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Antihyperglycaemic Activity of Cycloart-23-ene-3β, 25-diol Isolated from Stem Bark of *Pongamia pinnata* in Alloxan Induced Diabetic Mice

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Abstract: *Pongamia pinnata* (L.) Pierre (Fabacae) has been used in traditional medicine for treatment of diabetes. The aim of the research was to study the antihyperglycaemic activity of cycloart-23-ene-3β, 25-diol (code name compound B2) isolated by column chromatography method from stem bark of *Pongamia pinnata* in alloxan induced diabetic mice. The structure of compound B2 was elucidated by spectroscopical data. Diabetes was induced in mice by alloxan (80 mg kg^{-1}, i.v.). Compound B2 was administered orally. Serum glucose level was determined at 0, 2, 4, 6 and 24 h. The onset was at 2nd h; peak effect at 6th h and the antihyperglycaemic effect was sustained until 24th h. Results obtained in the present study indicated antihyperglycaemic activity of cycloart-23-ene-3β,25-diol (B2).

Key words: *Pongamia pinnata*, triterpene, cycloart-23-ene-3β, 25-diol (compound B2), antihyperglycaemic

INTRODUCTION

Pongamia pinnata (Linn.) Pierre (family Fabacae , synonym; *P. glabra* Vent., *Derris indica* (Lam.) Beunet, *Cystisus pinnatus* Lam.) popularly known as Karanj or Dittouri in Hindi and Indian beech, Pongam oil tree, Hongay oil tree in English (Krishnamurthi, 1998). It is a handsome flowering tree with drooping branches, having shining green leaves laden with lilac or pinkish white flowers and greyish green or brown bark (Joy *et al*., 1998). Different parts of the plant have been used in traditional medicine for the treatment of tumors, piles, skin diseases, wounds, bronchitis, whooping cough, rheumatic joints, ulcers and quench dipsia in diabetes (Kirtikar and Basu, 1987). Flowers were prescribed in glycosuria and remedy for diabetes (Chatterjee, 1992; Krishnamurthi, 1998). The traditional practitioners of Indian system of medicine. Ayurveda and Siddha boil the flowers of plant in water, cool and administer the decoction including marc for treatment of diabetes. Bark is useful as anthelmintic, elexteric and used for treatment of hemorrhoids, beriberi, ophthalmopathy, vaginopathy and diabetes (Joy *et al*., 1998). The phytochemicals like flavonoids, alkaloids, triterpenoids reported in flowers are also present in the bark (Asolkar *et al*., 1992).

Previous phytochemical investigation of this plant indicated the presence of pongamone A-E (Li *et al*., 2006); isopongaglabol, pongaflavonol (Yin *et al*., 2006a); dihydropyranoflavones (Yin *et al*., 2006b); lanceolatin B (Alam, 2004); pongaflavone, karanjin, pongapin, pongachromene, 3,7-dimethoxy-3', 4'-methylenedioxy flavone, millettocalyxin C; 3,3',4', 7-tetramethoxyflavone (Yin *et al*., 2004); pyranochalcones, β-sitosterol, steroids, terpenoids, triterpenes, volatile oils (Carcache-Blanco *et al*., 2003); two triterpenes i.e., cycloart-23-ene-3β, 25 diol and friedelin; dipeptide aurantinamide acetate (Joy *et al*., 1998; Chatterjee, 1992); flavonoids, furanoflavones

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(Tanaka *et al*., 1992); glabrachalcone (Pathak *et al*., 1983) isopongaglabol and 6-methoxyiso-pongaglabol (Talapatra *et al*., 1982). Pongamol and karanjin isolated from fruits of *P. pinnata* were reported to have antihyperglycaemic activity (Tamrakar *et al*., 2008).

Recently, we have reported the antihyperglycaemic activity of alcoholic (Badole and Bodhankar, 2008) and petroleum ether extract (Badole and Bodhankar, 2009) of *P. pinnata* (L.) in alloxan-induced diabetic mice and increased oral glucose tolerance in non-diabetic as well as diabetic mice. Maximum antihyperglycaemic activity was observed in petroleum ether extract (25, 50, 100, 200 and 400 mg kg^{-1}, p.o.) compared to alcoholic extract (100, 200 and 400 mg kg^{-1}, p.o.). LD_{50} of petroleum ether extract of *P. pinnata* was found to be more than 5000 mg kg^{-1} p.o., (Badole and Bodhankar, 2009). The objective of the present investigation was to isolate and determine structure of active antihyperglycaemic compound from stem bark of *Pongamia pinnata*. There was paucity of data on the nature and activity of compound B2 present the bark of *Pongamia pinnata*.

MATERIALS AND METHODS

General Experimental Procedure

IR spectra were recorded on a JASCO FT/IR-5300 infrared spectrophotometer. The ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a VARION, Model: Mercury plus (England). ESITOF-MS were recorded on a Micromass (Water U.K.) Model: Q-Tof micro (YA-105). HPTLC λ_{max} spectra were recorded on a Linomat IV, JASCO spectrophotometer. Silica gel (Spectrochem Pvt. Ltd. India, 100-200 mesh) was used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60 F-254, 0.2 mm) were used for analytical TLC.

Collection and Authentication of Plant

Pongamia pinnata (L.) Pierre bark was collected during May-June 2006 from hilly area of Bhandara, Bhandara District, Maharashtra State, India. The plant was identified and authenticated at Agharkar Research Institute, Pune, India and the voucher specimen was deposited at that Institute (voucher specimen sample No. AHMA-23892).

Extraction and Isolation

Petroleum ether extract of stem bark of *P. pinnata* was prepared according to previously reported method by Badole and Bodhankar (2009). Petroleum ether extract (30 g) was subjected to column grade silica gel (1500 g) borosil glass column chromatography (height, 120 cm; diameter, 7 cm) eluting with mobile phase containing n-hexane: chloroform: ethyl acetate (8:2:2). Polarity of mobile phase was increased by their polarity order and 11 fractions (50 mL each) were collected. All fractions were analyzed by HPTLC and fractions showed similar compounds were pooled together and labeled alphabetically A to K and tested for antihyperglycaemic activity.

Only fraction B showed significant antihyperglycaemic effect hence fraction B was further processed for isolation by preparative TLC. From fraction B the compounds obtained and their quantity were B1 (8.4 mg), B2 (27.9 mg), B3 (10.5 mg), B4 (61.4 mg), B5 (9.9 mg), B6 (19.2 mg), B7 (20.2 mg), B8 (21.5 mg), B9 (25.5 mg), B10 (49.20 mg) and B11 (98.00 mg). The compounds from fraction B were further evaluated for antihyperglycaemic activity. The compound B2 showed significant antihyperglycaemic activity compared to remaining compounds. Hence compound B2 was further analyzed by HPTLC for determination of R_f and λ_{max}. The chemical structure of isolated compound B2 was elucidated by FT-IR, ^{1}H-NMR, ^{13}C- NMR and ESITOF-MS spectroscopy.

B2 Compound

Yellow colored semisolid; HPTLC: silica gel 0.25 mm (mobile phase- n hexane: chloroform: ethyl acetate, 8:2:2) R_f= 0.3, λ_{max}: 340 um; melting point 196-198°C (not corrected); IR λ_{max} (KBr) cm^{-1}: 3441.32, 3040.23, 2930.14. ^{1}H-NMR (400 MHZ, $CDCl_3$): δ 1.25-1.415 (16H, s, CH_2-1, CH_2-2,

Table 1: ^{13}C-NMR (400 MHZ in $CDCl_3$) spectral data of compound B2

Position	^{13}C-NMR (δ, ppm)	Position	^{13}C-NMR (δ, ppm)
C-1	32.01	C-16	26.59
C-2	26.87	C-17	51.98
C-3	79.32	C-18	17.89
C-4	39.65	C-19	30.01
C-5	47.43	C-20	35.41
C-6	21.03	C-21	17.89
C-7	28.28	C-22	39.21
C-8	46.89	C-23	139.93
C-9	20.29	C-24	124.34
C-10	26.07	C-25	71.07
C-11	25.83	C-26	28.91
C-12	35.84	C-27	30.84
C-13	45.31	C-28	19.01
C-14	49.02	C-29	15.32
C-15	32.81	C-30	25.23

CH_2-6, CH_2-7, CH_2-11, CH_2-12, CH_2-15, CH_2-16), δ 1.33 (3H, s, CH-5, CH-8, CH-17), δ 3.5 (H, s, CH-3), δ 0.86 (2H, m, CH_2-19), δ 1.61-2.01 (6H, m, CH_2-20, CH_2-21, CH_2-22), δ 3.032 (2H, m, -CH-23, -CH-24), δ 2 (2H, s, OH-3, OH-25), δ 1.04 (12H, s, CH_3-18, CH_3-26, CH_3-27, CH_3-28), δ 0.97 (3H, s, CH-29), δ 0.81 (3H, s, CH-30); ^{13}C NMR (400 MHZ, $CDCl_3$): (Table 1); ESITOF-MS (positive mode) m/z : 443.38 $[M+H]^+$, 428 (M^+-methyl), 410 (M^+- methyl-H_2O), 316.52 (M^+-side chain) [calc. for $C_{30}H_{50}O_2$ 442.38].

Preliminary Phytochemical Screening

The preliminary phytochemical analysis for compound B2 was carried out for the alkaloid (Mayer's, Hager's, Dragendorff's and Wagner's test), flavonoids (Shinoda test), triterpenes (Liberman-Burchard test) and volatile oils (Kokate, 1991).

Drugs and Chemicals

Glyburide (Ranbaxy Pharma. Ltd., India), alloxan monohydrate (Spectrochem, India), glucose estimation kit (glucose oxidase/peroxidase kit) (Accurex Biomedical Pvt., Ltd., India), tween 80 (Research-Lab., India), petroleum ether, chloroform, n-hexane, ethyl acetate, ethanol (Merck, India) of GR grade were purchased from the respective vendors.

Animals and Research Protocol Approval

Swiss albino mice (25-30 g) of either sex were purchased from National Toxicology Centre, Pune, India. Animals were maintained at a temperature of 25±1°C and relative humidity of 45 to 55% under 12 h light: 12 h dark cycle. The animals had free access to standard food pellets (Chakan Oil Mills, Pune, India) and water was available *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) constituted in accordance with the rules and guidelines of the Committee for the Purpose of Control and Supervision on Experimental Animals (CPCSEA), India.

Induction of Diabetes

Diabetes was induced in Swiss albino mice by a single intravenous injection of aqueous alloxan monohydrate (80 mg kg^{-1}) solution and serum glucose was determined. Mice showing serum glucose level above 300 mg dL^{-1} (diabetic) was selected for the study (Badole and Bodhankar, 2009).

Antihyperglycaemic Activity of Fractions A to K and Compounds B1 to B11 in Alloxan Induced Diabetic Mice

The selected non-fasted mice were divided into following groups (n = 6) viz; Group I- vehicle (tween 80, 2%; 10 mL kg^{-1}), Group II- standard drug, glyburide (10 mg kg^{-1}), Group III to

Group XIII- Fractions A to K (25 mg kg^{-1}, p.o.) respectively. Serum glucose was determined at 0, 2, 4, 6 and 24 h after fractions administration. Isolated compounds from fraction B were selected further antihyperglycaemic study and processed by dividing animals in following groups i.e. Group I- vehicle (tween 80, 2%; 10 mL kg^{-1}), Group II- glyburide (10 mg kg^{-1}), Group III to Group XIII- B1 to B11 (10 mg kg^{-1}, p.o.), respectively.

Statistical Analysis

Data was expressed as Mean±SEM and statistical analysis was carried out by one-way ANOVA with post hoc Tukey test performed using GraphPad InStat version 3.00 for Windows Vista™ BASIC, GraphPad Software, San Diego California USA. The $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

Eleven fractions were labeled alphabetically A to K and evaluated for antihyperglycaemic activity. Only fraction B showed significant antihyperglycaemic activity (Table 2). Fraction B was further processed for preparative TLC. Eleven compounds were obtained and further tested for antihyperglycaemic activity. The compound B2 (Fig. 1) showed significant antihyperglycaemic activity compared to remaining compounds (Table 3).

Compound B2 was obtained as a yellow colored semisolid substance (27.9 mg). The yield was 0.93%. Melting point of compound B2 was 196-198°C and showed positive results in Lieberman-Buchard test for triterpenes. HPTLC of isolated sample showed a single peak with its R_f value 0.3 and absorption maxima (λ_{max}) at 340 nm, which is a characteristic absorption frequency of cycloart-23-ene-3β, 25-diol (Djerassi and McCrindle, 1962). The IR spectrum showed bands at 3441.32 cm^{-1} indicated presence of hydroxyl groups, cyclopropyl ring at 3040.23 cm^{-1} and aliphatic stretch at 2930.14 cm^{-1}. The ^{1}H-NMR ($CDCl_3$, 400 MHZ) spectrum of B2 compound showed a two proton downfield signal at integral value δ 2 which indicated presence of two chelated hydroxyls at the 3rd and 25th positions. The presence of cyclopropyl ring was confirmed by the presence of characteristic integral value δ 0.86 for non-equivalent protons at C-19. The multiple integral value of δ 3.032 at C-23 and C-24 for the vinylic two protons indicated the presence of double bond in the structure. ^{13}C NMR ($CDCl_3$, 400 MHZ) spectrum revealed that double bond is present between positions between C-23 at integral value δ 139.93 and C-24 at δ 124.34. The ESITOF-MS indicated a molecular ion with a large peak at [M^+] m/z 443.38 suggesting a possible formula of $C_{30}H_{50}O_2$. Further fragments were observed at, m/z

Table 2: Antihyperglycaemic activity of fractions (A to K) on serum glucose level in alloxan induced non-fasted diabetic mice

	Mean serum glucose level (mg dL^{-1})				
Treatments	0 h	2 h	4 h	6 h	24 h
Vehicle (10 mL kg^{-1})	549.81±11.23	556.270±12.5	554.89±12.73	557.12±12.26	544.97±14.86
Glyburide (10 mg kg^{-1},p.o.)	539.84±19.87	448.210±16.14**	419.00±15.54***	358.86±12.10***	521.71±34.27
A (25 mg kg^{-1},p.o.)	547.03±16.31	541.710±15.55	533.36±17.08	525.60±5.71	551.11±12.37
B (25 mg kg^{-1},p.o.)	551.27±11.27	443.899±22.38*	326.06±15.39***	284.03±17.04***	539.04±15.46
C (25 mg kg^{-1},p.o.)	515.41±11.01	483.170±15.65	499.83±12.08	482.31±17.66	514.35±13.88
D (25 mg kg^{-1},p.o.)	547.96±14.91	530.390±21.91	524.89±17.80	512.05±15.92	542.42±12.51
E (25 mg kg^{-1},p.o.)	483.99±16.37	462.190±16.17	466.82±22.51	466.70±16.15	473.17±12.29
F (25 mg kg^{-1},p.o.)	498.47±21.90	487.580±23.64	474.95±13.96	462.64±22.49	478.20±18.23
G (25 mg kg^{-1},p.o.)	489.20±25.97	480.340±30.16	452.64±38.83	477.00±34.97	502.73±32.83
H (25 mg kg^{-1},p.o.)	501.53±13.49	495.310±19.62	502.06±14.75	502.07±16.22	504.56±13.55
I (25 mg kg^{-1},p.o.)	503.98±19.98	489.260±18.74	482.27±20.65	478.81±20.45	503.46±25.64
J (25 mg kg^{-1},p.o.)	502.95±19.83	492.540±28.07	477.03±23.09	469.68±27.12	507.65±29.49
K (25 mg kg^{-1},p.o.)	510.45±18.36	489.450±26.22	496.47±24.07	499.11±12.18	527.21±18.30

Values are expressed as Mean±SEM; n = 6 in each group; Statistical analysis by one-way ANOVA followed by post hoc Tukey's test using Graphpad Instat software; *$p<0.05$; ***$p<0.001$ compared to vehicle treated group (Tween 80, 2%; 10 mL kg^{-1})

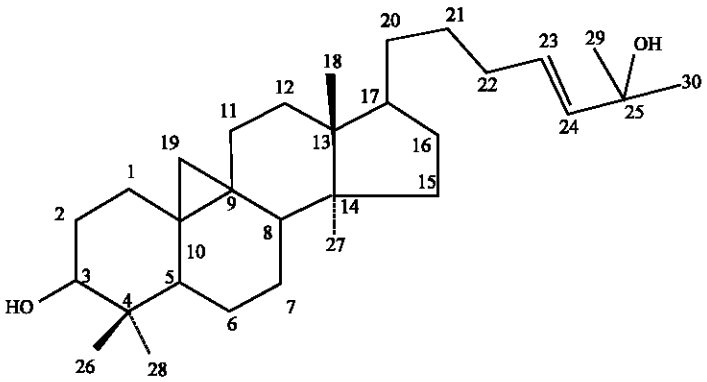

Fig. 1: Structure of cycloart-23-ene-3β, 25-diol (compound B2)

Table 3: Antihyperglycaemic activity of compounds (B1 to B11) from fraction B on serum glucose level in alloxan induced non-fasted diabetic mice

	Mean serum glucose level (mg dL^{-1})				
Treatments	0 h	2 h	4 h	6 h	24 h
Vehicle (10 mL kg^{-1})	549.81±11.23	556.27±12.5	554.89±12.73	557.12±12.26	544.97±14.86
Glyburide (10 mg kg^{-1},p.o.)	539.84±19.87	448.21±16.14**	419.00±15.54***	358.86±12.10***	521.71±34.27
B1 (10 mg kg^{-1},p.o.)	490.31±8.91	491.30±14.55	497.45±13.31	485.68±14.42	472.67±18.97
B2 (10 mg kg^{-1},p.o.)	493.28±8.72	253.86±8.78***	237.56±9.17***	200.73±7.07***	315.90±12.32***
B3 (10 mg kg^{-1},p.o.)	491.71±13.34	492.01±10.7	481.10±17.35	489.10±16.51	477.11±12.17
B4 (10 mg kg^{-1},p.o.)	493.73±12.98	480.21±19.02	481.30±17.17	485.57±13.72	497.02±10.34
B5 (10 mg kg^{-1},p.o.)	514.85±17.02	493.99±20.82	475.90±9.29	461.48±9.50	522.44±15.50
B6 (10 mg kg^{-1},p.o.)	492.38±11.14	452.26±13.17	442.79±8.94	427.82±14.87	496.30±14.06
B7 (10 mg kg^{-1},p.o.)	515.57±12.39	448.16±16.92	436.18±12.90	401.00±14.45	475.32±11.39
B8 (10 mg kg^{-1},p.o.)	515.57±12.39	448.16±16.92**	436.18±12.90**	401.00±14.45***	475.32±11.39
B9 (10 mg kg^{-1},p.o.)	496.25±16.90	469.03±12.14	442.26±16.39	423.16±15.46	500.00±15.13
B10 (10 mg kg^{-1},p.o.)	522.01±12.59	498.89±19.02	483.89±20.19	489.08±12.14	544.28±14.72
B11 (10 mg kg^{-1},p.o.)	505.12±11.83	483.11±13.3	451.67±11.41	447.39±13.97	530.00±8.58

Values are expressed as Mean±SEM, n = 6 in each group; Statistical analysis by one-way ANOVA followed by post hoc Tukey's test using Graphpad Instat software; **p<0.01; ***p<0.001 compared to vehicle treated group (Tween 80, 2%; 10 mL kg^{-1})

428 (M^+- CH_3), 410 (M^+- CH_3-H_2O), 316 (M^+- side chain). The peak observed at m/z 316 indicated the loss of entire substituent chain at C-17 as well as revealed that the second hydroxyl group and double bond were present in the side chain. On the basis of physical properties and spectroscopic data like IR, ^{1}H-NMR, ^{13}C-NMR and ESITOF-MS the compound seems to be triterpene compound identical to previously reported cycloart-23-ene-3β, 25-diol (Djerassi and McCrindle, 1962; Teresa *et al*., 1987; Madureira *et al.,* 2003).

Single dose administration of fraction B (25 mg kg^{-1}, p.o.) as well as glyburide (10 mg kg^{-1}, p.o.) significantly ($p<0.001$) reduced serum glucose level at 2nd, 4th and 6th h after administration. The reduction in serum glucose from basal value (before drug administration in each group) at 6th h after fraction B (25 mg kg^{-1}) and glyburide (10 mg kg^{-1}) was 267.27 and 180.80 mg dL^{-1}, respectively (Table 2). The onset of antihyperglycaemic effect of fraction B (25 mg kg^{-1}) and glyburide (10 mg kg^{-1}) was observed at 2nd h; peak effect at 6th h but the antihyperglycaemic effect waned at 24th h (Table 2). The other fractions (A, C, D, E, F, G, H, I, J and K) did not show significant antihyperglycaemic activity (Table 2).

The fraction B was further fractionated into 11 compounds by preparative TLC. The isolated compound B2 (10 mg kg^{-1}, p.o.) was administered in alloxan (80 mg kg^{-1}, i.v.) induced diabetic mice.

The reduction in serum glucose from basal value (before drug administration in each group) at 6th h after administration of compound B2 (10 mg kg^{-1}, p.o.) and B8 (10 mg kg^{-1}, p.o.) was 292.55 and 114.57 mg dL^{-1}, respectively (Table 3). The onset of antihyperglycaemic effect of compound B2 and B8 was observed at 2nd h; peak effect at 6th h. The antihyperglycaemic effect of compound B2 (10 mg kg^{-1}) was sustained at 24th h but the antihyperglycaemic effect of compound B8 (10 mg kg^{-1}) waned at 24th h (Table 3). The other compounds (B1, B3, B4, B5, B6, B7, B9, B10 and B11) did not show significantly antihyperglycaemic activity (Table 3).

CONCLUSION

Compound B2 (cycloart-23-ene-3β, 25-diol) was isolated from the petroleum ether extract of stem bark of *Pongamia pinnata*. Compound B2 (cycloart-23-ene-3β, 25-diol) when administered orally to alloxan induced diabetic mice reduced the blood sugar. The onset was 2h, peak 6h and after which the antihyperglycaemic effect gradually waned. It is concluded that cycloart-23-ene-3β, 25-diol is the active antihyperglycaemic compound of stem bark of *Pongamia pinnata*. The precise site(s) and the molecular and cellular mechanism(s) of this compound remain to be investigated.

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