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Subhabrata Sengupta [a]; Abhishek Mukherjee [a]; Riddhi Goswami [a]; Srabanti Basu [a]
[a] Department of Biotechnology, Heritage Institute Technology, Calcutta, India

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Hypoglycemic activity of the antioxidant saponarin, characterized as α-glucosidase inhibitor present in *Tinospora cordifolia*

SUBHABRATA SENGUPTA, ABHISHEK MUKHERJEE, RIDDHI GOSWAMI, & SRABANTI BASU

Department of Biotechnology, Heritage Institute Technology, New Chowbaga Road, Anandapur. P.O: East Calcutta Township, Calcutta 700107, India

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Abstract

Tinospora cordifolia, used in anti-diabetic herbal drug preparations, was reported [12] to contain an α-glucosidase inhibitor, characterized as saponarin (apigenin-6-C-glucosyl-7-O-glucoside). The leaf extract had appreciable antioxidant and hydroxyl radical scavenging activities and contained the flavonoid in the range of 32.1 ± 1.5–45.5 ± 3.5 mg/g of dry solid. Saponarin showed mixed competitive inhibition on activities of α-glucosidase and sucrase of different origins. IC_{50}, Ki and ki^{l} values determined were 48 μM, 8 μM and 19.5 μM respectively for intestinal maltase and 35 μM, 6 μM and 13 μM respectively for intestinal sucrase. When given orally to maltose-fed rat, saponarin showed hypoglycemic activity in the range of 20–80 mg/kg compared to 100–200 mg/kg for acarbose as reported [27].

Keywords: *Saponarin,* tinospora cordifolia, *non-insulin dependent diabetic, antioxidant, glucosidase inhibitor, sucrase inhibitor, maltase inhibitor*

Introduction

One of the most direct and beneficial types of therapy for non-insulin dependent diabetes is to control blood glucose level after meal by delaying glucose absorption [1]. Reversible inhibitors of α-glucosidase or maltase, present in intestinal membrane, regulate glucose absorption from small intestine into the blood stream. Many such inhibitor molecules like acarbose, iso-acarbose, cyclodextrins, acarviosine-glucose and hibiscus acid were isolated from natural sources [2–5]. Among those molecules, acarbose, valiolamine and miglitol (Figure 1) were successfully developed as anti-diabetic drugs.

Tinospora cordifolia Miers, belonging to *Meninspermaceae* family of plants, is found throughout tropical India and tropics of Asia, Africa and Australia. Ayurveda system of medical treatment in India prescribes aqueous extract of the plant *Tinospora cordifolia* for the treatment of various diseases like diabetes, debility, hepatitis, dyspepsia, jaundice and other liver diseases [6]. Oral administration of aqueous or alcoholic extract of stem or root was reported to reduce blood glucose level of alloxan diabetic rats, rabbits [7–10]. But the active principle present in *T. cordifolia*, which regulates blood sugar level, was not known. In processes patented by Sengupta et al. [11,12], it was reported that the aqueous extract of *Tinospora cordifolia* contained low molecular weight non-protein amylase inhibitor and an α-glucosidase-sucrase inhibitor molecule present in the extract was purified as apigenin-6-C-glucosyl-7-O-glucoside or saponarin (Figure 1).

We report that leaf extract of *Tinospora cordifolia* has appreciable antioxidant and hydroxyl radical scavenging activities and is a very rich source of saponarin. The molecule has relatively better hypoglycemic

Correspondence: S. Sengupta, Department of Biotechnology, Heritage Institute Technology, New Chowbaga Road, Anandapur. P.O: East Calcutta Township, Calcutta 700107, India. Tel: 91 33 2443 0454 56 57. Fax: 91 33 2443 0455. E-mail: profs_sengupta@yahoo.ca; biotech@heritageit.edu

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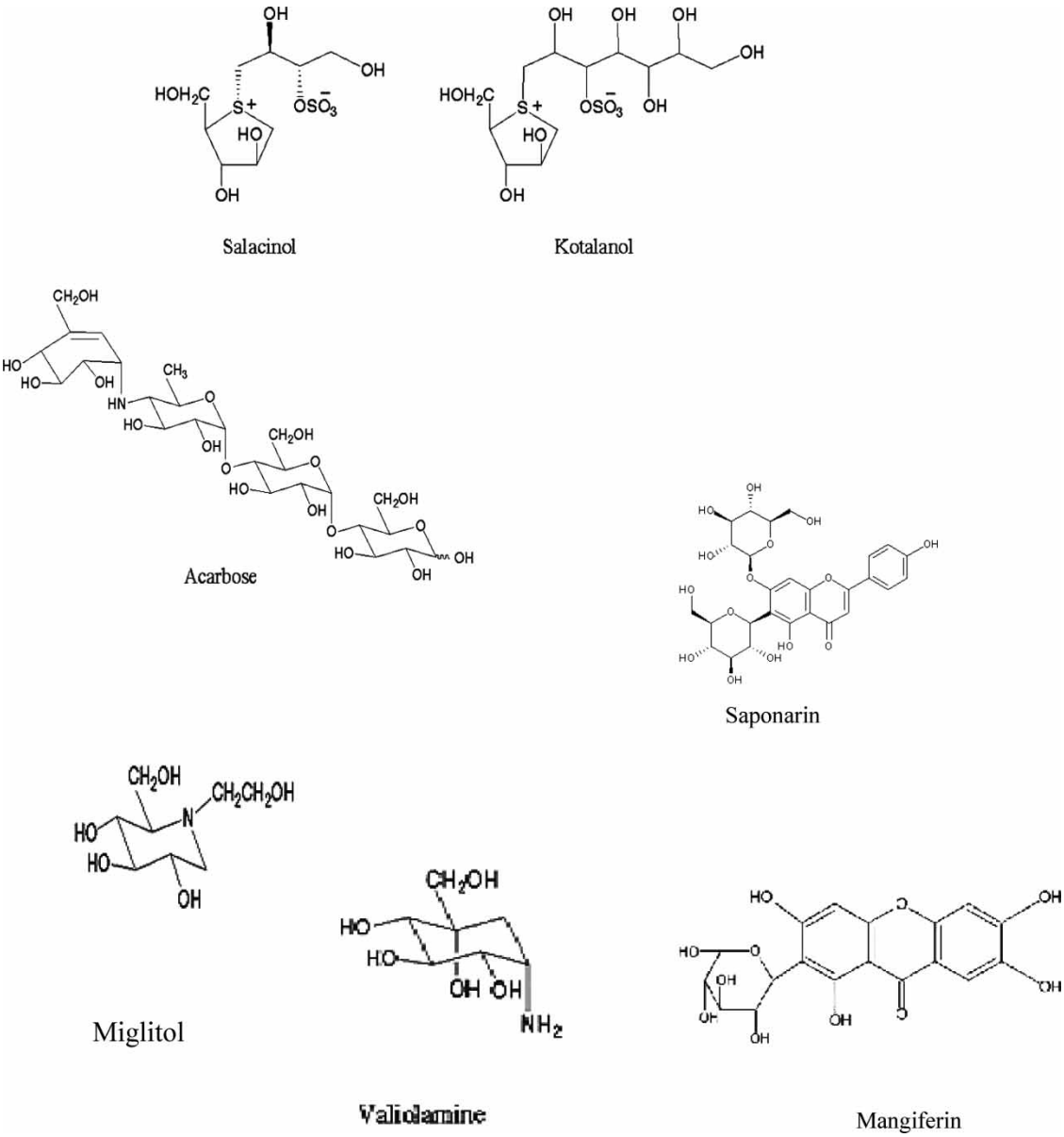

Figure 1. α-glucosidase inhibitors.

activity compared to its inhibitory activities on maltase and sucrase under *in vitro* condition.

Materials and methods

Materials

Saponarin was purchased from Chromadex. USA. Fungal maltase (*Aspergillus niger, M*-125), sucrase (Baker's yeast invertase I- 4504), rat intestinal acetone powder (I-1630), maltose, sucrose and other reagents were obtained from Sigma chemicals, USA.

Methods

Isolation of saponarin from T. cordifolia *leaves*. Saponarin was isolated from dry leaves of *Tinospora cordifolia* by the process as described in patent [12]. In the process, air-dried and milled leaves (150 gm) of *Tinospora cordifolia* were percolated with methanol (3 L) for 30 h at room temperature. The extract was concentrated to a small volume (100 mL) under reduced pressure at 50°C. The entire process was repeated twice to ensure complete extraction. The combined concentrates (300 mL) was further reduced in volume (75 mL), diluted with water (500 mL) with stirring and was washed three times with chloroform (200 mL). The aqueous layer was then filtered and the clear filtrate was five times thoroughly extracted with *n*-butanol (100 mL). The butanol layer was evaporated to dryness at 60°C under reduced pressure.

The brown residue (3.0 gm) was chromatographed over silica gel (60–120 mesh) column (3 × 90 cm).

Elution with 18% (v/v) methanol in chloroform yielded saponarin (200 mg).

Estimation of saponarin content of leaves. Saponarin content of leaves was determined by TLC method as reported by James et al. [13]. In practice, fresh and clean *T.cordifolia* leaves were weighed, air dried at 45–50°C for 24 h and crushed. One gm of dry leaf was homogenized in 5 mL of methanol containing 0.05%(v/v) HCl. The solution was centrifuged at 1000 × g and supernatant was dried at reduced pressure. Dried residue was dissolved in 0.5 mL of the same acidic methanol. The sample (0.1 mL) was quantitatively applied on silica gel plate. Chromatography was done using ascending solvent systems: a) tert- butanol- acetic acid – water (3:1:1, v/v) and b) 5% (v/v) aqueous acetic acid at right angle. Pure saponarin was used as standard. Saponarin was located by viewing the chromatogram under UV light. The spot was removed from the plate and the saponarin was eluted from the silica gel with 5 mL of methanol-water (4: 1, v/v) containing 0.05%(v/v) HCl. Saponarin was quantified by its absorbance ($\xi = 1.75 \times 10^4 M^{-1}cm^{-1}$) at 333 nm [13]. Elution of saponarin was confirmed by its characteristic λmax at 274 and 336 nm [14,15]. Presence of saponarin in *T.cordifolia* leaves was confirmed earlier with reference to m.p, TOF-MS, different NMR analysis etc with reference to authentic sample [12].

Assay of anti-oxidant activity of dried aqueous extract of leaves. Anti-oxidant activity was assayed using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) reagent [16]. Aqueous extract was prepared from fresh leaves and solution containing 5 mg solid/mL was used as source of antioxidant. Ascorbic acid (1 mg/mL) was used as reference antioxidant. The reaction mixture (1 mL) contained aqueous extract of leaf (10–100 μL) and DPPH solution (0.12 mg/mL). Incubation mixture containing no leaf extract was used as control. Mixtures were incubated at 37°C and loss of DPPH colour at 519 nm was observed at different time intervals till 30 min. The efficient concentration (EC_{50}) was estimated as the concentration of substrate that causes 50% loss of the DPPH colour [17]. Result was expressed in terms of ascorbic acid equivalent. Hydroxyl radical scavenging activity of leaf extract was determined by the inhibition of 2-deoxyribose degradation method as described by Patel et al. [18]. Reaction mixtures (1 mL) contained 2.8 mM 2-deoxy ribose, 15 mM phosphate buffer pH 7.4, 0.02 mM $FeCl_3$, 0.1 mM EDTA, 2.8 mM H_2O_2, 0.1 mM ascorbic acid and varied amounts of leaf extract. Reaction mixture was incubated at 37°C for 1 h and was terminated by the addition of 1 mL of 1% (w/v) thio-barbituric acid in 0.05 M NaOH and 1 mL of 2.8% (w/v) TCA solution. The clear reaction mixture was heated for 20 min in boiling water bath. The tubes were cooled to room temperature and colour was measured at 532 nm. Hydroxyl radical scavenging activity (IC_{50} value) was expressed in terms of the amount of dry solid capable of reducing change of color by 50%.

Kinetics of enzyme (maltase and sucrase) inhibition by saponarin. Maltase and sucrase were assayed by estimating the amount of glucose liberated from maltose or sucrose by glucose oxidase–peroxidase method [19]. One unit of enzyme activity was taken as the amount of enzyme which liberates one μmole of glucose from maltose or sucrose under the experimental conditions. Intestinal α-glucosidase and sucrase activities were assayed in 0.1 M phosphate buffer pH 6.8 at 37°C. Fungal maltase was assayed in 0.1 M sodium acetate buffer pH 5.0 at 50°C. Fungal sucrase was assayed in 0.1 M sodium acetate buffer pH 4.5 at 50°C. Saponarin was added at different concentrations in the reaction mixture. The reaction mixture (0.5 mL) contained different amounts of substrate in respective buffer, 5 milli-units of enzyme and 10-80 μM saponarin dissolved in DMSO(2 mg/mL). The reaction mixtures containing enzyme and inhibitor were pre-incubated for 10 min and the reaction was started by addition of the substrate. The incubation was continued for 20 min and reaction was terminated by keeping the same in a boiling water bath for 5 min. Liberated glucose was estimated by GOD-POD reagent (Span Diagnostic limited, India). The concentration of inhibitor required to inhibit 50% of enzyme activity (maltose/sucrose, 3.7 mM) under the above-mentioned conditions was taken as IC_{50} Value. Ki and ki′ values were determined from 1/V vs [I] (Dixon plot) and S/V vs [I] plots respectively. The potency of crude plant extract to inhibit intestinal enzymes was expressed in terms of Inhibitor unit (IU). One IU was considered as the amount, which could inhibit intestinal α-glucosidase by 50% (Maltose, 3.7 mM). Activity of leaf extract was compared with that of stem in terms of inhibitor units.

Table I. Saponarin content of *Tinospora cordifolia* leaves.

T.cordifolia leaves	Saponarin (mg /gm dried aqueous extract)
Newly emerged leaves	45.5 ± 3.5
Mature green leaves	36.2 ± 2.1
Yellow old leaves	32.1 ± 1.5

Methods for the estimation of saponarin have been described in the text. Results represent mean data obtained from three sets of experiments with one set of leaves collected at a time from one plant. On average, 60–65 gm of fresh leave yielded 1 gm of dry solid in aqueous extract and 400 mg in acidic methanolic extract according to protocol used in the study.

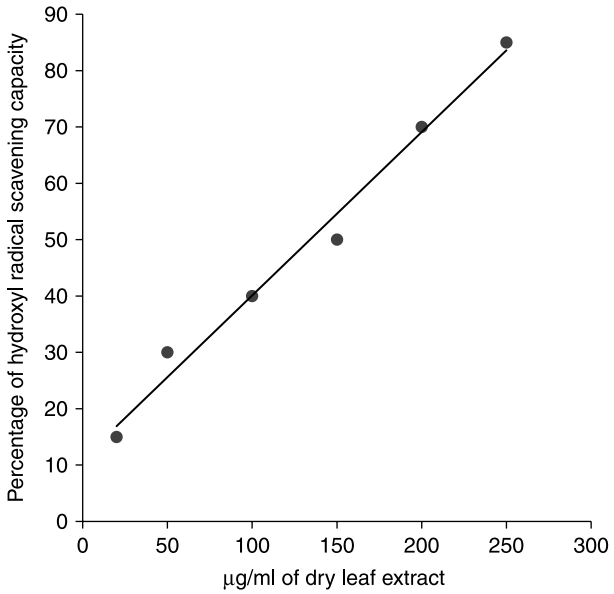

Figure 2. Hydroxyl radical scavenging capacity of dried aqueous extract of *Tinospora cordifolia* leaves. Methods for preparation of dried leaf extract and determination of hydroxyl radical scavenging activity were as described in text.

Determination of hypoglycemic activity of saponarin under in vivo *condition*. Hypoglycemic activity of saponarin was determined in rats according to Matsui et al. [20]. 6-week-old male rats (Sprague–Dawley) were fed a laboratory diet and given water *ad libitum*. All rats were housed for 1 week at 21-22°C under controlled light conditions. Food was withheld for 16 h before the experiment. Saponarin was orally administered to rats (n = 6, 220-225 gms in weight) at a single dose of 20, 40, 80 mg/kg body weight. After 5 min, maltose was fed to rats through the same route at the dose of 2 gm/kg body weight. Blood sample (20 μL) was collected from the tail vein at different time intervals till 2 h. Blood glucose was measured by a disposable glucose sensor (Accu-chek, Roche, Switzerland). Each set of experiment was carried out in triplicate. Animal experiments were done as per protocol laid down by the University of Burdwan, West Bengal, India.

Results and discussion

Flavonoids, which are widely present in plants, had been studied over a long periods of time. Many of them are potent antioxidant compounds. Although a

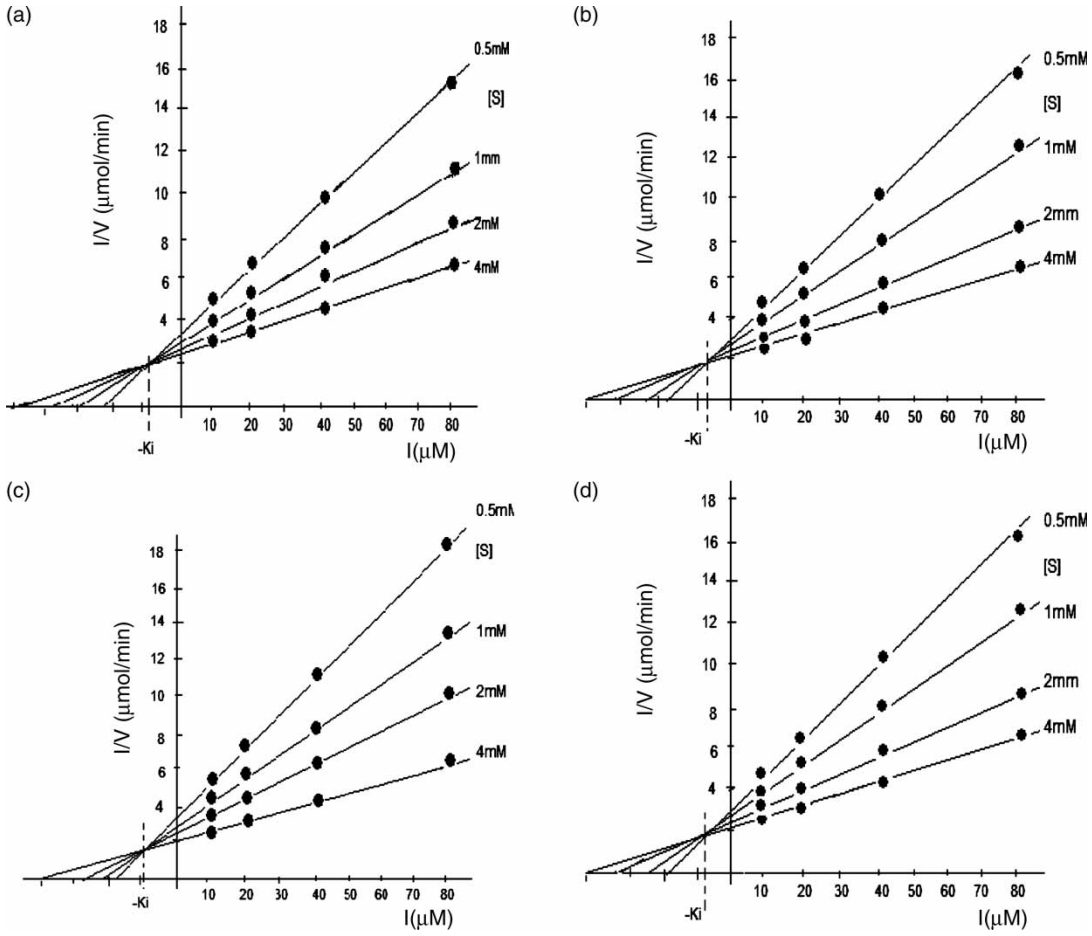

Figure 3. Determination of Ki of saponarin on maltase and sucrase. Dixon plot (1/V vs I) for inhibition of saponarin on activities of intestinal maltase (a), intestinal sucrase (b), fungal maltase (c) and fungal sucrase (d). Saponarin (10–80 μM) was added against different concentrations (0.5–4 mM) of maltose [S]. Enzyme activities were assayed by the method as described in text.

few flavonoids were reported to inhibit enzyme activities [21], but none to inhibit α-glucosidase or sucrase activity.

Saponarin, the antioxidant flavonoid was reported [13,22] to be present appreciably in barley leaf (0.122 mg/gm of fresh leaf). Commercial green barley leaf extract is valued for its saponarin and lutonarin contents [26]. Saponarin is also present in some mosses [23], aloe vera flowers [24], *cucumis sativus* [25] etc. Presence of the molecule in *Tinospora cordifolia* was not known before the activity based purification of the same as α-glucosidase inhibitor activity detected in the plant extract [12] Determination of saponarin content (Table I) of *T.cordifolia* leaves (acidic methanolic extract) revealed that it was present in much higher amounts than that reported to be present in barley leaf [13]. Although saponarin content of leaf did not change seriously with its age, but the amount was optimum in newly emerged leaves. Antioxidant activity of *T.cordifolia* leaf extract when compared to that of ascorbic acid by DPPH method was in the range of 125–135 mg of ascorbic acid equivalent per gram of dried leaf-extract. Normally 60–70 grams of fresh *T.cordifolia* leaves yielded 1 or 0.4 gm of solid in aqueous extract or in acidic methanolic extract respectively. The crude extract had appreciable hydroxyl radical scavenging activity. 50% scavenging (IC_{50}) was achieved at the

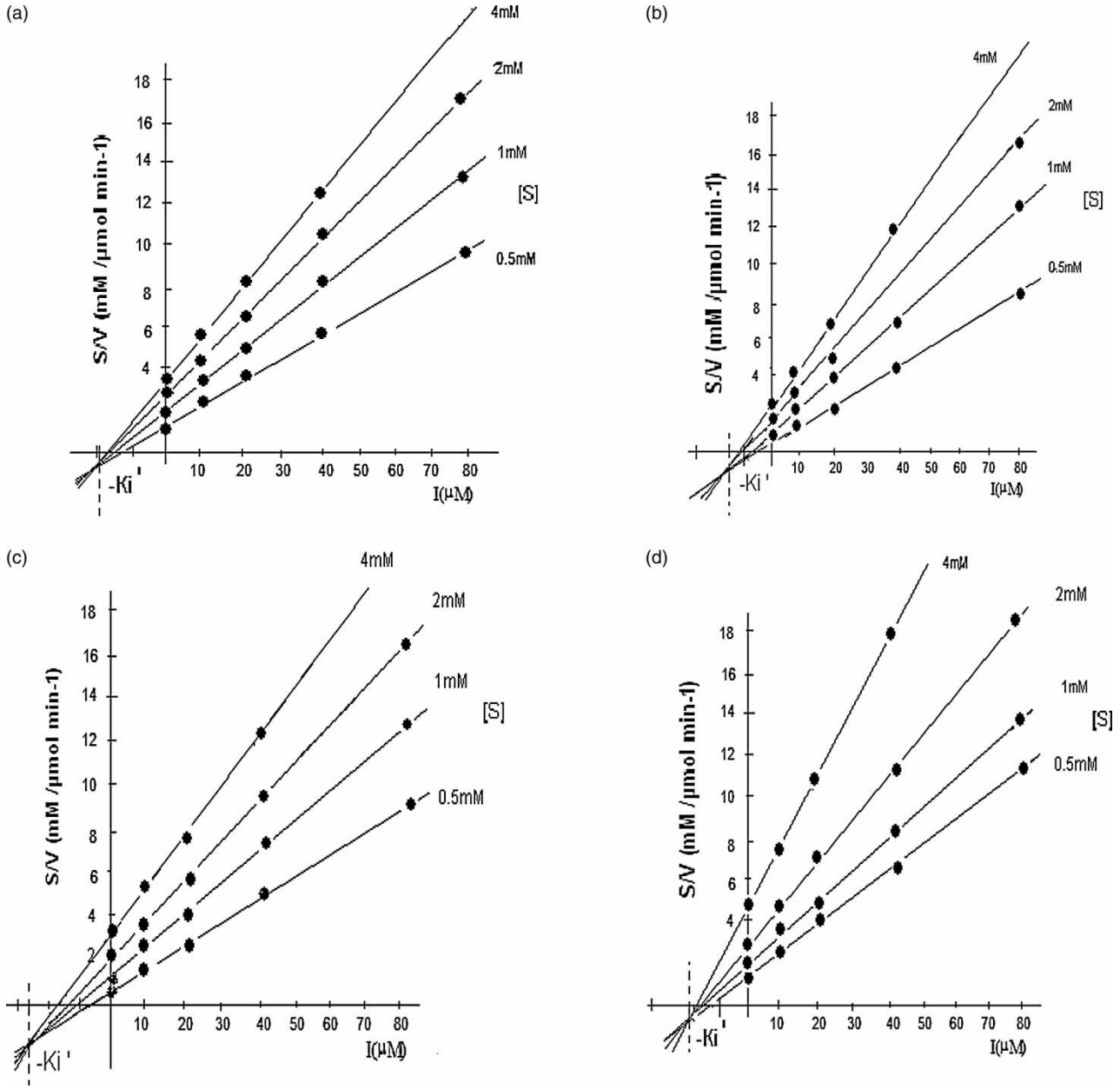

Figure 4. Determination of ki′ of saponarin on maltase and sucrase. S/V vs I plot for inhibition of saponarin on activities of intestinal maltase (a), intestinal sucrase (b), fungal maltase (c) and fungal sucrase (d). Saponarin (10–80 µM) was added against different concentrations (0.5–4 mM) of maltose [S]. Enzyme activities were assayed by the method as described in text.

concentration of approximately 140 μg of dried leaf extract/ml of dried leave extract/ml (Figure 2). Kinetic parameters for maltase and sucrase inhibitions by saponarin were determined from Dixon Plot (Figure 3) and S/V vs [I] Plot (Figure 4). Saponarin appeared to inhibit both enzyme activities in mixed competitive mode (Figure 4)

Table II shows IC_{50} and ki values and ki′ of saponarin on maltase and sucrase of both intestinal and microbial origins. As per data reported by Matsuda et al. [27], IC_{50} and Ki values of saponarin on intestinal maltase (48 μM and 8 μM) were higher than those of salacinol (9.58 μM/0.95 μM), kotanalol (6.58 μM/0.54 μM) and acarbose (2.0 μM/0.18 μM l) and voglibose (1.19 μM/0.11 μM). Similarly, IC_{50} and Ki of saponarin on intestinal sucrase (35 μM/6 μM) were also higher in comparison to those for salacinol (2.51 μM/0.95 μM), kotanalol (1.37 μM/0.42 μM), acarbose (1.7 μM/0.57 μM), and voglibose (0.22 μM/0.067 μM). Thus it appears that saponarin is less potent inhibitor under *in vitro* conditions than those mentioned above.

In the assessment of crude aqueous extracts from stem and leaves of *T.cordifolia*, inhibitor unit (IU) of the crude extracts were assumed to be the amount which could inhibit 50% of intestinal maltase activity as per assay protocol described in the text. The value coincides with IC_{50} value (48 μM) of pure saponarin on intestinal maltase. It was estimated that IU present per gm of dried aqueous extracts of leaf and stem were about 3000 ± 30 and 1200 ± 50 IU/gm respectively. However the same value, when calculated on the basis of saponarin content (Table I), appears to be 1500 (app) for leaves. The apparent higher value of the crude indicates that that leaves might contain, either other inhibitors or some factor(s) with synergistically stimulate activity of saponarin.

Blood glucose lowering activity of saponarin on maltose fed rat, was found to be encouraging (Figure 5) compared to its *in vitro* activity on α- glucosidase. The effective dose which could lower the blood glucose level was in the range of 20–80 mg/kg body weight. This range was lower compared to acarbose which was reported to be of 100–200 mg/kg [27].

Table II. Saponarin inhibitor constant (μM) for maltases and sucrases of different origins.

Enzymes	IC_{50}	Ki	Ki′
Maltase (Rat intestine)	48 ± 3.55	8 ± 0.15	19.5 ± 0.20
Sucrase (Rat intestine)	35 ± 1.95	6 ± 1.33	13 ± 0.13
Maltase (*Aspergillus niger*)	55 ± 3.53	9 ± 0.085	27 ± 0.195
Invertase (Baker's yeast)	48 ± 1.32	8 ± 0.072	20 ± 0.089

Values were determined from Dixon plots (Figure 3) and S/V vs I plot (Figure 4). Mean values presented were obtained from three similar sets of data obtained separately.

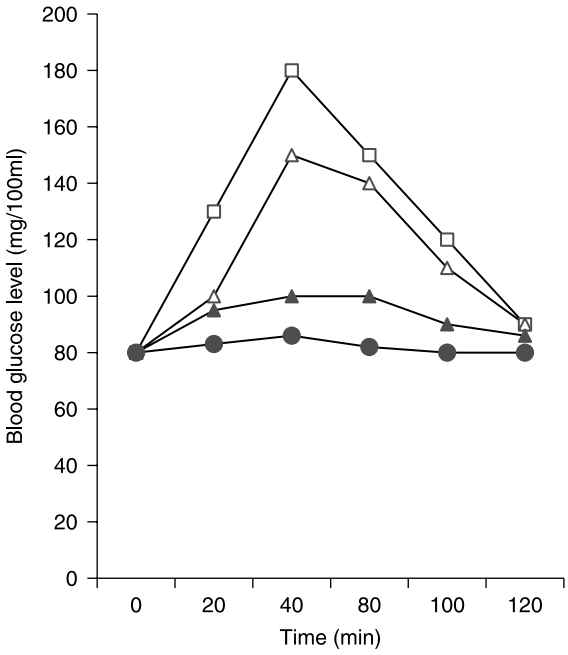

Figure 5. Hypoglycemic activity of saponarin. Saponarin (20–80 mg/kg body weight) was orally administered to the SD rats. Five minutes later, maltose (2 gm/kg body weight) was fed orally to the rats and the effect on blood glucose was recorded till 120 min. Controls were fed orally with equal volume of physiological normal saline. Results presented were mean values obtained from three sets of identical experiments. -□- Control; -△- 20 mg/kg; -▲- 40 mg/kg; -●- 80 mg/kg.

Numbers of potential molecules with α-glucosidase inhibitor activities have been isolated from various sources. Many of the compounds, found use as anti-diabetic drug, contain either sugar analogue molecule or sugar attached to different aglycon molecules. Acarbose (pseudo tetra-saccharide), miglitol (nitrogen containing sugar) and voglibose(aza-sugar) obtained from microbial sources have already found use as anti-diabetic drugs [1]. Similar inhibitors from plant sources like mangiferin with xanthinone aglycon and salacinol or kotalanol with thiosugar moieties have received attention for the development of anti-diabetic drug [27]. Although a large number of acylated flavone C-glycosides [21] have been characterized in plant, but none of them was reported to be a α- glucosidase inhibitor. Tadera et al. [28] extensively studied inhibitory activity of flavonoids (other than saponarin) on α-glucosidase activity and reported relatively poor inhibition of rat intestinal enzymes by the flavonoids. A few flavonoids were reported to inhibit NADH-oxidase or xanthine-oxidase activities [21]. Antioxidant molecule with potential enzyme inhibitory activity is very encouraging. Mangiferin, a glucosidase inhibitor, was reported to have antioxidant activity [29]. Recently it was reported that catechin analogues with alkyl side chains were potential antioxidant molecules with α-glucosidase inhibitor activities [30].

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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