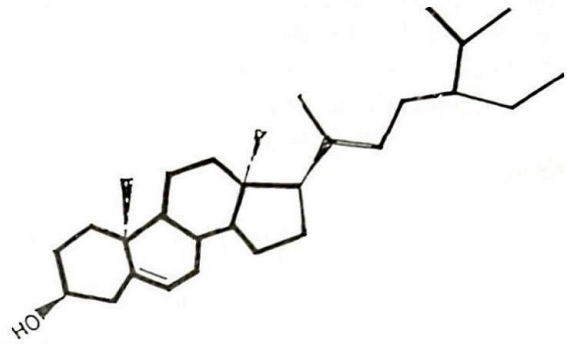
MS: (E1-MS): m/z 414 (M+, 100%), 396(34%), 303(28%), 275(7%), 231(12%), 213 (19%).

^{1}HNMR: (300MHz, $CDCl_3$; TMS = 0.000ppm): ä5.30 (IH, m, H-6), 3.5(m), 1.54(1H, m, H-8), 0.94(1H,m,H-9), 1.43 (2H, m, H-11), 1.69 (2H, m, H-12), 1.18(1H, m, H-14), 0.61 (3H, m, H-18), 0.99 (3H,s,H-19), 0.90 (3H, d, H-12), 1.60 (2H, m, H-22), 1.65 (2H, m, H-23), 1.58 (2H, m, H-24, 1.56 (2H, m, H-25), 0.78 (3H, d, H-26), 0.80 (3H, d, H-27), 1.52 (2H, m, H-28), 0.84 (3H, t, H-26), 0.80 (3H, d, H-27), 1.52(2H, m, H-28), 0.84(3H, t, H-29)ppm.

^{13}CNMR: ä37.30 (C1), 72.40 (C3), 42.20(C-4), 140.80(C-5), 122.56 (C-6), 50.20 (C-9), 36.42(C-10), 21.50(C-11), 40.08(C-12), 42.40(C-13), 56.80(C-14), 24.20(C-15), 56.38(C-17), 12.05(C-18), 19.40(C-19), 36.40(C-20), 18.90(C-21), 48.34(C-24), 18.80(C-26), 22.78(C-28), 12.25(C-29)ppm.

The spectra data correlated with data published for â-sitosterol [17,18] and also were identical with data available for same compound at the Spectra Database for Organic Compounds (SDBS) of the Institute of Advanced Science and Technology of the University of Newcastle Upon Tyne, UK. Compound AJP also co-chromatographed with authentic sample (Rf = 0.35, silica gel GF_{254}, Toluene/Ethylacetate (80.20). In addition, there was no significant difference in the á-amylase inhibition of isolated compound AJP (69.8%) and authentic â-sitosterol (69.5%, figure 1).

Figure 1; β - sitosterol
Main alpha - amylase inhibitor
isolated from bioactive TLC
band AJP from leaves of S. mombin



($C_{29}H_{50}O$; 16mg; 69·8% inhibition at 2µg/ml)

Fig. 1

Discussion

Phytotherapy is an important aspect of traditional medical practices in Africa. For centuries, herbal remedies have served as important sources of medicines for prevention and treatment of diseases [7,19-21]. Plants and plant products represent source of new drugs to complement the action of oral hypoglycaemic agents and as dietary adjuncts to existing therapies [22].

In 1995, Fathaiya *et al* concluded that plant sterols may act as micronutrients that play some role in regulation of physiological processes in the body [23]. Kotowaroo *et al* [24] attributed the á-amylase inhibitory activity of leaves of *Artocarpus heterophyllus* to presence of phytosterols containing cycloartenone, cycloartenol and â-sitosterol. Furthermore, â-sitosterol –D – glucoside was identified to be the á-glucosidase inhibitor from the ethylacetate fraction of *Arctium lappa* Linn [18] producing significant inhibition of 97.3% at concentration of 200µmol/ml. This activity-guided study of *S. mombin* Linn has shown that â-sitosterol (AJP) is the main á-amylase inhibitor isolated from this species and it is first report of this potent inhibitor from this medicinal plant.

â-sitosterol – D – glycoside has been reported to be hypoglycaemic constituents of root bark of

Ficus glomerata and *F. religiosa* [25]. Charantin or foetidin identified as the hypoglycaemic compounds from *Momordica charantia* [26,27] and *M. foetrda* [28] are steroid mixtures (50:50) of â-sitosterol- D – glucoside and 5, 25 – stigmastadiene -3 â- ol – glucoside [29]. Charantin was reported to be more potent than tolbutamide and acted through a central pancreatic and slight extrapancreatic mechanism, but did not heal diabetic patients [30]. Even though related steroids e.g. stigmast – 4- ene – 3 – ol and stigmast -4- ene-3-one isolated from bark of *Anarcadium occidentale* [31] and pods of *Parkia speciosa* [23] have been reported to exhibit significant hypoglycaemic activities, the exact role of plant sterols such as sitosterol in diabetes control became more obvious when Fathaiya *et al* [30] reported that the hypoglycaemic activity of seeds of *Parkia speciosa* was due largely to synergistic cation of â-sitosterol and stigmasterol. When tested individually â-sitosterol and stigmasterol showed no hypoglycaemic activity, indicating synergism between â-sitosterol and stimasterol was necessary to effect hypoglycaemic activity in *P. speciosa* seeds.

It is highly probable that the antidiabetic activity of leaves of *S. mombin* is due to synergistic effect of the isolated â-sitosterol (fig. 1) and other triterpenoids, enhanced by the potent á-amylase inhibitory action of this identified sterol. However, the totality of the mechanism of action is presently unclear.

Drugs or diet that reduce postprandial hyperglycaemia by suppressing hydrolysis of carbohydrate are helpful in controlling diabetes mellitus [32]. â-sitosterol from *S. mombin* significantly inhibited á-amylase activity. This was helpful in decreasing blood glucose in the diabetic rats. It appears *S. mombin* extracts could hold important therapeutic potential for diabetic patients since the extracts also showed ability to significantly lower serum cholesterol and protein levels of the diabetic rats [13]. This publication is also the first report of hypoglycaemic activity of leaf extracts of *S. mombin*. This study justifies the ethnopharmacological use of this species in recipes for the management of diabetes mellitus.

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