PTP 1B inhibitory action of a phytochemical propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl)

M. Venkatachalam[1], G. Singaravelu[2,*], K. Govindaraju[3] and Jong Seog Ahn[4]

[1]Department of Biochemistry, Institute of Veterinary Preventive Medicine, Ranipet 632 402, India
[2]Department of Zoology, Thiruvalluvar University, Vellore 632 115, India
[3]Centre for Ocean Research, Sathyabama University, Chennai 600 119, India
[4]Bio-Therapeutics Research Institute, Chemical Biology Research Centre, Korea Research Institute of Bioscience & Biotechnology, 52 Oeundong, Yusongku, Daejon 305-806, Republic of Korea

Bioassay-guided study of the *n*-butanol fraction of hydromethonolic flower extract of *Cassia auriculata*, propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl) has demonstrated potential as a protein tyrosine phosphatase 1B (PTP 1B) inhibitor. An *in vivo* study using Wistar rats has demonstrated that propanoic acid exerted a hypoglycemic action when given orally. The results highlight the green chemistry approach in developing novel PTP 1B inhibitor.

Keywords: *Cassia auriculata*, insulin resistance, propanoic acid, protein tyrosine phosphatase.

DIABETES MELLITUS (DM) is a global health issue and in response to this global health challenge, the World Health Organization (WHO) expert committee on DM recommended further evaluation of the folkloric methods of managing the disease because of high mortality and morbidity arising from its associated complications with the use of conventional antidiabetic drugs[1]. As the number of people with diabetes increases, the disease takes an ever-increasing proportion of national and international healthcare budgets. It is projected to become one of the world's main disablers and killers within the next 25 years. It is the most common serious metabolic disease. The number of people suffering from the disease is increasing at an alarming degree[2], with a probable 552 million people likely to be diabetic by 2030 as against 366 million estimated in 2011. Ninety per cent of these patients suffer from type 2 diabetes, which is characterized by a resistance to insulin. Regions with greatest potential are Asia and Africa, where DM rates could rise two- to threefold than the present rates. Pathophysiology of diabetes involves a complex cascade of several interrelated mechanisms resulting from malfunction in insulin secretion and/or insulin action, both responsible for causing impaired metabolism of glucose, lipids and proteins. Many studies showed that insulin resistance is one of the characteristic pathogenic signs for the development of type-2 diabetes and several drugs that increase the insulin sensitivity are currently in clinical use[3]. Studies have shown that protein tyrosine phosphatase 1B (PTP 1B) is a negative regulator of the insulin-signalling pathway and is considered a promising potential therapeutic target, in particular for the treatment of type-2 diabetes[4]. PTP 1B has been demonstrated to dephosphorylate insulin receptor in intact cells and thus act as a negative regulator of insulin signalling[5]. Inhibition of PTP 1B for the management of type-2 diabetes is gaining momentum. The present study demonstrates the PTP 1B inhibitory activity of a phytochemical.

In recent years search for novel types of natural drugs from several plant materials has received considerable attention. Further, WHO has recommended evaluation of traditional plant treatments for diabetes[6]. Moreover, there is an increasing demand by patients to use natural products with antidiabetic activity, because both insulin and oral hypoglycemic drugs possess undesirable side effects[7]. Throughout medical history, herbal medications have been used for a variety of ailments. In the last two decades the use of natural health products as complementary or alternative approaches to existing medications is growing in popularity[8,9] and many herbal medicines possess considerable anti-diabetic potential[10,11]. Plants and plant products both as extracts and derived compounds are known to be effective and versatile chemo-preventive agents against various types of diseases[12–14]. *Cassia auriculata* (Cesalpiniaceae) is one such plant widely used in Indian traditional medicine[15] and it has been proved to exhibit antidiabetic activity[16]. It is a shrub with bright yellow flowers found throughout southern, western and central India[17] and cultivated in other parts of the country as well. The various parts of plant, i.e. roots, leaves, flowers, bark and unripe fruits have medicinal value and are reported to have a number of therapeutic activities to manage diseases like leprosy, asthma, gout, rheumatism and diabetes[18]. It is also used as antipyretic, anticancer and treatment of skin infection[19], hepatoprotective[20], antihyperglycemic[21], hypolipidemic[22] and anti-microbial activity[23]. Here we report an active principle of the flower extract of *C. auriculata* possessing PTP 1B inhibitory activity.

The flower of *C. auriculata* L. was collected from the fields around Thiruvalluvar University Campus, Vellore, India. The plant was identified, authenticated by Botanical Survey of India, Coimbatore, India, and the specimen was prepared and maintained in Thiruvalluvar University. The collected flowers were shade-dried for 4–5 days and ground to coarse powder.

PTP 1B (human recombinant) was purchased from BIOMOL® International IP (USA). All the other chemicals used in this study were analytical grade and purchased from Fischer Chemicals Ltd, India and Merk India Ltd.

*For correspondence. (e-mail: gsvelu@gmail.com)

The dried coarse powder of the flowers of *C. auriculata* (500 g) was soaked in 2 litre of hydromethanolic (water : methanol, 1 : 1 ratio) solution for 48 h with shaking and filtered using Buchner funnel. It was concentrated using vaccum rotary evaporator (Heidolph, Germany). The hydromethanolic extract (20 g) was fractionated further with ethyl acetate and *n*-butanol. The *n*-butanol fraction having potent inhibitory effect against PTP 1B was freeze-dried at –20°C and stored at 4°C until use.

The dried *n*-butanol fraction was chromatographed on a silica gel column and successfully eluted with stepwise gradient of chloroform, methanol solvent system (100 : 0; 80 : 20; 60 : 40; 50 : 50; 20 : 80; 0 : 100). A dark brown sticky material was obtained in chloroform, methanol solvents in the ratio 60 : 40. It was checked on thin layer chromatography (TLC) and showed a single spot. It was subjected to gas chromatography–mass spectrometry (GC–MS) analysis.

The composition of the extract was established by GC–MS analysis. The analysis was performed on a JEOL GCMATE II GC–MS system in EI/CI mode equipped with a split/split less injector (220°C), at a split ratio of 1/10, using a VF-1MS fused-silica capillary column (30 m × 0.25 mm i.d.; film thickness: 0.25 mm). The oven temperature was programmed from 60°C (5 min) to 280°C at a rate of 4°C/min and held at the temperature for 10 min. Helium was used as a carrier gas at a flow rate of 0.8 ml/min.

PTP 1B inhibitory activity was carried out as described earlier[24,25]. The enzyme activity was measured by adding 2 mM *p*-nitrophenol and PTP 1B (0.05–0.1 μg) in a buffer containing 50 mM citrate (pH 6.0), 0.1 M NaCl, 1 mM EDTA and 1 mM dithiothreitol (DTT) with or without test compounds. Following incubation at 37°C for 30 min, the reaction was terminated by adding 1 N NaOH. The amount of produced *p*-nitrophenol was estimated by measuring the absorbance at 405 nm. The non-enzymatic hydrolysis of 2 mM *p*-nitrophenol was corrected by measuring the increase in absorbance at 405 nm obtained in the absence of PTP 1B enzyme.

Antidiabetic studies were made using healthy male Wistar rats (180–200 g) procured from the Institute of Veterinary Preventive Medicine (IVPM), Ranipet, India. The experiments were conducted according to the Institutional Ethical Committee, India (IAEC). The rats were housed in an air-conditioned room at 22°± 1°C with a lighting schedule of 12 h light and 12 h dark. The animals were fed with commercial rat diet (Hindustan Lever, Mumbai) and water *ad libitum*. They were allowed to fast for 12 h and were administered with freshly prepared alloxan (120 mg/kg body weight). After 48 h, rats with moderate diabetes having persistent glycosuria and hyperglycemia (blood glucose > 200–300 mg/dl) were considered diabetic. The isolated compound was administered orally through orogastric tube. Animals were divided into four groups, consisting of a minimum of four animals each as follows: (i) group I, normal control; (ii) group II, diabetic control; (iii) group III, diabetic rats with glibenclimade; (iv) group IV, diabetic rats with isolated compound[26].

Blood samples were collected from the retro orbital venous plexus with heparinized capillary tubes. The plasma was separated from the blood cells by refrigerated centrifugation at 2000 *g* for 15 min at 4°C. Blood glucose levels were measured by an auto analyser[27].

Diabetes is a metabolic disorder resulting due to insulin resistance or deficiency[28]. Recently, PTP-1B has emerged as a promising attractive target for the treatment of type-2 diabetes[29,30]. Inhibition of PTP 1B results in sensitization to insulin signalling and protection against diet-induced obesity. This dual effect of PTP 1B makes it an exciting target in the treatment of type-2 diabetes.

Here we demonstrate an active compound from *C. auriculata* flowers having PTP 1B inhibitory activity. The GC–MS analysis revealed that the spectra obtained from *n*-butanol fraction was propanoic acid 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl). The molecular weight of the compound was found to be 355.0756 Da, which has been compared with MS spectra library (Figures 1 and 2).

The PTP 1B inhibitory activity assay was carried out with ethyl acetate and *n*-butanol fractions and the IC_{50} values were found to be 96.27 and 35.5 μg/ml respectively. Based on the observations, *n*-butanol fraction was chromatographed and eluted. The eluted fraction was subjected to PTP 1B inhibitory action. The result was found promising and the IC_{50} value was recorded at 3.55 μM/ml (Table 1). A known phosphatase inhibitor, RK-682 (IC_{50} of 4.4 μM/ml) was employed as a positive control in the assay. When compared, the isolated compound was found to possess significant PTP 1B inhibitory activity. The PTP super family comprises more than 100 enzymes[31]. The aberrant of PTP activity contributes to several human pathologies, such as diabetes, obesity, cancer and immune disorders[32–34]. Moreover, PTP 1B is a key member in the down-regulation of the insulin and leptin signalling pathway by dephosphorylating the insulin receptor[35], insulin receptor substrates (IRS)[36]. Recent studies have shown that PTP 1B inhibitors have emerged as potential therapeutics for treatment of type-2 diabetes and obesity[37].

In vivo studies were also made to assess the antidiabetic activity of the isolated compound. Plasma glucose levels were measured in normal and experimental rats after a single day and at the end of 15 and 28 days of treatment (Table 2). Alloxan-treated diabetic rats showed significant increase in the levels of blood glucose compared to normal rats. The alloxan-treated diabetic rats were given 5 mg/kg body weight of the isolated compound. The level of blood glucose returned to near normal concentrations in diabetic rats treated with isolated compound and glibenclamide. Isolated compound showed effects comparable to that of glibenclamide.

Table 1. Comparison of the inhibitory activity of RK-682 (positive control), extracts of *Cassia auriculata* and compound isolated from *C. auriculata* against PTP 1B

Plant extracts/compound (*C. auriculata*)	PTP 1B (IC_{50})[#]
Ethyl acetate fraction (μg/ml)	96.27 ± 0.3
n-Butanol fraction (μg/ml)	35.5 ± 0.3
Propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl) (μM/ml)	3.55 ± 0.5
RK-682* (μM/ml)	4.4 ± 0.5

[#]IC_{50} values were determined by regression analyses and expressed as means ± SD of three replicates.
* Positive control.

Table 2. Glucose levels of Wistar albino rats treated with propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl) of *C. auriculata*

Group	First day	15th day	28th day
Normal control	83.32 ± 1.89	87.23 ± 2.21	92.35 ± 1.92
Diabetic control[$]	295.25 ± 5.82	303.21 ± 5.42	307.24 ± 5.21
Diabetic rats with Glibenclamide[#]	97.35 ± 2.62	98.35 ± 2.31	99.12 ± 2.14
Diabetic rats with propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl[#]	127.32 ± 4.32	92.36 ± 3.96	91.26 ± 4.13

All values are expressed as mean ± SD (n = 6). [$]Diabetic control compared with control. [#]Diabetic control treated with isolated compound; diabetic control treated with glibenclamide treated compared with diabetic control. *P < 0.001.

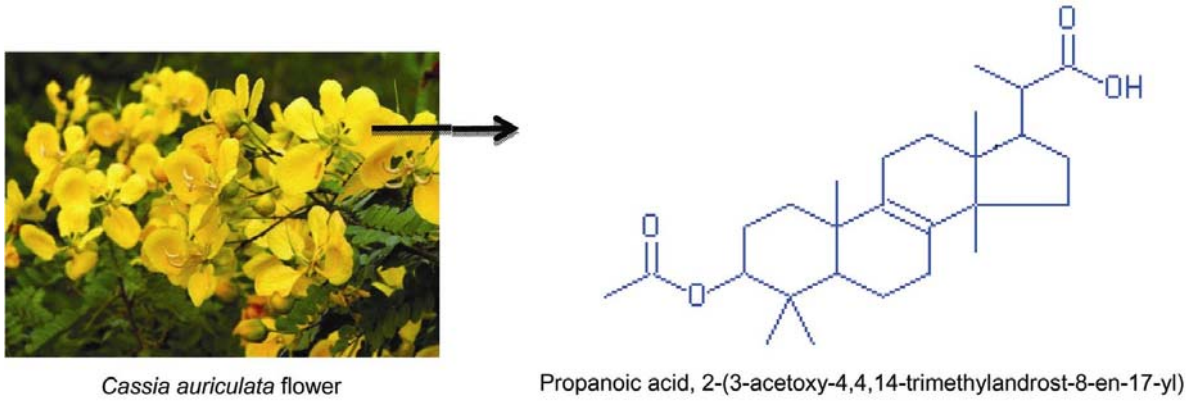

Figure 1. Propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl) isolated from *Cassia auriculata*.

Development of PTP 1B inhibitors from natural products or synthetic counterparts is one of the biggest issues. Chinese crude drug 'Sang-Bai-Pi'(*Morus* root bark) was fractionated and found to possess PTP 1B inhibitory activity[25]. A compound dammaranes isolated from *Gynostemma pentaphyllm* had PTP 1B inhibitory activity[38]. Butanol soluble fraction of *Psidium guajava* exhibited significant inhibitory effect on PTP 1B[39]. Bioassay-guided fractionation resulted in the isolation of five PTP 1B inhibitory triterpenes, including a new one 3α, 24-dihydroxyolean-12en.27-oic acid from rhizomes of *Astilbe koreana*[40]. We had reported earlier that guavanoic acid (isolated compound from *P. guajava* leaf extract)-mediated gold nanoparticles possess PTP 1B inhibitory activity[41]. Na *et al.*[42] demonstrated that CH_2Cl_2 soluble extract of the roots of *Acanthopanase koreanum* was found to inhibit PTP 1B activity (72% inhibition at 30 μg/ml). Hoang *et al.*[43] described, isolation and characterization of six compounds from a $CHCl_3$ extract of *Morus bombysis* as PTP 1B inhibitory with IC_{50} values ranging from 2.7 to 13.8 μM. Aquastatin A, a metabolite derived from the marine fungus *Cosmospora* sp. SF-5060 was found possess PTP 1B inhibitory activity[44] with IC_{50} value of 0.19 μM. The structural features of PTP 1B molecule relevant to the interaction with inhibitors have been reviewed[45]. PTP 1B inhibitors also exert beneficial systemic effects such as circulating HbA1c and the reduction of fructosamine levels[46], insulin sensitivity, plasma metabolic profile reinstallation[44] and reduction of serum insulin and leptin levels[47].

Liu *et al.*[48] pointed out lack of selectivity and bioavailability in most of the reported PTP 1B inhibitors. One

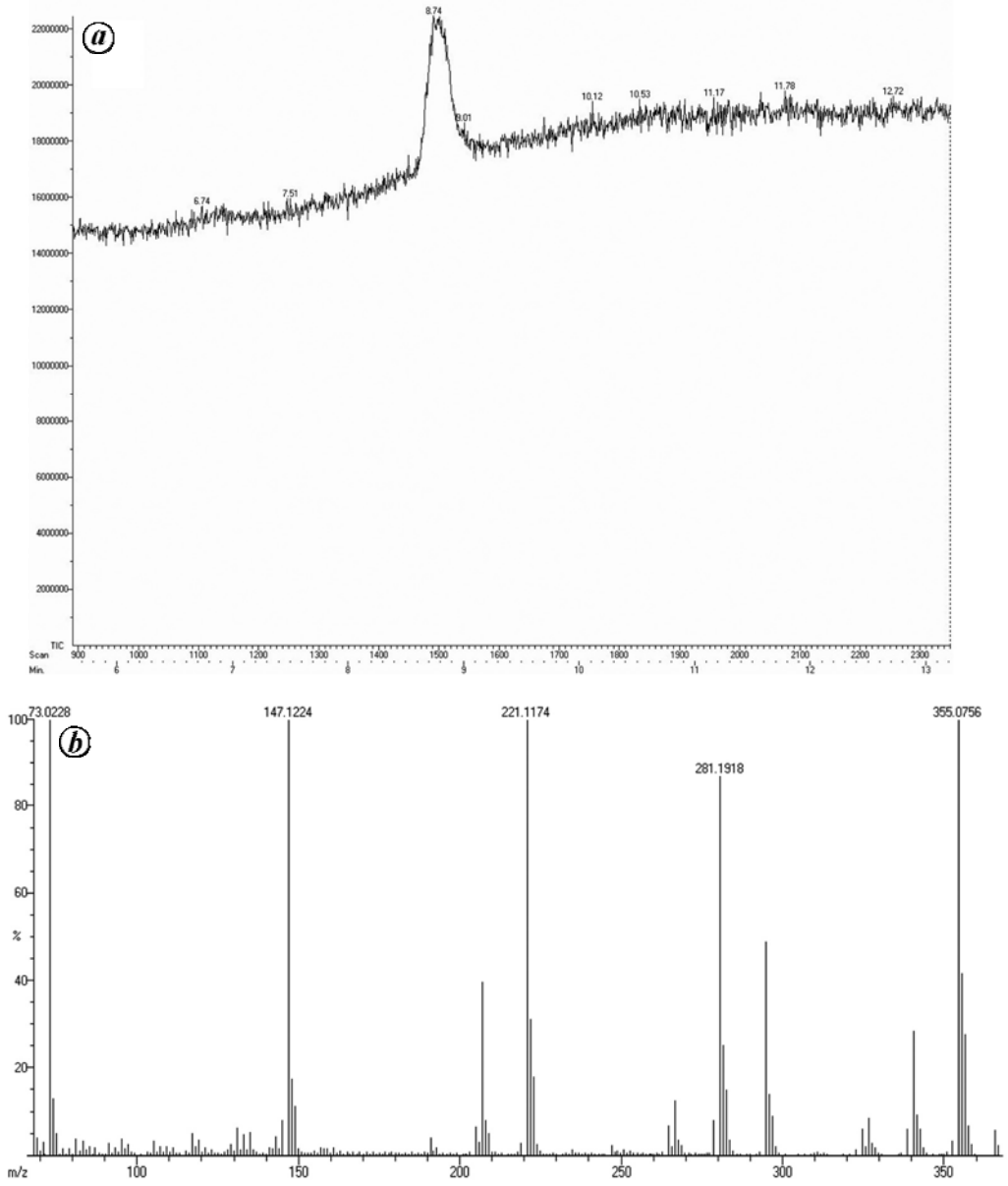

Figure 2. Gas chromatography (***a***) and mass spectrum (***b***) of propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl) isolated from *C. auriculata*.

reason for this is that most of the compounds were developed to target the positively charged active site of PTP 1B, thus leading to low cell permeability[48]. Second, most PTPs are known to share a highly conserved catalytic domain that can be broadly inhibited by non-specific inhibitors[49]. Therefore, the development of novel PTP 1B inhibitors with improved target specificity and bioavailability is still necessary[45]. PTP 1B is known to have several binding sites such as electrostatic, hydrophobic and hydrogen-bonding sites and also several N-terminals favourable for binding to the acidic site[38]. The molecular features of the isolated compound propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl) might facilitate the hydrophobic interaction and the hydroxyl group in propanoic acid, maybe presumed to form hydrogen bonds.

The power of phytochemicals, which initiate a variety of chemical transformations within biological systems, is well known[50–53]. For example, the high level of genistein found in soybean is both a phytoestrogen and antioxidant and has been extensively used to treat conditions affected by estrogen levels in the body[54,55]. Polyphenolic flavanoid in tea, of which epigallocatechin gallate is the major constituent, has anticarcinogenic activity[56,57]. Cinnamon, a common household spice is known to have potential properties to treat diabetes mellitus[58,59]. Although several health benefits exist due to the chemical cocktails present in tea, soya and cinnamon, the actual

application of the chemical reduction power of the myriad of chemicals present in herbs and spices is still in its infancy.

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Model for determining geometry of wetted soil zone under subsurface drip irrigation

D. K. Singh[1], R. M. Singh[2,*] and K. V. R. Rao[3]

[1]Vegetable Production Division,
Indian Institute of Vegetable Research, Varanasi 221 305, India
[2]Department of Farm Engineering, Institute of Agricultural Sciences,
Banaras Hindu University, Varanasi 221 005, India
[3]Irrigation and Drainage Engineering Division,
Central Institute of Agricultural Engineering, Bhopal 462 041, India

A model was developed using dimensional analysis approach for determining geometry of wetted soil zone under point sources of water application with subsurface drip irrigation (SDI). The predicted values of wetted depth and width were compared with those obtained through field experiments conducted in black vertisols at the Central Institute of Agricultural Engineering, Bhopal, India. Experimentation included determination of maximum depths and widths of wetted soil zone after 0.5, 1, 2, 3 and 5 h of water application through SDI laterals placed at 0.05, 0.10, 0.15, 0.20 and 0.25 m depths below the soil surface. The effect of discharge, depths of placement of laterals and duration of water application on wetted width and depth were observed. Statistical analysis revealed no significant difference between predicted and observed values of wetted width and depth. Predictability of model expressed in terms of model efficiency was found to be 88.7% and 93.3% for wetted width and depth respectively. Therefore, the developed model could be used to simulate wetted depth and width under SDI with point source of water application.

Keywords: Dimensional analysis, model efficiency, soil water content, subsurface drip irrigation.

TRADITIONALLY surface method of irrigation being used in major areas in India has low field-level application efficiency of only 35–40% because of huge conveyance and distribution losses[1,2]. Whereas about 2 million hectare (m ha) land under horticulture and vegetable crops is being irrigated through both sprinkler and drip irrigation. It improves[2] irrigation control with smaller frequent application, supplies nutrients to the crop as needed; results in less weed growth and improved crop yields by 50%, water saving by 315% and water-use efficiency by 119%. Drip irrigation may achieve field-level application efficiency of 80–90%, as surface run-off and deep percolation losses are minimized[3–5]. Area under drip irrigation is likely to increase, to realize enhanced water-use efficiency and crop yield as well as sustainable management of irrigation water[6,7]. It has vast potential of 27 m ha in India[8,9]. It is suitable for areas that are presently under

*For correspondence. (e-mail: mandirsingh@rediffmail.com)