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Partial regeneration of β-cells in the islets of Langerhans by Nymphayol a sterol isolated from *Nymphaea stellata* (Willd.) flowers

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ABSTRACT

Reduction of the β-cell mass is critical in the pathogenesis of diabetes mellitus. The discovery of agents which induce regeneration of pancreatic β-cells would be useful to develop new therapeutic approaches to treat diabetes. The present study was aimed at identifying a new agent for the control of diabetes through regeneration of pancreatic β cells and insulin secretory potential. *Nymphaea stellata* flower chloroform extract (*NSFCExt*) showed significant plasma glucose lowering effect. Further *NSFCExt* was utilized to isolate and identify the lead compound based on bioassay guided fractionation; we found Nymphayol (25,26-dinorcholest-5-en-3β-ol) a new crystal [space group $P2_1$ (No. 4), a = 9.618(5), b = 7.518(5), c = 37.491(5)]. It was purified by repeat column. The structure was determined on the basis of X-ray crystallography and spectral data. Oral administration of Nymphayol for 45 days significantly ($p < 0.05$) lowered the blood glucose level and more importantly it effectively increased the insulin content in diabetic rats. In addition, Nymphayol increased the number of β cell mass enormously. Islet-like cell clusters in the islets of Langerhans were clearly observed based on histochemical and immunohistochemical study.

1. Introduction

Diabetes mellitus (DM) is a common endocrine disorder which is characterized by hyperglycemia resulting from the absence or inadequate pancreatic insulin secretion with or without concurrent impairment of insulin action.[1] This illness affects approximately 150 million people worldwide and its incidence rate is expected to double during the next 20 years.[2] Epidemiological studies and clinical trials strongly support the notion that insulin deficiency results in hyperglycemia and long lasting hyperglycemia eventually leading to coronary artery disease, cerebrovascular disease, renal failure, blindness, limb amputation, neurological complications and pre-mature death.[3] In diabetic patients the blood glucose concentration is controlled by multiple injection of insulin, a principal hormone regulating glucose metabolism. Administrations of oral hypoglycemic agents are often used to treat type 2 diabetes. When therapy with oral hypoglycemic agents is ineffective insulin can also be used to treat type 2 diabetes.[4]

An alternate approach is possible for the treatment of type 2 diabetic patients. If formation of newly differentiated β cells can be stimulated by activation of the stem or progenitor cells of the pancreas it would increase the β cell mass and thereby reverse diabetes. There are many growth factors and differentiation factors known to promote differentiation or regeneration of pancreatic β cells.[5] These include nicotinamide, glucagon-like peptide, gastrin, activin A, betacellulin, Reg protein and hepatocyte growth factor (HGF). Chemical ligands that induce insulin secretion and β-cell regeneration may be useful as new therapeutic agents for both type 1 and type 2 diabetes.[6] From a practical point of view, low molecular weight compounds are favorable because such agents are not immunogenic and may be effective even when administered orally. For example, treatment of STZ-induced diabetic animals with (−)-epicatechin and *N*-acetyl-L-cysteine (NAC) (well-known phytoprinciples) prevented hyperglycemia through reduced oxidative stress and restored β-cell function.[7,8]

A wide array of plant derived active principles representing numerous chemical compounds has demonstrated activity consistent with their possible use in the treatment of DM. In an attempt to obtain effective new compounds to reduce blood glucose levels via insulin secretory potential we isolated a new sterol 25,26-dinorcholest-5-en-3β-ol (Nymphayol) from *Nymphaea stellata* (willd.) flower chloroform extract (Fig. 1) associated with antidiabetic activity by bioassay-guided fractionation. Previously we have reported antidiabetic activity of Nymphayol (20 mg/kg bw) in streptozotocin induced diabetic rats.[9] The aim of this study was to examine the effect of Nymphayol on regeneration of pancreatic β-cell mass and stimulation of insulin secretion in streptozotocin induced diabetic rats.

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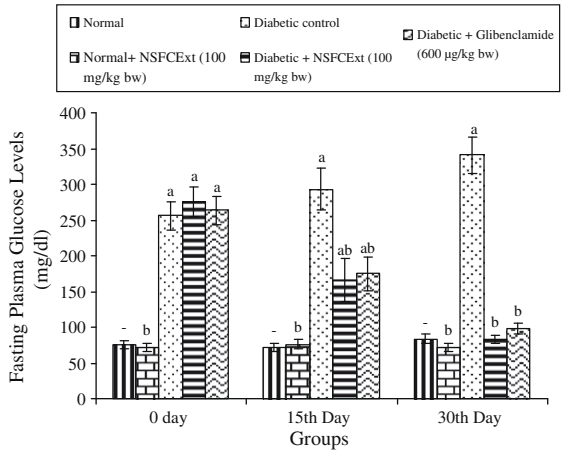

Figure 1. Effect of *Nymphaea stellata* flower chloroform extract (*NSFCExt*) (100 mg/kg bw) on normoglycemic and STZ-induced hyperglycemic rats. Each value is mean ± SD for 6 rats in each group. (a) $p < 0.05$ by comparison with normal rats. (b) $p < 0.05$ by comparison with streptozotocin induced diabetic rats. –: Not significant.

2. Results and discussion

2.1. X-ray structure description

Figure 2 shows ORTEP representation of the isolated compound. Figure 3 represents packing of the compound in the unit cell. The compound crystallizes in space group $P2_1$ with two molecules and a water molecule in the asymmetric unit. The two molecules have an enantiomeric relationship, even though there are no crystallographic mirrors relating the two. The 2-hexyl chains show extended conformation in both molecules. In one of the molecules terminal-CH_3 group is disordered. The molecule and its screw-translation equivalent are connected through O–H···O (O(1)–H(1A) ···O(2)#1, 2.06 Å, 167.9°; Symm: #1, 1 – *x*, y – ½, –*z* + 1) hydrogen bonds to form weak hydrogen bonded dimers in the solid state. Further, the packing is stabilized through van der Waals interactions.

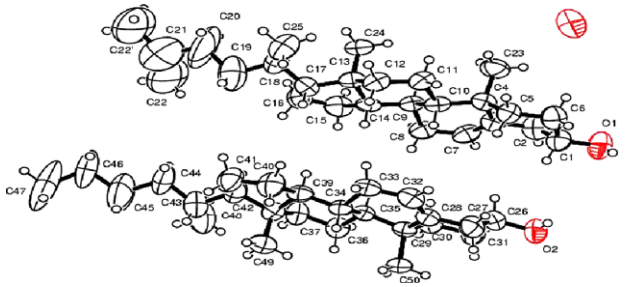

Figure 2. The ORTEP representation of molecule showing numbering scheme. The asymmetric unit consists of two molecules of Nymphayol (25,26-dinorcholest-5-en-3β-ol) and one water molecule.

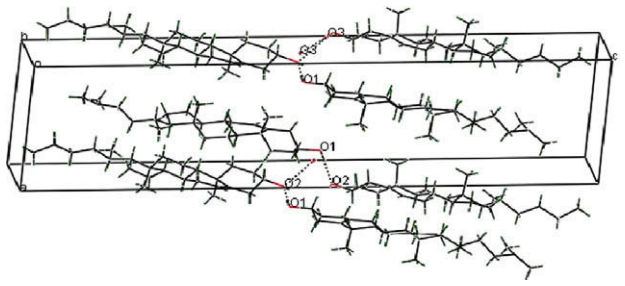

Figure 3. Packing of the molecule Nymphayol (25,26-dinorcholest-5-en-3β-ol) in the unit cell.

2.2. ^{1}H NMR, ^{13}C NMR, IR and MASS spectra

^{1}HNMR (δ, $CDCL_3$, 500 MHz): 0.63 (3H, S,H-18), 1.02 (3H, S, H-19), 0.84 (3H, t, J = 6.5 Hz, H-25), 0.93 (3H, d, J = 6.5 Hz, H-21), 3.54 (^{1}H, m, H-3), 5.37 (^{1}H, br s, H-6). (Fig. 4a); ^{13}C NMR (δ, $CDCL_3$, 125 MHz): 29.16 (C-1), 31.9 (C-2), 71.82 (C-3), 40.47 (C-4), 140.75 (C-5), 121.72 (C-6), 31.65 (C-7), 29.69 (C-8), 50.14 (C-9), 37.20 (C-10), 22.84 (C-11), 37.25 (C-12), 45.84 (C-13), 56.06 (C-14), 28.90 (C-15), 28.24 (C-16), 56.87 (C-17), 20.20 (C-18), 23.07 (C-19), 36.14 (C-20), 19.39 (C-21), 36.83 (C-22), 29.69 (C-23), 24.30 (C-24), 12.23 (C-25). (Fig. 4b); IR**:** IR γ KBr/max cm^{-1}: 3433 (hydroxyl); 2936, 2866, 1645 (trisub double bond); 1464, 1377, 1231, 1054, 801 (trisub double bond) (Fig. 5a); MASS: EIMS (*m/z*): 358 $[m]^+$, 343 $[m^+–me]$, 273 [m-side chain]$^+$, 325 $[m–CH_3–H_2O]$, 287 $[m^+–$Ring B clevage$]$, 231 [m-side chain-ring D cleavage]$^+$, 329 $[m–CH_3–CH_2]^+$, 315 $[m–CH_3–(CH_2)]^+$ (Fig. 5b). Molecular formula is $C_{25}H_{42}O$. Based on the above spectral data the isolated compound was confirmed as 26, 27-dinorcholest-5-en-3-β-ol, a sterol (Fig. 6).

2.3. Biochemical study

Streptozotocin (STZ) induced diabetes results in degenerative and lytic changes in the islets of Langerhans of the pancreas. DNA damaging substances such as superoxide (O·2) and nitric oxide (N·O) are produced in inflammatory processes by cytotoxic agents such as STZ. When DNA is damaged, poly (ADP-ribose) synthetase polymerase (PARP) senses the nicks and autopoly (ADP-ribosyl)-ates itself for DNA repair. Autopoly (ADP-ribosylation) of PARP inhibits the formation of Reg gene transcriptional complex and transcription of this gene stops.[10] Phytochemicals with antioxidant properties and free radical scavengers in particular prevent autopoly(ADP-ribosyl)-ation of PARP by stabilizing Reg gene transcriptional complex resulting in the regeneration of β-cells and protecting pancreatic islets against cytotoxic effects of STZ.[11]

Diabetes mellitus caused a disturbance in the uptake of glucose as well as glucose metabolism. The use of a lower dose of STZ (55 mg/kg bw) produced an incomplete destruction of pancreatic β-cells even though the rats became permanently diabetic.[12] The increased levels of plasma glucose in STZ-diabetic rats were lowered (78.05%) by Nymphayol administration (Fig. 7). The antihyperglycemic action of Nymphayol resulted from the potentiation of insulin release from existing β-cells of the islets of Langerhans (Fig. 8). The plasma glucose lowering activity was compared with glibenclamide a standard hypoglycemic drug. Glibenclamide has been used for many years to treat diabetes by stimulating insulin secretion from pancreatic β-cells.[13] The results of the present study indicated the glucose lowering effect of Nymphayol due to the plasma insulin release from pancreatic β-cells of the islets of Langerhans. Nymphayol augmented the release of insulin many folds probably through β-cells stimulation resembling direct insulin secretagogue effect.

Insulin secretory effect of Nymphayol is directly related to the glucose level in STZ-diabetic rats. The mechanism of action of sterols might stimulate the beta islets to secrete insulin and increase the sensitivity of insulin to uptake glucose.[14] Similarly Ng et al.[15] have reported that Charantin, a steroidal saponin, obtained from *Momordica charantia* is known to have an insulin-like activity and also stimulated the release of insulin and blocks the formation of glucose in the bloodstream. Achrekar et al.[16]

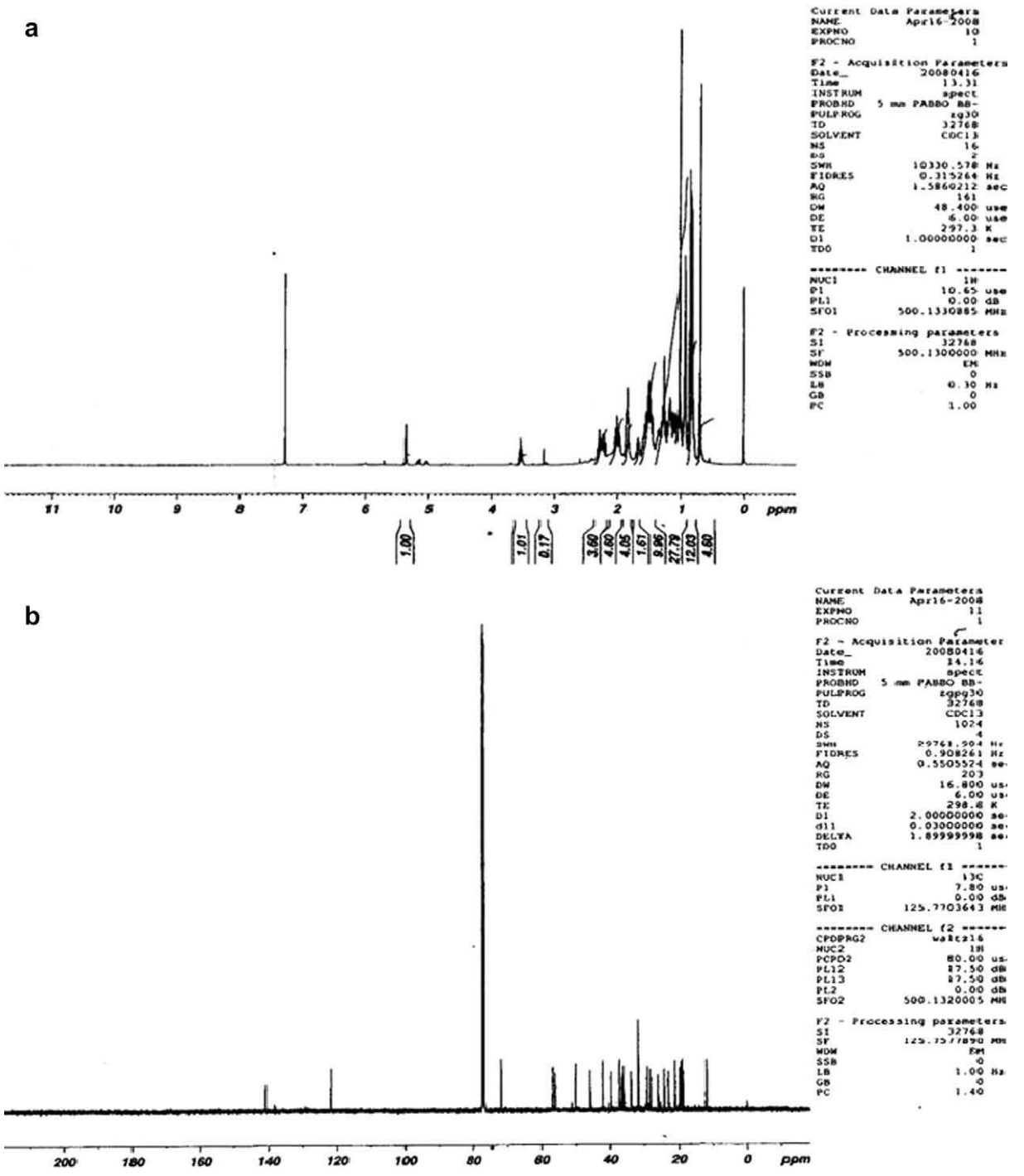

Figure 4. ^{1}H NMR and ^{13}C NMR spectrum of isolated compound.

reported that water extract of *Eugenia jambolana* pulp stimulated the release of insulin both in vivo and in in vitro studies. Similarly Bansal et al.[17] reported that the increase in plasma insulin by seeds of *E. jambolana* was due to proinsulin leading to insulin conversions possibly by pancreatic cathapsin B, and/or its secretion.

2.4. Histology and immunohistochemical study

In STZ-diabetic rats the islet is considerably reduced and shrunken. There is destruction of some β-cells with central hyalinization; a few cells showed pyknotic nuclei and the number of cells were decreased (Fig. 9c).[18–20] After 45 days of oral administration with Nymphayol, when blood glucose came down to normal level, islets of Langerhans also showed improvement in the beta cell granulation (Fig. 9f). Sharma et al.[21] have also reported improvement in islet cells after one month of oral feeding with *E. jambolana* seed powder and some alkaloids. Regeneration of islet cells by dietary components and stimulation of insulin secretion by different plant extracts have been reported.[22,23]

Immunocytochemical staining of the islets of control rats for experimental diabetes revealed that the islets are well granulated

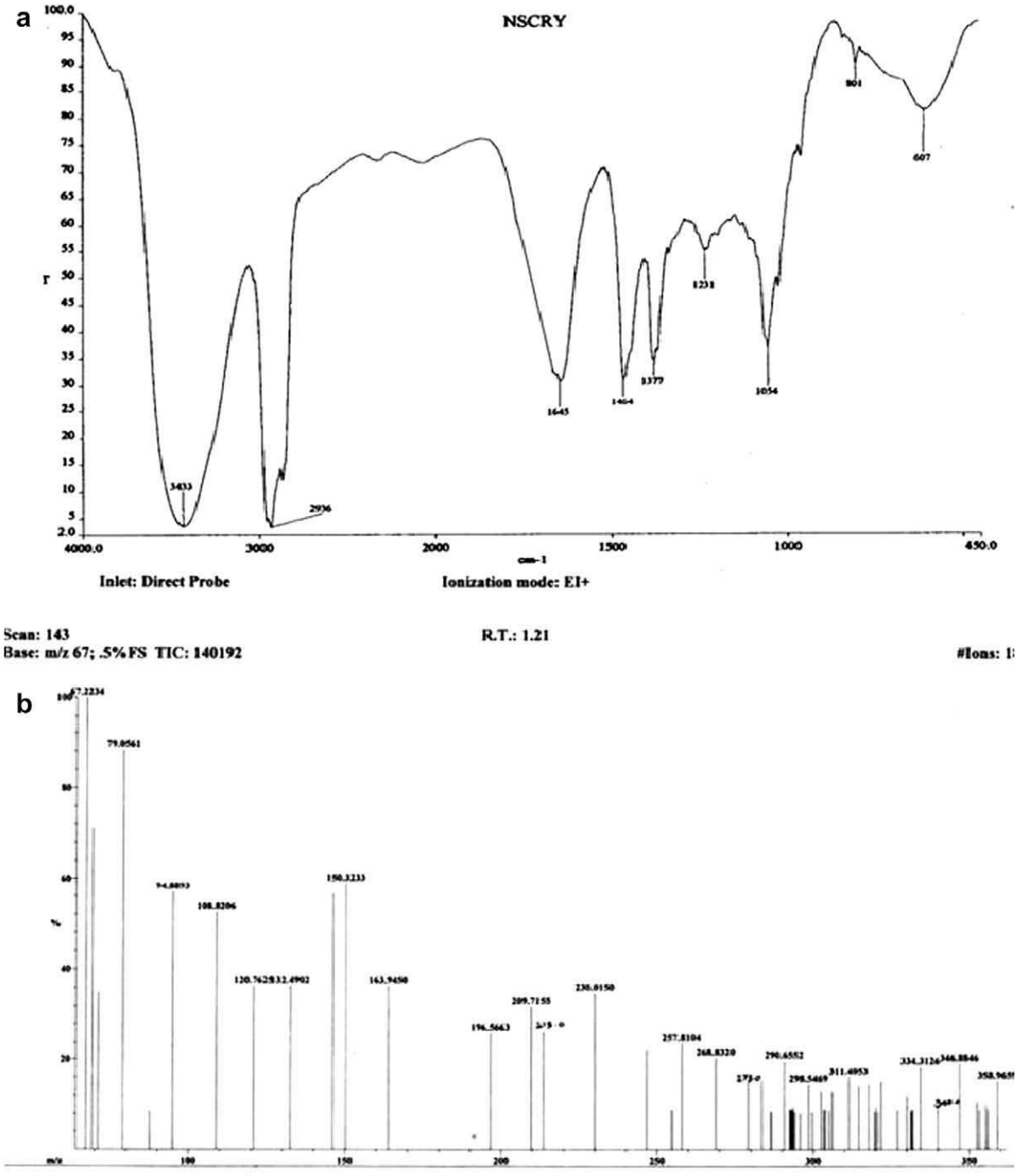

Figure 5. IR and MASS spectrum of isolated compound.

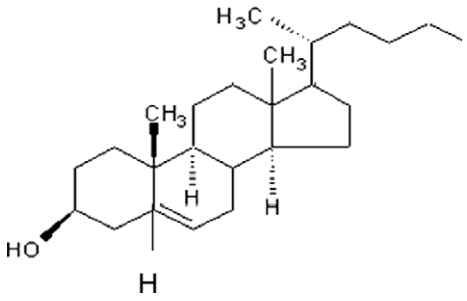

Figure 6. Chemical structure of Nymphayol.

and the insulin positive β-cells form the majority of the islet cells located at the center.[24] Islets from the diabetic animals showed lack of insulin response with degenerated β-cells.[25,26] In the present study islets from untreated rat showed well granulated β-cells with a majority of the β-cells being insulin-positive (Fig. 10a). Diabetic rat did not show any insulin-positive cells in the islets (Fig. 10b). Oral administration of Nymphayol to STZ-diabetic rats showed several insulin positive β-cells in the islet of Langerhans (Fig. 10c). This resulted in the preservation of β-cell mass and insulin secretory granules which further potentiated the efficacy of Nymphayol. These apparently regenerated β-cells as evident from immunohistochemistry may have secreted insulin and alleviated diabetes.

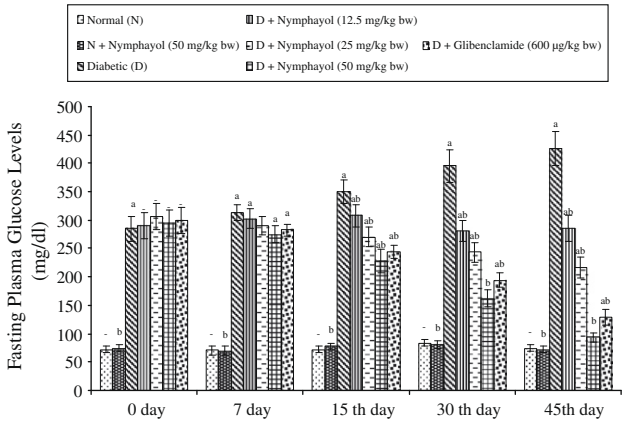

Figure 7. Effect of different doses of Nymphayol on fasting plasma glucose levels (mg/dl) in streptozotocin induced diabetic rats. Each value is mean ± SD for 6 rats in each group. a: $p < 0.05$ by comparison with normal rats. b: $p < 0.05$ by comparison with streptozotocin induced diabetic rats. -: Not significant.

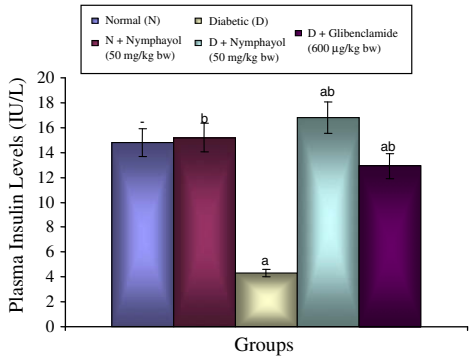

Figure 8. Effect of Nymphayol (50 mg/kg bw) on plasma insulin levels (IU/l) in normal and streptozotocin induced diabetic rats. Each value is mean ± SD for 6 rats in each group. a: $p < 0.05$ by comparison with normal rats. b: $p < 0.05$ by comparison with streptozotocin induced diabetic rats. -: Not significant.

3. Conclusion

Oral administration of Nymphayol for 45 days significantly restored the plasma glucose levels and increased the plasma insulin levels to near normal in STZ-diabetic rats. Light microscopy and immunocytochemical staining of Nymphayol treated diabetic pancreas also revealed increased number of insulin positive β-cells. The mode of action of Nymphayol appears to be the reversal of the damaged endocrine tissue and thereby stimulating the secretion of insulin in β-cells as revealed by insulin assay. The active principle Nymphayol enhances the antioxidant defense against reactive oxygen species produced under hyperglycemic condition and thus protects the pancreatic β-cells against loss.

4. Experimental

4.1. Chemicals and Biochemical measurements

Streptozotocin was procured from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals were of analytical grade. All spectrophotometric measurements were carried out using UV2010 Spectrophotometer (Hitachi, Germany).

4.2. Plant collection and identification

Fresh *N. stellata* willd. flowers were collected from Thiruvallur district, Tamil Nadu, India. The species was identified and authenticated by Dr. D. Narasimhan, Taxonomist, Department of Botany, Madras Christian College, Chennai and the voucher specimen (MPC-186) was deposited at the institute herbarium.

4.3. Extraction and screening for antihyperglycemic effect

Shade dried and coarsely powdered flowers of *N. stellata* (3 kg) were sequentially extracted successively with Hexane, Chloroform, Ethyl acetate and Methanol at room temperature for 48 h respectively. All the extracts were filtered and concentrated under reduced pressure using rotary evaporator to get completely dried extracts. Remaining residue of flower powder was soaked in H_2O for 24 h, then filtered and concentrated to get aqueous extract. The yield of the *N. stellata* flower crude extracts were 12 g of hexane extract, 45 g of chloroform extract, 29 g of ethyl acetate extract, 60 g of methanol extract and 24 g of aqueous extract. All the extracts were screened for antihyperglycemic effect.

4.4. Bioassay-guided fractionation of active crude extract

The active *N. stellata* flower chloroform extract (*NSFCExt*) (25 g) was chromatographed over silica gel column (Acme's silica gel, 100–200 mesh size, 750 g, 3.5 id × 60 cm) and successively eluted with stepwise gradient of hexane, hexane:chloroform and hexane:ethyl acetate system (0%, 5%, 10%, 20%, 30%, 50%, 70%, and 100%). 74 fractions (each 150 ml) were collected; each fraction was spotted on a precoated Silica Gel 60 F_{254}, 0.25 mm thick TLC plate (Merck) and eluted in hexane:ethyl acetate (4:1) system and fractions with similar R_f values in TLC pattern were pooled together. Finally 17 major fractions were obtained and a crystal (30:70—hexane:ethyl acetate) was obtained from fraction 12 (4.8 g). The fractions and crystal were assayed for plasma glucose lowering effect in STZ-induced diabetic rats.

4.5. X-ray crystallography

4.5.1. Crystal data

$(C_{25}H_{42}O)_2{\cdot}H_2O$, *Mr* 735.19, monoclinic, space group $P2_1$ (No. 4), a = 9.618(5), $b = 7.518(5)$, $c = 37.491(5)$ Å, $\beta = 94.483(5)°$, $V = 2703(2)$ Å^3, $Z = 2$, $Dc = 0.903$ Mg/m^3, $F(000) = 820$, $\mu(M_0\ K\alpha) = 0.054$ mm^{-1}, crystal size = 0.3 × 0.1 × 0.1 nm (Table 1).

4.5.2. Data collection and reduction

A crystal of suitable size was inspected for single crystallinity using LEICA DMLSP polarizing microscope and mounted on Kappa Apex2, CCD diffractometer equipped with graphite monochromated Mo (Kα) radiation, ($\lambda = 0.71073$ Å). The unitcell parameters were obtained using reflections scanned from three different zones of the reciprocal lattice. The intensity data were collected using ω and φ scan with frame width of 0.5°. The frames integration and data reduction were performed using Bruker SAINT-Plus (Version 7.06a) software.[27] Multiscan absorption corrections were applied to the data using SADABS (Bruker axs) software.[28]

4.5.3. Structure solution and refinement

The structure was solved using SIR92 and refined using SHELXL-97 programmes.[29,30] All the non-hydrogen atoms were refined with anisotropic displacement parameters. All the hydrogens could be located in the different Fourier maps. However, they were relocated at chemically meaningful positions and were given riding model refinement. For hydrogen of tertiary CH_3 groups C–H was fixed at 0.96 Å with $U_{iso} = 1.2\ U_{eq}$ of parent carbon. For primary CH group C–H = 0.98 Å and $U_{iso} = 1.2\ U_{eq}$ of the parent carbon. The hydroxyl hydrogen (OH) was fixed at 0.82 Å and C–O–H angle tetrahedral. The structure was full matrix least squares refined with 5337 unique reflections. The weighting scheme employed was

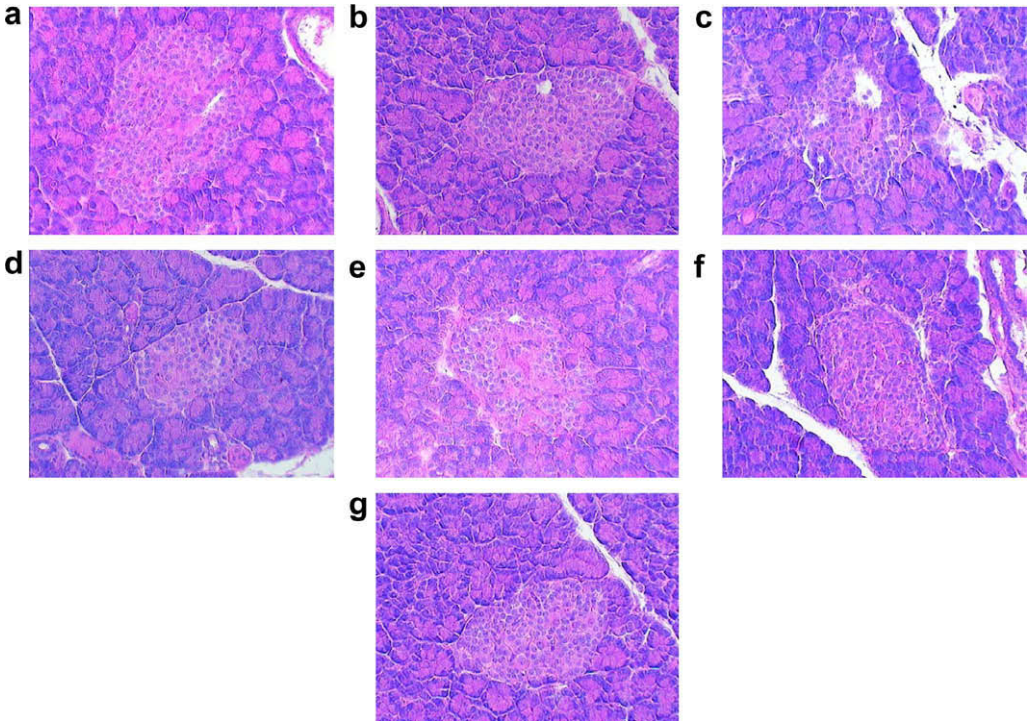

Figure 9. Histopathological observation of normal and experimental rats (H&E, 400×). (a) normal (Native architecture of pancreatic islets); (b) normal + Nymphayol 50 mg/kg bw (Native architecture of pancreatic islets); (c) diabetic control (Presence of pancreatic acini, small atrophic islet cells); (d) diabetic + Nymphayol 12.5 mg/kg bw (Expansion and dilated islet cells); (e) diabetic + Nymphayol 25 mg/kg bw (Mild expansion and absence dilations); (f) diabetic + Nymphayol 50 mg/kg bw (Moderate expansion pancreatic islets, shows prominent hyperplastic islet); (g) diabetic + Glibenclamide 600 μg/kg bw (Absence of dilation and prominent hyperplastic of islets).

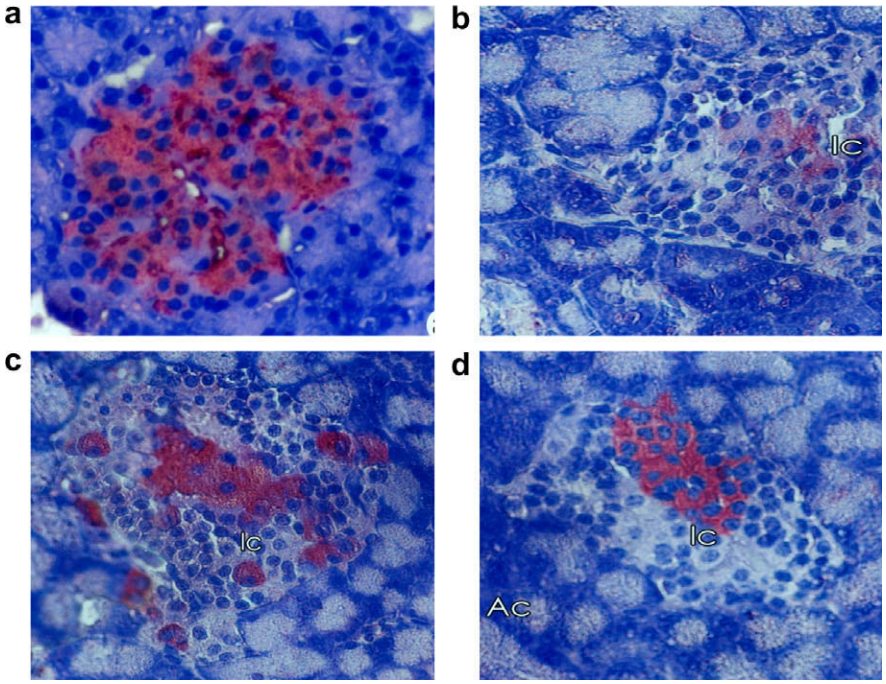

Figure 10. Immunohistochemical study of normal and experimental rat pancreas. (a) Immunocytochemical staining of β-cells of the islet of an untreated rat. Ac, acinar cells; Ic, islet cells (400×); (b) islet of a diabetic rat which has not reacted positively for insulin containing β-cells. Ac, Acinar cells; Ic, Islet cells. (400×); (c) immunocytochemical staining of nymphayol treated diabetic rat (Ac, acinar cells; Ic, islet cells) (400×) showing positive for insulin-containing β-cells; (d) immunocytochemical staining of glibenclamide treated diabetic rat (Ac, acinar cells; Ic, islet cells) (400×) also showed insulin-containing β-cells.

$w^{-1} = (\sigma^2(F_o^2) + 0.20P^2)$, where $P = (F_o^2 + 2F_c^2)/3$. The refinement was continued until shift/esd converged 0. The goodness of fit factor for refinement was 1.105. The highest residual electron density peak was 0.404 e/Å^3. The crystal was rather poorly diffracting at higher Bragg angles. There were practically no reflections present for Bragg angles >20.5°. The final residual factor (RCF) was 0.0842.

Table 1
Crystal data and structure refinement for Nymphayol (25,26-dinorcholest-5-en-3β-ol)

Empirical formula	$C_{50}H_{86}O_3$
Formula weight	735.19
Temperature	293(2) K
Wavelength	0.71073 Å
Crystal system, space group	Monoclinic, $P2_1$
Unit cell dimensions	a = 9.618(5) Å α = 90.000(5)°
	b = 7.518(5) Å β = 94.483(5)°
	c = 37.491(5) Å γ = 90.000(5)°
Volume	2703(2) Å^3
Z, calculated density	2, 0.903 Mg/m^3
Absorption coefficient	0.054 mm^{-1}
F(000)	820
Crystal size	0.3 × 0.1 × 0.1 mm
Theta range for data collection	2.12–20.45°
Limiting indices	$-9 \leqslant h \leqslant 9$, $-7 \leqslant k \leqslant 7$, $-36 \leqslant l \leqslant 36$
Reflections collected / unique	16593/5337 [R(int) = 0.0406]
Completeness to θ = 20.45	99.2%
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.9560 and 0.9140
Refinement method	Full-matrix least-squares on F^2
Data/restraints/parameters	5337/11/484
Goodness-of-fit on F^2	1.105
Final R indices [I > 2sigma(I)]	R_1 = 0.0842, wR_2 = 0.2505
R indices (all data)	R_1 = 0.0991, wR_2 = 0.2687
Absolute structure parameter	−2(4)
Extinction coefficient	0.0013(17)
Largest difference in peak and hole	0.404 and −0.218 e A^{-3}

4.6. Spectral analysis

The isolated compound was subjected to spectral analysis like, ^{1}H NMR, ^{13}C NMR, IR and MASS. Tetra methyl saline (TMS) was used as standard, which shows chemical shift value at zero on the δ scale. ^{1}H and ^{13}C NMR spectra were recorded with a JEOL 300 MHz FT NMR spectrometer (H^1) 75 MHz (C^{13}) and chemical shifts were recorded in ppm. IR spectra were recorded in Shimadszu by KBr pellet method. IR spectra were taken on a Perkin Elmer FT–IR (Spectrum One) spectrophotometer. High resolution Electron Impact Mass Spectroscopy (EI–MS) was performed. Mass spectra were taken on a Jeol JMS-DX30 spectrometer.

4.7. Biochemical estimation

4.7.1. Estimation of plasma glucose and insulin

Fasting plasma glucose was estimated colorimetrically using commercial diagnostic kits (Sigma Diagnostics (I) Pvt. Ltd, Baroda, India.[31] Plasma insulin concentrations were determined by radioimmunoassay kit (Pharmacia, Uppsala, Sweden) with a beta metric counter (Cronex, Dupont, France). The kit included human insulin as standard and ^{125}I-labeled human insulin antibody, which cross-reacts similarly with rat insulin.

4.7.2. Immunohistochemical analysis

Tissues were dehydrated in graded series of alcohol, embedded in paraffin, sectioned at 5 μm thickness and used for immunostaining. All sections were de-paraffinized in xylene bath. The slides were placed in two changes of absolute alcohol for 3 min each. The same procedure was repeated with 90% alcohol. The slides were placed in blocking reagent in order to block the endogenous peroxidase activity for five min which was pre-diluted with 5 volumes of 100% ethanol. The slides were placed in two changes of 70% alcohol for 3 min each. The excess alcohol around the sections was removed and the slides were quickly immersed in Tris buffer (pH 7.6), for 5 min. Two drops of tissue conditioner were added and the sections were incubated for 5 min and then rinsed in buffer solution. Pre-diluted primary polyclonal anti-guinea pig antibody to insulin (1:1,000), raised against human insulin, was added to the sections and incubated for 1 h. The secondary antibody for insulin was anti-rabbit polyclonal antibody. After incubation for half an hour, the sections were rinsed with Tris buffer and peroxidase solution was added, incubated for 30 min and later rinsed with the buffer. AEC (3-amino, 9-ethyl carbazole) chromogen substrate was added to the sections and incubated for 15 min and rinsed with distilled water. The sections were observed under a Leitz diaplan microscope (Leica, Germany) and photographed.

Supplementary data

Crystallographic data (without structure factors) for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Center as supplementary publication No. CCDC 681081; e-mail: deposits@ccdc.cam.ac.uk; Website: www.ccdc.cam.ac.uk. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.02.021.

References and notes

1. Ortiz-Andrade, R. R.; Garcıa-Jimenez, S.; Castillo-Espana, P.; Ramırez-Avila, G.; Villalobos-Molina, R.; Estrada-Soto, S. *J. Ethnopharmacol.* **2007**, *109*, 48–53.
2. Cohen, P.; Goedert, M. *Nat. Rev.* **2004**, *3*, 479–487.
3. Lopez-Candales, A. *J. do Medico* **2001**, *32*, 283–300.
4. Schoenfelder, T.; Cirimbelli, T. M.; Citadini-Zanette, V. *J. Ethnopharmacol.* **2006**, *107*, 456–459.
5. Nielsen, J. H. *J. Mol. Med.* **1999**, *77*, 62–66.
6. Takatsuna, H.; Umezawa, K. *Biomed. Pharmacotherapy* **2004**, *58*, 610–613.
7. Sheehan, E. W.; Zemaitis, M. A. *J. Nat. Prod.* **1983**, *46*, 232–234.
8. Takatori, A.; Ishii, Y.; Itakagi, S.; Kyuwa, S.; Yoshikawa, Y. *Diabetes/Metabol. Res. Rev.* **2004**, *20*, 211–218.
9. Ignacimuthu, S.; Subash-Babu, P. *The Patent Office Journal*, 1567/CHE/2007A. **2007.**
10. Akiyama, T.; Takasawa, S.; Nata, K.; Kobayashi, S.; Abe, M.; Shervani, N. J.; Ikeda, T.; Nakagawa, K.; Unno, M.; Matsuno, S.; Okamoto, H. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 48–53.
11. Szkudelski, T. *Physiological Res.* **2001**, *50*, 536–546.
12. Aybar, M.; Sanchez Riera, A. N.; Grau, A.; Sanchez, S. S. *J. Ethnopharmacol.* **2002**, *74*, 125–132.
13. Tian, Y. M.; Johnson, G.; Ashcroft, J. H. *Diabetes* **1998**, *47*, 1722–1726.
14. Li, W. L.; Zheng, H. C.; Bukuru, J.; De Kimpe, N. *J. Ethnopharmacol.* **2004**, *92*, 1–21.
15. Ng, T. B.; Wong, C. M.; Li, W. W.; Yeung, H. W. *J. Ethnopharmacol.* **1986**, *15*, 107–117.
16. Achrekar, S.; Kaklij, G. S.; Pote, M. S.; Kelkar, S. M. *In vivo* **1991**, *5*, 133–148.
17. Bansal, R.; Ahmad, N.; Kidwai, J. R. *Indian J. Biochem. Biophys.* **1981**, *18*, 377–381.
18. Bora, P. S.; Srivastava, L. M.; Bhatt, S. D. *Indian J. Exp. Biol.* **1985**, *23*, 23–26.
19. Shanmugasundaram, K. R.; Paneerselvam, C.; Samudram, P.; Shanmugasundaram, E. R. B. *J. Ethnopharmacol.* **1983**, *7*, 205–234.
20. Kavalali, G.; Tuncel, H.; Goksel, S.; Hatemi, H. H. *J. Ethnopharmacol.* **2003**, *84*, 241–245.
21. Sharma, S. B.; Nasir, A.; Prabhu, K. M.; Murthy, P. S.; Dev, G. *J. Ethnopharmacol.* **2003**, *85*, 201–206.
22. Hellerstrom, C.; Anderson, A.; Gunnarsson, R. *Acta Endocrinol.* **1976**, *83*, 145.
23. Shanmugasundaram, K. R.; Panneerselvam, C.; Samudram, P.; Shanmugasundaram, E. R. B. *Pharmacol. Res. Commun.* **1981**, *13*, 475.
24. Rebecca, S. S.; Pulimood, A.; Gunasekaran, S. *Indian J. Med. Res.* **2004**, *119*, 38–44.
25. Sasaki, M.; Arai, T.; Usui, T.; Oki, Y. *Veterinary Pathology* **1991**, *28*, 405–497.
26. Higdon, H. L.; Parnell, P. G.; Hill, J. E.; Spitzer, J. C. *Veterinary Pathology* **2001**, *38*, 715–720.
27. Bruker-Nonius APEX-II *and* SAINT-PLUS *(Version 7.06a)*; Bruker AXS: Madison, Wisconsin, USA, 2004.
28. Bruker *SADABS*; Bruker AXS: Madison, Wisconsin, USA, 1999.
29. Altormare, A.; Gascarano, G.; Giacovazzo, C.; Guagliardi, A. *J. Appl. Crystallogr.* **1993**, *26*, 343–350.
30. Sheldrick, G. M; SHELXL-*97*; University of Göttingen, Germany; Spek, A. L. *Acta Crystallogr., Sect. A* **1997**, *46*, C34.
31. Trinder, P. *Ann. Clin. Biochem.* **1969**, *6*, 24–27.