Rhizobium rhizogenes -elicited hairy root cultures of Sida acuta Burm.f.: extracts attenuate streptozotocin-induced hyperglycaemic effects in Wistar rats

Somanatha Jena
Utkal University
Kedar K. Rout
Odisha University of Agriculture and Technology
Sagar K. Mishra
Utkal University
Pradeep Kumar Chand
pkchanduubot@rediffmail.com
Utkal University

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Abstract

Genetically transformed root (TR) clones (rhizoclones) were developed of an ethno-medicinal plant species, *Sida acuta* mediated by *Rhizobium rhizogenes*. Various key factors (bacterium × plant × culture environment) were optimized for the transformation event *per se* and hairy root growth. RT-PCR amplification demonstrated transcription activity of opine biosynthetic genes and opine production was confirmed in rhizoclones. Anti-hyperglycemic activity of the methanolic/aqueous extracts of a selected *rolB* ORF-positive fast-proliferating TR clone was investigated *vis-á-vis* natural roots (NR) employing streptozotocin-induced Wistar diabetic rats. Compared to root extracts of naturally occurring donor plants, aqueous extract of the TR clone caused a more pronounced reducing effect on fasting blood glucose level and normalization of biochemical indices (serum and urine) as hepatic/renal functional markers, Metabolic attenuation was evident in respect of insulin and hepatic glycogen content as well as biochemical levels of blood serum, liver-specific enzymes and urine-specific biochemicals. Aqueous TR extracts, and the purified β-sitosterol isolate from such extracts, at optimal levels, demonstrated anti-hyperglycemic activity, to a considerable extent, which was comparable with that of the commercial drug glibenclamide towards restitution of biochemical homeostasis in the diabetic rats. Our finding on fast-renewable transformed root culture extract, capable of a noteworthy glycemic control in diabetic rats, ushers a possibility of designing and developing potent bioactive anti-diabetic drug(s) to be implicated in human healthcare.

Key message

Therapeutically effective anti-hyperglycemic activity aptly demonstrated by extracts of optimally established transformed root cultures of *Sida acuta* could help develop complementary medicines for controlling diabetes.

Introduction

Healthy animals are able to maintain a controlled steady state of normoglycemia owing to a balanced and coordinated regulation of carbohydrates, protein and fat metabolism. Lapses at checkpoints of glucose metabolic pathways through glycolysis, gluconeogenesis, glycogenesis or glycogenolysis and over-exercising of the body system in attenuating the incongruity often cause a disruption of the endocrine control (Mul et al., 2015). Such a disorder is associated with complete or relative insufficiency of insulin secretion and/or action, a situation that leads to chronic hyperglycemia, the hallmark of diabetes. Insulin-dependent *diabetes mellitus* (Type 1 diabetes) is an auto-immune disorder caused by destruction of insulin-producing pancreatic β-cells by T cells of the immune system, whereas Type 2 *diabetes mellitus* is a more complex metabolic disorder resulting from peripheral insulin resistance and relative insulin inadequacy/deficiency (Burrack et al., 2017). Therapies in current practice for controlling diabetes include administration of insulin or various synthetic hypoglycaemic drugs such as sulfonylureas, biguanides, thiazolidinedione and glinides. Unfortunately, in several cases these drugs have either failed to fully restore glycaemic control or were unable to correct the course of diabetic complications. Besides, they have limited applications being associated with adverse health effects often caused by long-term intake (Chaudhury et al., 2017). This has tempted researchers to shift their interest to safer and more effective natural drugs from plants (Kolhe and Rachh 2018). Anti-hyperglycemic activity of medicinal plants is mainly due to their ability to restore the function of pancreatic tissues causing an increase in insulin output or inhibit the intestinal absorption of glucose or facilitation of metabolites in insulin dependent processes (Kooti et al., 2016).

Plants naturally produce a diverse array of secondary (specialized) metabolites such as alkaloids, terpenoids, flavonoids, quinones, sterols, saponins, etc. An insight into their diversity, classification, biosynthesis, biological properties and their multidimensional prospective in plant life have been aptly reviewed (Ahmed et al., 2017). These plant-derived natural products are implicated in various therapeutic activities because of their pharmaceutical relevance and exploited as precursors to new innovative drug development targeting diseases, such as cancer and diabetes (Newman and Cragg 2016; Thomford et al., 2018; Buyel 2018; Agarwal 2019). The scope of harnessing *in vitro* plant cell and tissue culture systems as “green cell factory” for beneficial products has been considerably expanded over the last decade (Marchev et al., 2020). Though a few anti-diabetic plants have been identified, the quest for new effective species, especially their key bioactive phytocompound(s) responsible for alleviating hyperglycaemic effects would certainly be rewarding.

Sida acuta Burm.f. (Family: Malvaceae) is a woody perennial undershrub with a tap root system possessing ethno-medicinal significance (Tcheghebe et al., 2017). The plant species is native to Mexico and Central America and is also spread throughout the tropics and subtropics including the hotter parts of India. The plant species is known to contain several bioactive compounds, such as various alkaloids, flavonoids, phytosterols, etc., namely betaine, β-phenethylamine, β-sitosterol, cryptolepine, ephedrine, evofolin, quindolinone, scopoletine, vasicine, vasicinone, vomifoliol, etc. in aerial parts as well as roots, which are implicated in human healthcare (Karou et al., 2007; Jena 2016). Abat et al. (2017) have reviewed the ethno-medicinal, phytochemical and ethno-pharmacological aspects of four different plant species of Family: Malvaceae including *Sida acuta*, the one which reportedly possessed a range of ethno-pharmacological activities, such as anti-inflammatory, anti-ulcer, anti-bacterial, anti-viral, anti-fungal, anti-plasmodial, anti-pyretic, analgesic, hepato-protective, neuro-protective, etc. (Abat et al., 2017). Unfortunately, seeds of this plant germinate only during the rainy season and the plants are naturally available between July and December. This restricts the continuous availability of its valuable phyto-constituents to the benefit of mankind especially in off-season, precisely during the summer. Besides, chemotypic variations between plants owing to environmental factors, particularly edaphic factors including soil microflora affecting plant growth and metabolism, which are prevalent at different geographical locations pose a constraint on obtaining the key secondary compounds from their roots in desirably uniform quantity and quality. To cater to the growing demand for incessant accessibility to root parts of *S. acuta* throughout the year for preparation of herbal drugs, a

biotechnological intervention was worth exploring aimed at applying a reproducible technique for *in vitro* root tissue proliferation as a viable alternative.

In the past few decades, various biotechnological approaches such as de-differentiated cell suspension cultures raised from plant tissues have been exploited to enhance the quantity of the bioactive phytoconstituents. However, success in this direction has been inadequate and restricted owing to a very low-level accumulation of secondary metabolites, unwanted somaclonal variation causing genetic and biochemical instability of cells, and requirement of morphogenic differentiation for production of the organ-specific bioactive compound(s). In this context, a considerable interest has been addressed to *in vitro* transformed root cultures which are, genetically and biochemically more stable compared to dedifferentiated cell cultures. The pathogenic-molecular interaction between a symbiotic Gram-negative soil bacterium *Agrobacterium rhizogenes* (renamed as *Rhizobium rhizogenes*) and plant cells results in an inter-kingdom conjugational transfer of T-DNA, mediated through a cascade of signal transduction events, from the large Ri plasmid of the infecting *R. rhizogenes*, carrying characteristic gene sequences which code for auxin biosynthesis (*iaa*M, *iaa*H) and trigger root induction (*rol*A-D). This is followed by the integration of T-DNA borne genes into the host root genome and finally their over-expression resulting in transformed roots (TR) endowed with auxin-autotrophy. These TR are characterized by high growth rate with profuse incidence of lateral branching, copious hairiness, negative geotropism, phytohormone autotrophy, genetic stability of the cells and higher biosynthetic capacity. *In vitro* cultures of TR (often referred to as 'hairy roots') are considered as a sustainable source of raw materials for pharmaceutical industry to manufacture selective drug types of therapeutic relevance (Srivastava et al., 2017). Hairy root cultures (HRC) are viewed as a suitable platform for optimized production of important bioactive compounds and tailor-made molecules, such as specialized metabolites, recombinant proteins, etc. (Gutierrez-Valdes et al., 2020; Li and Wang 2021; Roy 2021). Over the years, HRCs have been exploited as a biotechnological tool for production of value-added products using advanced techniques of molecular biology (Shi et al., 2021). Our focus of the present investigation was on evaluating the extracts of a selected high biomass-generating TR clone *vis-à-vis* natural roots of *Sida acuta* and the efficacy of purified β-sitosterol isolate, as one of the key bioactive principles in such extracts, for attenuating diabetic effects in induced experimental rat systems.

Materials and methods

Plant transformation and establishment of rhizoclones

Genetic transformation of *S. acuta* (Voucher Specimen No. 7298/24.4.09; Regional Plant Resource Centre, Department of Forests & Environment, Government of Odisha) via co-cultivation of explants from outdoor-grown plants or *in vitro*-multiplied shoot cultures with *Rhizobium* (*Agrobacterium) rhizogenes* strains followed by establishment of transformed root clones (rhizoclones) were according to the published procedure (Jena et al., 2015).

Evaluation of key factors influencing transformation

Relevant bacterial × plant-specific parameters were assessed for which each experiment included ten replicate jars each containing four explants. Experiments were repeated at least three times each. The parameters included the source of explants (harvested from naturally grown garden plants or *in vitro*-grown axenic cultures), type of explants (internode or leaf), bacterial strains (A4, A4T, LBA9402), bacterial growth phase (OD at 660 nm [A_{660}] – 0.4, 0.6, 0.8, 1.0), acetosyringone concentrations (50, 100, 150, 200 µM) for bacterial culture pre-treatment, methods of wounding of explants (injection, immersion after pricking & immersion without pricking), and the co-cultivation period (3, 4, 5, 6, 7, 8 d). Acetosyringone (Sigma, USA; 100 mM stock solution in DMSO) was added to the culture medium (used for growing overnight bacterial suspension) 1h prior to infection. Following co-cultivation, explants were transferred to agar-solidified MS0 media supplemented with a bactericidal antibiotic, cefotaxime (Hi-Media Laboratories Ltd., Mumbai, India), at a range of concentrations (250, 500, 750, 1000 µg ml^{-1}). Control cultures, containing similar explants but wounded with the hypodermic needle without bacteria, were maintained under similar light and temperature conditions as for inoculated cultures. Transformation efficiency was determined as the percentage of explants, treated with *R. rhizogenes*, capable of forming roots.

Determination of root growth in transformed rhizoclones

After two successive sub-cultures a single root segment (*ca.* 4–5 cm long) was excised from each of the transformed rhizoclone and transferred onto MS medium devoid of growth regulator supplement (MS0) and kept inside the culture room (25 ± 1 ^{o}C) under diffused light with a low PFD (10–15 µmol m^{-2} s^{-1}). The initial fresh weight (IFW; *ca.* 100 mg) was recorded at the time of inoculation. Root length and biomass of hairy roots were recorded at every 10-day interval up to 50 days. These roots were taken out from the culture vessel, washed with double-distilled water, blotted dry and weighed to determine the final fresh weight (FFW). Root growth index (RGI) in terms of biomass gain over a culture period was calculated as (FFW-IFW) / IFW.

Evaluation of factors influencing growth of hairy roots in culture

Several factors affecting the growth and proliferation of *in vitro* raised transformed hairy roots were evaluated. This included light intensity (0 [dark], 10, 20, 30, 40, 50 µmol m^{-2} s^{-1} photon flux density [PFD]), culture matrix (agar gel concentration 0 [liquid], 0.4, 0.6, 0.8, 1.0 g l^{-l}) and growth regulator

(auxin) concentration (IAA/IBA/NAA; 0.1, 0.25, 0.5, 0.75, 1.0 mg l^{-1}). Assessments were made with respect to root length (cm) and root growth index (RGI).

Molecular analysis of rhizoclones for detection of T_L-DNA/T_R-DNA genes and opine synthase expression

Five fast-proliferating TR clones (rhizoclones) incited by *R. rhizogenes* A4T strain (ST2D1, ST3E3, ST5F4, ST7G6 and ST8H7) were tested for the presence of pRi T_L-DNA– specific *rol*B ORF in rhizoclonal DNA by PCR amplification (Swain et al., 2012) To detect transcription of *Agrobacterium* T_R-DNA borne agropine synthase gene (*ags*) and manopine synthase gene (*mas2*), total RNA were isolated from selected fast-growing root somaclones of *S. acuta* using RNA isolation kit (RNeasy Mini Kit, Cat. No 74104, Qiagen, USA). Reverse Transcription Polymerase chain reaction (RT-PCR) was conducted using the Quanti Tect Reverse Transcription kit (Qiagen, USA, Cat. No 205310) following the manufacturer's instructions. The primers for amplifying *mas2* (FP 5´- GCG CAT CCC GAG GCG ATG – 3´; RP 5´ - AGG TCT GGC GAT CGC GAG GA-3´) and *ags* (FP 5´ - CGG AAA TTG TGG CTC GTT GTG GAC – 3´; RP 5´- AAT CGT TCA GAG AGC GTC CGA AGT T- 3´) genes are expected to give cDNA products of 512 bp or 1.6 kb product, respectively. Total RNA isolated from natural roots of the naturally grown plant (without *R. rhizogenes* treatment) were subjected to RT-PCR under similar conditions to serve as a negative control. The housekeeping gene actin was used as a positive control (loading control) to indicate the amount of starter RNA reflecting the level of expression. The primer sequences were designed from tobacco (*Nicotiana tabacum)* actin gene (GenBank accession no. X63603) as FP 5'- CGC GAA AAG ATG ACT CAA ATC-3' and RP 5'-AGA TCC TTT CTG ATA TCC ACG-3', which would be expected to generate a 533-bp product of cDNA. The RT-PCR products were electrophoresed on 1.5% agarose gel, detected by ethidium bromide staining and photographed using a Gel Documentation unit (Bio-Rad, USA). The presence of opines in the transformed roots was analyzed by paper electrophoresis employing the tested method (Morgan et al.,1987).

Studies on anti-hyperglycemic activity

Preparation of root extracts

Freshly collected roots from *in vivo* donor plant were washed, air-dried, powdered and extracted (48h) with methanol (Merck, Mumbai, India) or double-distilled water. Of several rhizoclones resulted via genetic transformation, a fast-proliferating rhizoclone ST5F4, incited by *R.rhizogenes* A4T strain, was selected for investigating anti-hyperglycemic activity *vis-à-vis* natural roots (NR). ST5F4-TR were similarly extracted with methanol/water as for NR. The extracts were filtered separately and concentrated in a rotary evaporator. Methanolic extract (MEt) and aqueous extracts (AEt) of TR/NR were then separately suspended in vehicle solution (saline plus carboxymethyl cellulose [CMC]) to be used in experiments for evaluating anti-hyperglycaemic activity.

Purification and quantification of β-Sitosterol

β-sitosterol (β-STL) was isolated from dichloromethane–methanol extract of NR/TR (rhizoclone ST5F4) by column chromatography and recrystallized from methanol. Preparation of sample and standard solution, chromatography & HPTLC scanning, validation methods and quantification of β-STL was as per Rout et al. (2014). The purified compound was characterized by ^{1}H-NMR and ^{13}C-NMR analysis (JEOL, 400 MH_z, Japan). The obtained spectral value of β-STL was further confirmed by comparing that with published literature (Pateh et al., 2008). β-STL content was determined by normal-phase HPTLC using CAMAG TLC plate heater; scanning performed using a CAMAG TLC Scanner 3 (winCATS software version 1.4.2) in absorption–reflection scan mode. The scanner slit dimension was set at 5.0 × 0.45 mm (micro) with 100 µm per step data resolution and 20 mm s^{-1} scanning speed. β-Sitosterol was quantified at its maximum absorbance of wavelength of 414 nm, and the quantity was determined using linear calibration via peak areas.

Experimental animals

Healthy adult Wistar albino rats of both sexes (*ca*. 4-month old, 200–220 g each) were used as test animals for acute toxicity and anti-hyperglycemic studies. The animals were housed in clean polypropylene cages under the standard vivarium conditions (12 h photoperiod, 25 ± 2^{o}C, 35–60% R.H.) with free access to balanced pellet diet (Hindustan Level Ltd, Bangalore, India) and water *ad libitum*.

Acute toxicity study

To determine the safety of extracts administered on long term basis overnight-fasted animals were fed once daily with TR/NR extracts (500–4000 mg/kg body weight) or β-STL (100–200 mg/kg) for 28 days. There was neither mortality nor gross behavioural changes in animals treated with TR/NR extracts (methanolic/aqueous) or purified β-STL of *S. acuta* up to a level of 2000 mg/kg b.wt. or 100 mg/kg body weight respectively. One-tenth of LD_{50} value of root extracts/β-STL (4000 mg/kg b.wt./200 mg/kg) were the screening dose of crude root extracts/β-STL for experiments.

Test of innate hypoglycaemic effect of root extracts on normo-glycaemic healthy rats

The rats were grouped randomly (six each). Group-I (Normal Control, NC) were fed daily with saline plus CMC (2 ml/kg b.wt.). Group-II rats were treated with the commercial anti-diabetic drug glibenclamide (Daonil, Safoni India Ltd.; 10 mg/kg b. wt.) (GLBC) to serve as a positive standard. Root extracts (AEt/MEt, 400 mg/kg b.wt.) were administered to remaining groups; TR to Groups III & V rats while NR to Group IV& VI. Group-VII animals were given purified β-STL (20 mg/kg. b.wt.). Blood samples were collected by tail veinipuncture and fasting blood glucose (FBG) levels measured (Clever Chek TD-4230, TailDoc Technology Corporation, Taiwan) at 0 h, 2h, 4 h, and 8 h for all treatments.

Studies on streptozotocin (STZ) induced diabetic rats

Induction of diabetes

Fasted (24 h) rats received a single intra-peritoneal injection (65 mg/kg b.wt.) of 2-deoxy-2-(3-methyl-3-nitrosoureido)-D-glucopyranose (Streptozotocin; STZ, Sigma Chemical Co., St. Louis, Mo, USA), freshly dissolved in acidified saline with 50 mM sodium citrate buffer (pH 4.5). After 72 h of STZ administration, rats showing FBG level > 220 mg/dl were considered diabetic.

Oral Glucose Tolerance Test (OGTT)

OGTT was conducted in which Group-II rats (glucose control) and STZ-induced T2 diabetic rats (Diabetic Control, DC; Group-III) were fed with glucose (2 g/kg b.wt.). Diabetic rats of remaining groups (Groups IV-IX) were fed with glucose 30 min after having received respective treatments (GLBC/TR/NR/β-STL). Postprandial blood glucose (PBG) levels were measured at 30, 60, 90 and 120 min after glucose loading.

Test of anti-hyperglycemic activity

Randomized grouping of rats and treatments were similar to that observed for glucose-loaded healthy rats, except that Group-II rats (Diabetic Control, DC) were treated with STZ in lieu of glucose. Diabetic animals of all remaining groups had been administered with STZ before receiving root extract/ β-STL treatments. Root (TR/NR) extracts (400 mg/kg b. wt.), purified β-STL isolate (10/20 mg/kg b. wt.) or GLBC (10 mg/kg b. wt.) were administered orally between 12:00–02:00 P.M. on daily basis for a period of 0–40 days. FBG levels were measured at 0th, 10th, 20th, 30th and 40th day.

Determination of serum/urine biochemicals

Weight of individual animals was measured gravimetrically between 09:00–11:00 A.M. on every 10th day up to 40 days. Blood samples were collected on the 40th day (9:00–11:00 A.M) by cardiac puncture and serum separated for biochemical estimations of serum glutamate pyruvate transaminase (SGPT, E.C.2.6.1.2.) and serum glutamate oxaloacetate transaminase (SGOT, E.C.2.6.1.1.) (Reitman and Frankel 1957). Alkaline phosphatase (ALP, E.C. 3.1.3.1) was estimated using commercially available kit (Crest Biosystems, India) (Kind and King 1954). Serum triglycerides, total cholesterol, creatinine, total protein, albumin & globulin content were determined by published protocols (Rice 1970; Parekh and Jung 1970; Owen et al., 1954; Reinhold 1953). Liver glycogen was determined in sliced liver samples flash-frozen in liquid nitrogen (Carroll et al., 1956). Serum insulin was measured using RIA assay kit for rats (Linco Research, Inc., USA). Blood urea nitrogen (BUN) was assayed (Wybenga et al., 1971) using commercial diagnostic kit (Sigma Diagnostics [I] Pvt. Ltd, Baroda, India). Urine biochemical parameters, such as urine sugar, urea, uric acid, creatinine and albumin were estimated by commercial kit-based methods (Miller 1959; Fawcett and Scott 1960; Caraway 1955; Tietz 1987).

Statistical analysis

Data were analyzed by one-way ANOVA and individual comparisons performed by Duncan's Multiple Range Test (DMRT) (Duncan 1957) with levels of significance at $p \leq 0.05$.

Results

Optimization of bacterium × plant transformation factors

Explants obtained from outdoor plants were more susceptible to the infecting *Rhizobium rhizogenes* than those from *in vitro*-grown cultures in respect of rooting response. Root development occurred in 13–16 days along internode explants from outdoor-grown plants, whereas 15–19 days were required for rhizogenesis in that from the axenic source. Of the two types of explants used in transformation experiments, internodes were more responsive compared to leaves in terms of an early root emergence, percentage rhizogenesis and number of putative transformed roots per explant (Table 1).

Table 1 Hairy root induction following co-cultivation of explants from *in vivo* plants/*in vitro* regenerated axenic shoot cultures with different *R.rhizogenes* strains

R. rhizogenes strain	Days for root emergence		% Root emergence		Roots per explant	
	OE	IE	OE	IE	OE	IE
Internode segments						
A4 (pRi A4)	18-22	22-26	21.65^{b}	13.59^{b}	3.9^{b}	1.7^{b}
A4T (pRi A4)	13-16	15-19	36.98^{a}	24.77^{a}	5.9^{a}	4.1^{a}
LBA 9402(pRi 1855)	21-25	25-30	11.87^{c}	6.84^{c}	2.4^{c}	1.2^{c}
Leaf segments						
A4 (pRi A4)	22-24	26-28	16.45^{b}	8.39^{b}	2.2^{b}	1.8^{b}
A4T (pRi A4)	18-20	22-24	21.08^{a}	15.77^{a}	3.9^{a}	2.6^{a}
LBA9402(pRi 1855)	25-30	32-35	5.32^{c}	3.4^{c}	1.6^{c}	1.1^{c}

OE - outdoor explants, IE - in vitro explants, Data pooled from a total of 3 separate co-cultivation experiments each comprising of 10 replicate jars containing 1-4 explants per jar.Mean values within column followed by different alphabets in superscript are significantly different (p < 0.05; Duncan's New Multiple Range Test)

A4T strain carrying pRiA4 was the most virulent, adjudicated on the basis of the minimum period required for root emergence (13–16 days for *in vivo* internodes; 18–20 days for *in vivo* leaves). Overall, the extent of infectivity varied with strains in a descending order as follows: A4T (pRiA4) → A4 (pRiA4) → LBA9402 (pRi1855). In order to determine the right stage of bacterial growth for high efficiency transformation, four different growth phases were examined. At late log phase corresponding to A_{660} (OD at 660 nm) ≅ 0.6 maximum transformation occurred with A4T (36.98%) followed by A4 (21.65%) and LBA9402 (11.87%) (Fig. 1a). Extensive tissue damage occurred at OD values > 1.0 because of the bacterial overgrowth.

Of a range of concentrations of acetosyringone (AS) evaluated (50–200 µM), 100 µM was the most effective with respect to transformation frequency, irrespective of the strain employed (Fig. 1b). AS concentrations below or beyond the threshold were less effective.

Among the three different methods adopted for infecting the explants with bacteria, the one in which explants were pre-pricked prior to immersion in bacterial suspension was the most suitable and gave the maximum number of transformants (*ca.* 36.98% with A4T), followed by direct inoculation (*ca.* 28.34% with A4T) (Table 2). Transformation percentages were negligible with unwounded explants regardless of the explant type/source or the bacterial strain employed.

Table 2
Effect of explant infection methods on *R.rhizogenes*-mediated genetic transformation

R.rhizogenes strains	Immersion without pricking				Injection				Immersion after pricking			
	% Root emergence		Roots per explant		% Root emergence		Roots per explant		% Root emergence		Roots per explant	
	OE	IE	OE	IE	OE	IE	OE	IE	OE	IE	OE	IE
A4 (pRi A4)	9.44^{b}	7.49^{b}	1.4^{b}	1.2^{a}	16.88^{b}	10.66^{b}	3.3^{b}	1.5^{b}	21.65^{b}	13.59^{b}	3.9^{b}	1.7^{b}
A4T (pRi A4)	12.54^{a}	9.77^{a}	1.9^{a}	1.2^{a}	28.34^{a}	21.87^{a}	4.2^{a}	3.9^{a}	36.98^{a}	24.77^{a}	5.9^{a}	4.1^{a}
LBA9402 (pRi 1855)	4.77^{c}	0	1.2^{c}	0	10.64^{c}	8.88^{c}	2.1^{c}	0.9^{c}	11.87^{c}	6.84^{c}	2.4^{c}	1.2^{c}

OE - outdoor explants, IE - in vitro explants, Data pooled from a total of 3 separate co-cultivation experiments each comprising of 10 replicate jars containing 1–4 internode explants per jar. Mean values within column with different superscript alphabets are significantly different (p < 0.05; Duncan's New Multiple Range Test)

The maximum transformation efficiency was achieved in case of internodal explants from outdoor-grown plants after 5 days of co-cultivation with *R.rhizogenes* (*ca.* 36.98% with A4T, *ca.* 21.65% with A4, *ca.* 11.87% with LBA9402) (Fig. 1c); longer periods were unsuitable because of uncontrollable overgrowth of bacteria. Most of the internode or leaf explants from *in vitro* shoot cultures failed to survive beyond 10 days of co-cultivation.

Reduction in the overgrowth of bacteria, adhering to the explants or proliferation in the culture medium, was crucial following co-cultivation. In comparison with two other bactericidal antibiotics namely carbenicillin and sporidex, cefotaxime was most effective to keep the bacterial growth at check. Of a range of concentrations of cefotaxime evaluated, 500 µg ml^{-1} was most efficient (Fig. 1d). Higher concentrations were phytotoxic particularly for *in vitro* culture derived explants, regardless of bacterial strain employed.

Optimization of factors influencing hairy root growth

Bacterial strains

Compared to the hairy root somaclones (rhizoclones) resulted from infection with A4 or LBA9402 strains, those incited by A4T strain grew more luxuriously with fast and extensive lateral branching within 6–8 weeks generating higher biomass gain as denoted by root growth index (RGI) measurements (Table 3).

Table 3
Evaluation of growth and proliferation of selected rhizoclones incited by different *A. rhizogenes* strains on MS0 medium at day 45

Strain	Rhizoclone	Root length (cm)	Root Growth Index (RGI)
A4	SA1A2	16.3^{fg}	27.3^{ghi}
	SA2B5	17.8^{f}	35.2^{f}
	SA4C4	15.9^{fgh}	29.9^{g}
	SA5E7	15.5^{fghi}	25.8^{i}
	SA6H1	15.1^{fghij}	29.4^{gh}
A4T	ST2D1	29.7^{b}	36.9^{cde}
	ST3E3	28.4^{bcde}	39.3^{c}
	ST5F4	32.1^{a}	45.3^{a}
	ST7G6	29.2^{bcd}	38.5^{cd}
	ST8H7	29.5^{bc}	41.6^{b}
LBA9402	SL3B6	8.1^{kl}	11.2^{kl}
	SL5A5	7.5^{klm}	10.2^{klm}
	SL7C8	8.9^{k}	23.9^{j}
	SL8D9	7.3^{klmn}	12.5^{k}
	SL9E4	7.0^{klmno}	9.9^{klmn}

Data pooled from a total of 3 separate experiments each comprising of 10 replicate jars. Mean values within column followed by different alphabets in superscript are significantly different ($p \leq 0.05$; Duncan's New Multiple Range Test).

Illumination

Light intensity played an important role in the percentage rhizogenesis of the inoculated explants. The highest root growth in terms of RGI and root length was achieved at the lowest illumination (10 µmol m^{-2} s^{-1} PFD). At this illumination the highest values of RGI i.e. 45.3, 35.2 and 23.9 as well as maximum root length i.e. 32.1, 17.8, and 8.9 cm were recorded for ST5F4 (A4T), SA2B5 (A4) and SL7C8 (LBA9402) rhizoclones, respectively (Table 4). Interestingly, transformed rhizoclones demonstrated retrogression in root growth in response to an incremental higher regime of illumination (20–50 µmol m^{-2} s^{-1} PFD). When root cultures were grown under higher light conditions for a longer time (more than 30 days), the roots turned brown and their growth was markedly retarded. On the contrary, under complete dark conditions, the roots grew luxuriantly during the initial phase but the growth decelerated later through subsequent subcultures.

Table 4
Effect of light intensity on the growth of transformed roots of different rhizoclones incited by different *R. rhizogenes* strains on MS0 at day 30

Light intensity (µmol $m^{-2}s^{-1}$)	Root length (cm)			Root Growth Index (RGI)		
	SA2B5 (A4)	ST5F4 (A4T)	SL7C8 (LBA9402)	SA2B5 (A4)	ST5F4 (A4T)	SL7C8 (LBA9402)
Dark	14.6^{b}	22.76^{b}	7.21^{b}	27.25^{b}	31.87^{b}	19.76^{b}
10	17.8^{a}	32.1^{a}	8.9^{a}	35.2^{a}	45.3^{a}	23.9^{a}
20	11.09^{c}	15.43^{c}	5.01^{c}	25.87^{c}	29.89^{c}	16.99^{c}
30	9.65^{d}	12.43^{d}	3.98^{d}	13.01^{d}	13.87^{d}	9.98^{d}
40	7.44^{e}	10.65^{e}	2.77^{e}	9.67^{e}	11.32^{e}	6.33^{e}
50	5.29^{f}	7.88^{f}	1.83^{f}	7.44^{f}	9.78^{f}	3.99^{f}

Data pooled from a total of 3 separate experiments each with 10 replicates. Mean values within column followed by different alphabets in superscript are significantly different ($p \leq 0.05$; Duncan's New Multiple Range Test).

Culture matrix

In liquid MS0 medium maintained either at a stagnant state or under slow agitation (20 rpm), root growth was minimum. Therefore, it was mandatory to use the medium (MS0) gelled with agar in order to support root cultures. Of the varying concentrations tested 0.6% (w/v) agar-agar gave the ideal matrix in promoting luxuriant root growth (Fig. 2a-d). In this culture matrix, highest values of RGI i.e. 45.3, 35.2 and 23.9 as well as maximum root length i.e. 32.1, 17.8 and 8.9 cm were recorded for rhizoclones ST5F4 (A4T), SA2B5 (A4) and SL7C8 (LBA9402), respectively (Fig. 3).

Plant growth regulators

Neither of the auxins (IAA/NAA) added to the co-cultivation medium had a stimulatory effect on induction of hairy roots (root emergence period, percentage rhizogenesis, number of roots per explant). Transformed root clones cultured on MS supplemented with IBA proliferated rapidly and eventually generated a higher biomass. Of the range of IBA concentrations evaluated, 0.25 mg l^{-1} was the optimum, exceeding which a negative effect on root growth was noticed. In MS + 0.25 mg l^{-1} IBA, the highest RGI (45.3) and longest root length (32.1 cm) was attained by the rhizoclone ST5F4 (A4T), followed by SA2B5 (A4) and SL7C8 (LBA9402), retrogressively (Table 5).

Table 5
Effect of plant growth regulators (PGRs) in MS medium on the growth of transformed roots of different rhizoclones (incited by different *R. rhizogenes* strains) at day 30

PGR (mgl^{-1})		Root length (cm)			Root Growth Index (RGI)		
		SA2B5 (A4)	ST5F4 (A4T)	SL7C8 (LBA9402)	SA2B5 (A4)	ST5F4 (A4T)	SL7C8 (LBA9402)
NIL (0)		12.54^{b}	24.21^{b}	5.43^{b}	24.87^{b}	32.99^{b}	9.21^{b}
IAA	0.25	4.99^{cde}	7.65^{f}	2.21^{de}	5.43^{ef}	7.53^{e}	2.22^{def}
	0.5	2.06^{fgh}	3.21^{h}	1.18^{g}	2.65^{gh}	3.21^{g}	1.29^{h}
	1.0	1.01^{fghij}	2.43^{hij}	0.87^{h}	0.98^{j}	1.11^{j}	0.65^{i}
IBA	0.25	17.8^{a}	32.1^{a}	8.9^{a}	35.2^{a}	45.3^{a}	23.9^{a}
	0.5	7.65^{c}	20.1^{c}	5.21^{bc}	18.99^{c}	27.29^{c}	7.76^{bc}
	1.0	2.87^{f}	14.87^{d}	2.22^{d}	11.91^{d}	18.6^{d}	3.18^{d}
NAA	0.25	5.87^{cd}	10.81^{e}	3.76^{c}	6.99^{e}	6.98^{ef}	2.66^{de}
	0.5	2.81^{fg}	6.45^{fg}	2.11^{e}	3.12^{g}	3.11^{gh}	1.87^{g}
	1.0	1.89^{fghi}	2.76^{hi}	1.22^{f}	1.99^{i}	1.49^{i}	0.43^{j}

Data pooled from a total of 3 separate experiments each with 10 replicates. Mean values within column followed by different alphabets in superscript are significantly different ($p \leq 0.05$; Duncan's New Multiple Range Test)

Molecular characterization of rhizoclones

Genomic DNA isolated from five independent transformed fast-proliferating hairy root clones, incited by *R. rhizogenes* A4T strain (ST2D1, ST3E3, ST5F4, ST7G6 and ST8H7), capable of showing auxin independent growth, revealed the expected amplicon of 206 bp, specifying *rol*B ORF following PCR amplification (Fig. 4a). This indicated the presence of the *R. rhizogenes rol*B gene as part of the pRi T_L-DNA transferred to the recipient plant genome via genetic transformation. Amplification product was not detected in DNA from non- transformed plants when subjected to PCR amplification with the gene primers specific to the *rol*B gene sequences. The transcript expression was confirmed by RT-PCR using the primer designed for pRi T_R-DNA specific *mas*2 gene (encoding mannopine synthase) or *ags* gene (encoding agropine synthase), which gave the predictable amplified cDNA products of 512 bp (Fig. 4b) or 1.6 kb (Fig. 4c) respectively. Opine production in rhizoclones were detected by silver nitrate positive blackish-brown spots along the electropherogram (Fig. 4d). All the selected rhizoclones contained a considerable amount of compounds which had the same mobility as authentic agropine in addition to those of a lower mobility representing a combination of mannopine and mannopinic acid, both being the opine chain precursors of agropine. Neither of these compounds were observed in natural root extracts of non-transformed *in vivo*-grown plants.

Antihyperglycemic activity studies

The fast-proliferating rhizoclone ST5F4, resulted because of genetic transformation via treatment with *R.rhizogenes* A4T strain, was selected for investigating anti-hyperglycemic activity *vis-à-vis* natural roots (NR).

β-sitosterol characterization and content

β-sitosterol (β-STL) was obtained as a colourless amorphous powder (30 mg), m.p. 109°C; R_f: 0.44 (n-hexane–ethyl acetate; 96:4); IR (KBr, cm^{-1}): 3360, 3222, and 1665. NMR (^{1}H-NMR and ^{13}C-NMR) analysis revealed the following spectral value which in corroboration with that of published literature (Pateh et al., 2008) confirmed its structure elucidation as $C_{29}H_{50}O$.

^{1}H NMR ($CDCL_3$, 200 MHz)	:	**δ 5.37 (brs, 1H, H-6), 3.52 (m, 1H, J = 5.2 Hz, H-3), 1.02, 0.68 (s, each 3H, H-19, H-18), 0.94 (d, 3H, J = 6.8 Hz, H-21), 0.83 (d, 3H, J = 6.4 Hz, H-27), 0.80 (d, 3H, J = 6.2 Hz, H-26), 0.91 (t, 3H, J = 6.8 Hz, H-29).**
^{13}C NMR ($CDCL_3$, 300 MHz)	:	δ36.90 (C_1), 29.26 (C_2), 70.82 (C_3), 40.22 (C_4), 140.78 (C_5), 120.88 (C_6), 31.49 (C_7), 31.19 (C_8), 49.70 (C_9), 36.13 (C_{10}), 20.65 (C_{11}), 39.39 (C_{12}), 41.89 (C_{13}), 56.34(C_{14}), 23.95 (C_{15}), 27.83 (C_{16}), 55.58 (C_{17}), 11.47 (C_{18}), 19.11 (C_{19}), 35.70 (C_{20}), 18.45 (C_{21}), 33.51 (C_{22}), 25.60 (C_{23}), 45.36 (C_{24}), 28.72 (C_{25}), 18.80 (C_{26}), 19.70 (C_{27}), 22.63 (C_{28}), 11.55 (C_{29}).
Mass (m/z)	:	414 [M+], 396, 381, 213, 145, 105, 81, 55.

The HPTLC method applied with appropriate solvent system (*n*-hexane–acetone (7.8:2.2, *v/v*) gave a high resolution of β-sitosterol [R_f 0.46; Fig. 5a] and enabled a good separation from other components of the extract (significantly differing in R_f values) of natural roots (NR) (Fig. 5b) and cultured TR clone ST5F4 (Fig. 5c) of *S. acuta*. Scanning of bands (R_f 0.46 individually at 490 nm) of TR extract gave super-imposable spectra with standard β-STL suggesting a remarkable homology in composition (Fig. 5d). Validation of the HPTLC method used for quantification of β-STL from *S. acuta* TR extracts was carried out as per the ICH guidelines (CPMP/ICH/381/95, CPMP/ICH/281/95) with respect to specificity, sensitivity, linearity, accuracy, precision, LOD & LOQ, repeatability and robustness (Jena 2016) (*data not presented*). Six replicates for each determination were made, and the average content of β-STL was calculated. HPTLC analysis revealed an approximately 3.5-fold higher content of β-STL in the selected rhizoclone ST5F4 that averaged 46.77 mg/100 g d.wt. root tissue in comparison with that in natural root (NR) extracts (13.43 mg/100 g d.wt. root tissue).

Root extract-induced effects in normoglycemic and glucose-loaded diabetic rats

TR/NR extracts (methanolic/aqueous) or purified β-STL caused no significant changes in FBG level of normal healthy rats (Groups III-VII) through different tested hours (0–8 h) (Suppl. Table S1). Contrarily, the commercial anti-diabetic drug GLBC had a significant hypoglycemic effect showing a marked reduction (> 50%) in the FBG level after 8 h. The variation in FBG level between different test groups failed to display any statistical significance when compared with the normal control (NC).

In glucose-loading experiments, compared to STZ-induced DC rats without extract-administration (Group-III) where PBG level continued to elevate throughout (30–120 min) a reduction became evident following crude extract/β-STL treatment (Suppl.Table S2). TRAEt-400 was most effective causing 10.91% and 17.30% reduction in PBG at 90 and 120 min respectively. While GLBCshowed 11.00% and 18.35% reduction in PBG (Group-IV rats), β-STL-treated Group-IX diabetic rats had reduced PBG by 10.22% and 16.17% after 90 and 120 min respectively.

Change in FBG levels of STZ-induced diabetic rats

In STZ-diabetic rats, treatment with extracts (aqueous/methanol) of TR/NR (T2-T5) or β-STL isolate (T6) showed a significant reduction in FBG level at day 40 (Fig. 6a,b). The corresponding percentage reduction in FBG by TRAEt-400 (T2) were calculated to be 20.82%, 36.24%, 63.23% and 74.49% at day 10, 20, 30 and 40 respectively. GLBC (T1) showed percentage reduction in FBG (Group-III) by 22.86%, 38.90%, 65.99% and 75.13% for the

respective durations. Group-VIII rats, following β-STL treatment (T6), exhibited a retrogression in FBG *at par* with T2-treated Group-IV rats; nevertheless, β-STL had no effect on FBG level of NC rats (Group-IX, T7).

Effect of TR extracts and purified β-STL isolate on serum/urine biochemical parameters in STZ induced diabetic rats

Serum insulin and liver glycogen content were significantly low in STZ-induced DC rats (av. 7.33 µU/ml, 7.01 mg/g) compared to that in NC rats (av. 17.06 µU/ml, 14.72 mg/g). However, the FBG level was much higher in diabetic control rats (av. 255.88 mg/dl) than NC (av. 81.23 mg/dl). A significant enhancement in serum insulin and liver glycogen content concomitant with a remarkable reduction in FBG levels was evident in STZ-induced diabetic Groups IV-IX following treatment with TR extracts or β-STL isolate (Fig. 7). After administering β-STL-20 to Group-VII rats serum insulin, liver glycogen and FBG levels were dramatically altered averaging 12.86 µU/ml, 10.31 µU/ml and 90.33 mg/dl respectively. However, there were no significant changes in respect of these in NC treated with β-STL regardless of dosage.

A 40-day treatment with TRAEt-400 or β-STL-20 caused a significant reduction in triglycerides, total cholesterol, creatinine, BUN content and liver specific enzyme activity (SGPT, SGOT, ALP) in STZ-induced diabetic rats tending to values obtained with that of NC (Table 6). The protein content resumed near-normalcy following such extract treatment. β-STL, purified from TR extracts, exerted a dramatic effect on each of the blood serum biochemicals examined. An enhancement in mean albumin and globulin content up to 3.68 g/dl and 2.31 g/dl was also recorded in Group-VII diabetic animals treated with β-STL. TRAEt-400/β-STL-20 treated rats had a substantially enhanced urea, uric acid and creatinine levels, but reduced urine sugar and albumin content, an effect comparable with that of the commercial anti-diabetic drug GLBC (Table 7).

Table 6
Effect of *S. acuta* aqueous TR extracts and purified β-STL isolate on some biochemical parameters of blood serum in STZ-induced diabetic rats

Animal Groups	Treatment	% reduction in FBG at day 40	SGPT (IU/l)	SGOT (IU/l)	ALP (IU/l)	Total protein (mg/dl)	Triglycerides (mg/dl)	Totalcholesterol (mg/dl)	Creatinine (mg/dl)	BUN (mg/dl)	A/G* ratio
Gr.I	NC	(-) 4.11 ± 0.44^{e}	32.51 ± 3.12^{g}	113.21± 4.77^{g}	77.33 ± 4.18^{g}	7.46 ± 0.88^{a}	85.55 ± 5.14^{g}	68.6 ± 4.51^{g}	0.38 ± 0.03^{d}	27.83 ± 2.96^{c}	1.61^{a}
Gr.II	DC	(-) 9.05 ± 0.76^{f}	77.59 ± 4.52^{a}	171.55± 6.42^{a}	160.86 ± 7.33^{a}	5.04 ± 0.45^{c}	223.15 ± 11.12^{a}	153.83 ± 8.96^{a}	1.16 ± 0.07^{a}	54.41 ± 3.69^{a}	1.34^{b}
Gr.III	D + GLBC (10)	73.33 ± 2.99^{a}	38.16 ± 3.19^{e}	122.37± 7.72^{f}	83.22 ± 5.14^{f}	6.95 ± 0.52^{b}	93.66 ± 4.36^{f}	74.11 ± 4.12^{f}	0.41 ± 0.04^{c}	29.88 ± 2.41^{b}	1.59^{a}
Gr.IV	D + TRAEt (200)	57.48 ± 1.87^{c}	47.87 ± 2.23^{b}	145.33 ± 3.44^{b}	121.58 ± 4.77^{b}	6.55 ± 0.37^{b}	109.87 ± 2.66^{c}	94.65 ± 2.76^{b}	0.55 ± 0.03^{b}	33.63 ± 1.87^{b}	1.64^{a}
Gr.V	D + TRAEt (400)	69.67 ± 2.81^{a}	40.67 ± 1.26^{d}	130.08 ± 2.89^{d}	90.46 ± 2.62^{d}	6.79 ± 0.50^{b}	96.83 ± 3.52^{e}	80.16 ± 1.13^{e}	0.44 ± 0.02^{c}	31.09 ± 1.82^{b}	1.61^{a}
Gr.VI	D + β-STL (10)	55.78 ± 2.11^{c}	43.16 ± 2.34^{c}	133.19± 2.57^{c}	96.12 ± 2.55^{c}	6.47 ± 0.40^{b}	112.5 ± 2.79^{b}	86.6 ± 2.29^{c}	0.51 ± 0.01^{b}	35.17 ± 1.82^{b}	1.62^{a}
Gr.VII	D + β-STL (20)	62.76 ± 2.65^{b}	37.33 ± 1.44^{f}	126.46 ± 2.88^{e}	88.33 ± 2.11^{e}	6.58 ± 0.45^{b}	101.65 ± 2.69^{d}	83.56 ± 1.23^{d}	0.47 ± 0.01^{c}	33.01 ± 1.71^{b}	1.59^{a}
Gr.VIII	N + β-STL (10)	1.21 ± 0.08^{d}	29.54 ± 2.42^{h}	108.88± 4.11^{h}	74.88 ± 3.11^{h}	7.31 ± 0.29^{a}	82.81 ± 5.97^{h}	64.98 ± 1.39^{h}	0.38 ± 0.02^{d}	26.65 ± 1.92^{c}	1.63^{a}
Gr.IX	N + β-STL (20)	2.16 ± 0.35^{d}	26.88 ± 2.17^{i}	102.63 ± 3.23^{i}	71.92 ± 2.44^{i}	7.12 ± 0.59^{a}	80.02 ± 3.18^{i}	61.29 ± 2.09^{i}	0.37 ± 0.01^{d}	25.44 ± 1.68^{c}	1.65^{a}

Data pooled from 3 experiments each with five replicates as recorded on the 40th day after treatment. Each treatment group contained six animals.Values (Mean ± SEM)

within columns (between rows) followed by different alphabets in superscript are significantly different ($p \leq 0.05$; Duncan's New Multiple Range Test)

NC: Normal control; DC: Diabetic control (STZ-induced diabetic rats without extract treatment); β-STL: β-sitosterol; GLBC: Glibenclamide; TRAEt: Transformed Root - Aqueous Extract;BUN: Blood Urea Nitrogen; N: Normal rats; D: Diabetic rats.Values given in parenthesis in the Column-1 (Treatment) denote dosage (mg/kg b. wt.) ;* Given A/G ratios were based on Mean values of albumin and globulin.

Table 7
Effect of *S.acuta* TR aqueous extracts and purified β-sitosterol isolate on some urine biochemical parameters

Animal Groups	Treatment	Urine sugar (g/day)	Urea (mg/dl)	Uric acid (mg/dl)	Creatinine (mg/dl)	Albumin (µg/dl)
Gr. I	NC	ND	150.99 ± 7.23^{b}	8.21 ± 1.19^{b}	2.90 ± 0.38^{a}	151.35 ± 4.45^{d}
Gr. II	DC	7.34 ± 0.96^{a}	94.44 ± 3.21^{f}	4.12 ± 0 .85^{f}	1.63 ± 0. 31^{e}	317.48 ± 5.62^{a}
Gr. III	D + GLBC (10)	ND	141.56 ± 3.99^{c}	7.90 ± 1.03^{c}	2.74 ± 0.36^{b}	160.11 ± 4.53^{c}
Gr. IV	D + TRAEt (200)	ND	132.67 ± 2. 47^{d}	7.34 ± 0.65^{d}	2.42 ± 0.17^{c}	180.07 ± 2.56^{b}
Gr. V	D + TRAEt (400)	ND	139.83 ± 2.81^{c}	7.82 ± 1.08^{c}	2.68 ± 0.22^{b}	166.98 ± 2.64^{c}
Gr. VI	D + β-STL (10)	ND	126.91 ± 1.88^{e}	6.96 ± 0.73^{e}	2.28 ± 0.17^{d}	178.88 ± 2.71^{b}
Gr. VII	D + β-STL (20)	ND	134.67 ± 1.71^{d}	7.64 ± 0.82^{d}	2.53 ± 0.15^{c}	172.11 ± 2.97^{b}
Gr. VIII	N + β-STL (10)	ND	152.86 ± 1.68^{b}	8.33 ± 0.56^{b}	2.96 ± 0.14^{a}	147.21 ± 1.44^{d}
Gr. IX	N + β-STL (20)	ND	158.88 ± 1.86^{a}	8.74 ± 0.81^{a}	3.14 ± 0.17^{a}	143.87 ± 2.53^{d}
in STZ-induced diabetic rats						
Data pooled from 3 experiments each with five replicates as recorded on the 40th day after treatment. Each treatment group contained six animals.Values (Mean ± SEM) within columns (between rows) followed by different alphabets in superscript are significantly different ($p \leq 0.05$; Duncan's New Multiple Range Test).ND: Not detected						
NC: Normal control; DC: Diabetic control (STZ-induced diabetic rats without extract treatment);GLBC: Glibenclamide; TRAEt: Transformed Root - Aqueous Extract; β-STL: β-sitosterol. Values given in parenthesis in the Column-1 (Treatment) denote dosage (mg/kg b. wt.) ;N: Normal rats; D: Diabetic rats						

Discussion

The present study was undertaken to (i) optimize specific factors (transforming bacterium × plant × *in vitro* culture environment) crucial to *Rhizobium rhizogenes*–mediated hairy root formation of an important ethnomedicinal plant species *S. acuta* and maintenance of hairy root cultures (HRC), (ii) evaluate the anti-hyperglycemic activity of methanol/aqueous extracts of natural roots (NR) in comparison with that of transformed root (TR) cultures and a bioactive phytocompound β-STL, purified from such cultures on streptozotocin (STZ) induced diabetic rats. Interestingly, unlike the commercial anti-diabetic drug (GLBC) neither root (TR/NR) extracts nor β-STL isolate exerted any innate hypoglycemic effect on normoglycemic animals, instead significantly reduced the elevated FBG level resulting from induced diabetes. This implies that *S. acuta* root extracts, particularly the phytocompound β-STL may possibly serve as a safer and effective therapeutic drug because its use would evade undesirable side effects resulting from hypoglycemia.

Administration of either TR/NR root extracts or purified β-STL isolate significantly reduced FBG level which indicates either reversal of insulin resistance or stimulation of insulin secretion possibly by revitalizing damaged pancreatic β-cells in STZ-induced diabetic rats. Being known as an insulin-sensitive organ, liver plays a pivotal role in the metabolism of glucose by strategically regulating the equilibrium between glucose utilization (glycolysis), its conversion to glycogen (glycogenesis) and its reconversion (glycogenolysis/gluconeogenesis). Decreased FBG levels may be correlated with decreased gluconeogenic activity or inhibition of glycogenolysis as indicated by increased liver glycogen. Our results correlating a fall in blood glucose content with a rise in liver glycogen accumulation corroborate with a similar observation resulted from treatment of caffeic acid in db/db mice (Hamideh et al., 2017) or isolated fraction of *Prosopis cineraria* against STZ-induced diabetic rats (Sonia et al., 2018).

The reduction in serum protein, particularly albumin, might be due to proteinuria, albuminuria or enhanced protein breakdown which is the clinical hallmark in diabetic nephropathy. Treatment of diabetic rats with aqueous TR extract or β-STL, resulted in an alteration in total protein and A/G ratio to near-normal level which might be related to an increase in the insulin-mediated amino acid uptake, elevated protein synthesis or reduced protein degradation.The ability of the aqueous extract of NR/TR to restore normalcy in body weight and urine passage implies glycaemic control and restoration of kidney function.

Our observations of elevated levels of blood (serum) urea nitrogen (BUN) and creatinine in diabetic subjects might be reflections of renal dysfunction caused by disturbed glucose regulation or excessive glycosylated protein levels. Urea being the major nitrogen-containing product of protein catabolism, the observed hyper-accumulation of BUN in the diabetic control rats was perhaps because of the enhanced breakdown of liver and plasma proteins. Quite likely, there was an elevated efflux/elimination of urea nitrogen resulting from imbalance in nitrogen homeostasis in the hepatocytes. Protein glycation in diabetics often lead to muscle wasting and enhanced release of purine as a source of uric acid as well as hyperactivated xanthine oxidase, lipid peroxidation, increased triacylglycerol and cholesterol levels. The impaired renal function in diabetic rats was revived following treatment with TR extracts, particularly the purified phytoconstituent β-STL. This was indicated by a marked reduction in renal

functional markers such as BUN and creatinine content in the blood serum of treated rats. The drop in BUN level was indicative of a reduced proteolysis, leading to a remarkable gain in body weight following the phytochemical treatment.

Raised activity of liver biomarker enzymes such as SGPT, SGOT and ALP in the blood serum of experimental diabetic control rats indicated a possibility of hepatocellular necrosis and mobilization of these enzymes from the liver cytosol into the blood stream –a condition that reflects hepatoxicity associated with hyperglycemic drugs such as STZ. Normalization of activity of these enzymes in diabetic rat groups treated with TR extract/β-STL implied correction of liver dysfunction, perhaps by extract-mediated regeneration of new hepatic cells or rejuvenation of damaged ones. Excessive hepatic glycogenolysis caused during insulin deficiency is known to account for over-contribution of glucose, leading to hyperglycemia. As the elevation in such liver-specific enzyme activity was attributed to decreased blood insulin level (Petersen et al., 2017), their restoration in extract-treated groups could be due to insulinotropic property of *S. acuta* TR extracts, especially β-STL constituent, which warrants further investigation. Our observation of a marked reduction in triglycerides and cholesterol levels in the blood serum of treated diabetic rats is believably because of treatment with TR extracts/β-STL, the latter known for its ability to attenuate lipid peroxidation and restore histo-architecture in rats with 1,2-dimethylhydrazine-induced colon cancer (Baskar et al., 2012).

Elevated excretion of proteins and sugar associated with reduced urea, uric acid and creatinine in diabetic subjects indicate kidney dysfunction. Perhaps, the underlying mechanism was the lowering of GFR as well as reduced blood flow to the glomerulus in combination with acute and chronic tubular damage as an effect of diabetes (Vivek et al., 2013). Our data showed that the effective dosage of aqueous TR extract /purified β-STL enhanced uric acid, urea, and creatinine, while reduced the albumin levels in urinary discharge of diabetic rats, thereby implicating the role of *S. acuta* TR phytoconstituents in attenuating diabetes-triggered renal damage.

The role of *rol* genes (especially *rol*B and *rol*C), transferred through pRi T-DNA of *R. rhizogenes* to the recipient plant cells, as potential activators of secondary metabolism in the TR cultures is well documented (Bulgakov 2008). Enhancement in production of secondary metabolites in tune of 2.5–fold plumbagin in *Plumbago* zeylanica (Nayak et al., 2015), 4–fold taraxerol in *Clitoria* ternatea (Swain 2012), 12–fold tropane alkaloids in *Atropa belladonna* (Bonhomme et al., 2000) and 100-fold resveratrol in *Vitis amurensis* (Kiselev et al., 2007) have been demonstrated in TR cultures compared to natural roots (NR] of respective plant species. In the present investigation, pronounced anti-hyperglycemic effects of TR (*rol*B–positive) compared to that of NR (*rol*B–negative) of *S. acuta* on experimental diabetic animals could be implicated to the significantly enhanced (*ca*.3.5–fold) accumulation of β-STL in the former compared to the latter. The *rol*B gene of *R. rhizogenes* was shown to be involved in the activation of the transcription factors of most specialized metabolites in HRCs as well as on the expression of chaperone-type proteins (Bulgakov et al., 2016; Bulgakov 2018).

It has been reported that β-sitosterol and β-sitosterol glucoside, which exist in a variety of plants, regulate the proliferation and activities of peripheral blood lymphocytes and natural killer cells, blood cholesterol level, anti-pyretic activity, and insulin release (Bouic et al., 1996; Fernandez and Vega-Lopez 2005; Gupta et al., 1980; Ivorra et al., 1988). These reports indicate that β-sitosterol and β-sitosterol glucoside have possible therapeutic effects on carcinogenesis, cardiovascular disease, pulmonary tuberculosis and immune-mediated disorders (Bouic and Lamprecht 1999). β-sitosterol and β-sitosterol glucoside are believed to be important molecules in immune-associated abnormalities such as allergic inflammation. β-sitosterol is reported to have anti-tumour, anti-microbial and immuno-modulatory activities (Yuk et al., 2007). It also causes an effective displacement of cholesterol from micellar binding by increasing hydrophobicity and diminished cholesterol absorption (Heinemann et al., 1991). β-sitosterol is also known to be responsible for reduction of cholesterol level in plasma (MacLatchy et al., 1995), and improvement in liver function activity (GDP, GOP) (Zak et al., 2005).

β-STL, besides being shown to be an immunomodulator and useful in coronary artery disease, hypercholesterolemia, rheumatoid arthritis, tuberculosis and cervical/breast/prostate cancers (Saeidnia et al., 2014), the isolate from the stem bark of *Solanum surattense* also demonstrated a promising antioxidative potential in STZ-induced experimental hyperglycemia (Gupta et al., 2011). Among various phytosterols, β-STL is the major compound in plants and has been shown through several *in vitro* and *in vivo* studies to possess various biological actions such as anxiolytic & sedative effects, analgesic, immunomodulatory, antimicrobial, anticancer, anti–inflammatory, lipid lowering effect, hepatoprotective, protective against NAFLD and respiratory diseases, wound healing effect, antioxidant and anti-diabetic activities (Babu and Jayaraman 2020). The chemotherapeutic activity of β-STL could be associated with the induction of apoptosis through blockade of multiple cell signaling mechanisms (Bin Sayeed and Ameen 2015). β-STL was reported to have triggered apoptosis through ROS dependent pathway, which was mediated through Trx/TrxR1 signaling pathway (Rajavel 2018). The underlying mechanism of the alleviating effect of β-STL, purified from transformed root cultures, on the blood/urine biochemistry of hyperglycemic rats could be based on their anti-oxidative activity (*data not shown*) (Jena 2026). β-STL is believed to play a role in scavenging free radicals and preventing protein damage caused by oxidative insult which is involved in pancreatic β-cell degeneration (Kowluru 2020).

In conclusion, the anti-hyperglycemic effect of genetically transformed *Sida acuta* transformed root (TR) culture extracts on STZ-induced diabetic rats, observed in the present study, perhaps owe to stimulation of β-cell regeneration mediated through improved protein synthesis and/or attenuation of the potential deleterious repercussion of ROIs on cellular metabolism. Additionally, compared to natural roots, TR would be a more efficient, stable, ecology/season-invariant and fast-proliferating renewable resource that will ensure round-the-year tissue availability for drug preparation. The global pharmaceuticals-based therapeutic scenario is now changing in favour of green chemistry harnessing natural plant products for innovative drug development and delivery in form of silver/gold nanocomplexes. In this context, in the near future, designing of effective drug(s)

from TR culture extracts of *S. acuta*, especially based on one of its key bioactive principles, such as β-STL could, perhaps, be explored as an efficient bio-prospective strategy towards developing an alternative or complementary nanomedicine for the control of diabetes.

Declarations

Author Contributions SJ: Organization of work elements**,** Data analysis. KKR: Data curation (supporting), critical feedback and β-sitosterol validation as per ICH guidelines. SKM: Experimental design for anti-hyperglycaemic activity studies, original draft preparation. PKC: Conceptualization, supervision, review and editing of manuscript.

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Data Availability: Data used to support the findings of this study are available from the Supplementary Files (Suppl. Tables S1 and S2) as well as from the corresponding author upon request.

Ethics Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. A scanned copy of the certificate issued by the Institutional Animal Ethical Committee" of Dadhichi College of Pharmacy *(Regd. No.1200/AC/08/CPCSEA; Ministry of Environment and Forests, Government of India)*, duly signed by the appropriate authority, that bears testimony to the ethical approval of the work, is available for reference.

Conflict of Interest All authors declare that they have no conflict of interest. Authors have read and accepted the final version of the manuscript and agreed for communicating the manuscript to the journal "Plant Cell, Tissue and Organ Culture (PCTOC): Journal of Plant Biotechnology" for consideration of publication.

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Figures

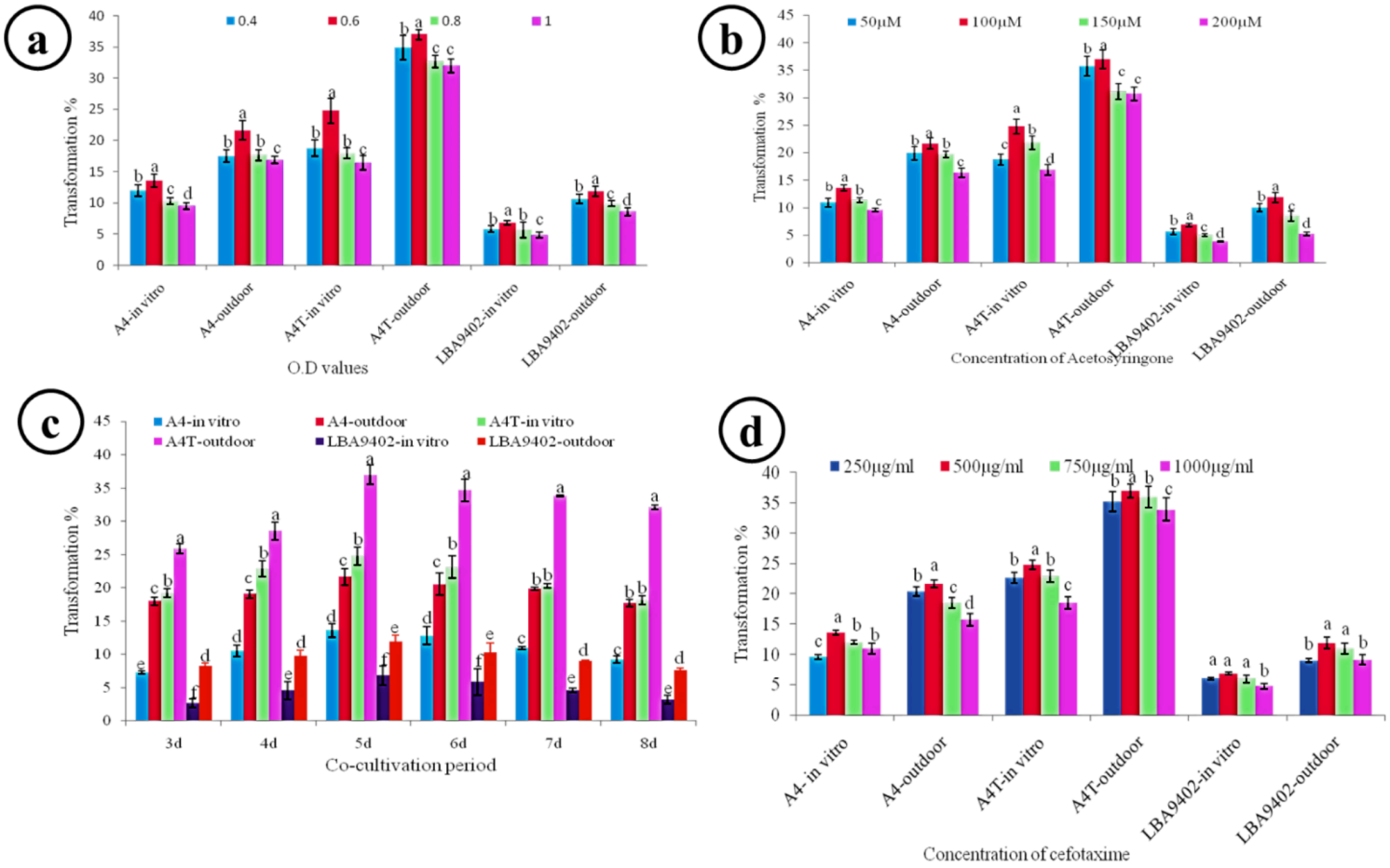

Figure 1

Optimization of bacterium × plant transformation factors for hairy root induction.

(a) Relation between growth phase of *R. rhizogenes* and transformation efficiency

(b) Relation between acetosyringone concentrations and transformation efficiency

(c) Relation between co-cultivation period and transformation efficiency

(d) Relation between bactericidal antibiotic cefotaxime and transformation efficiency

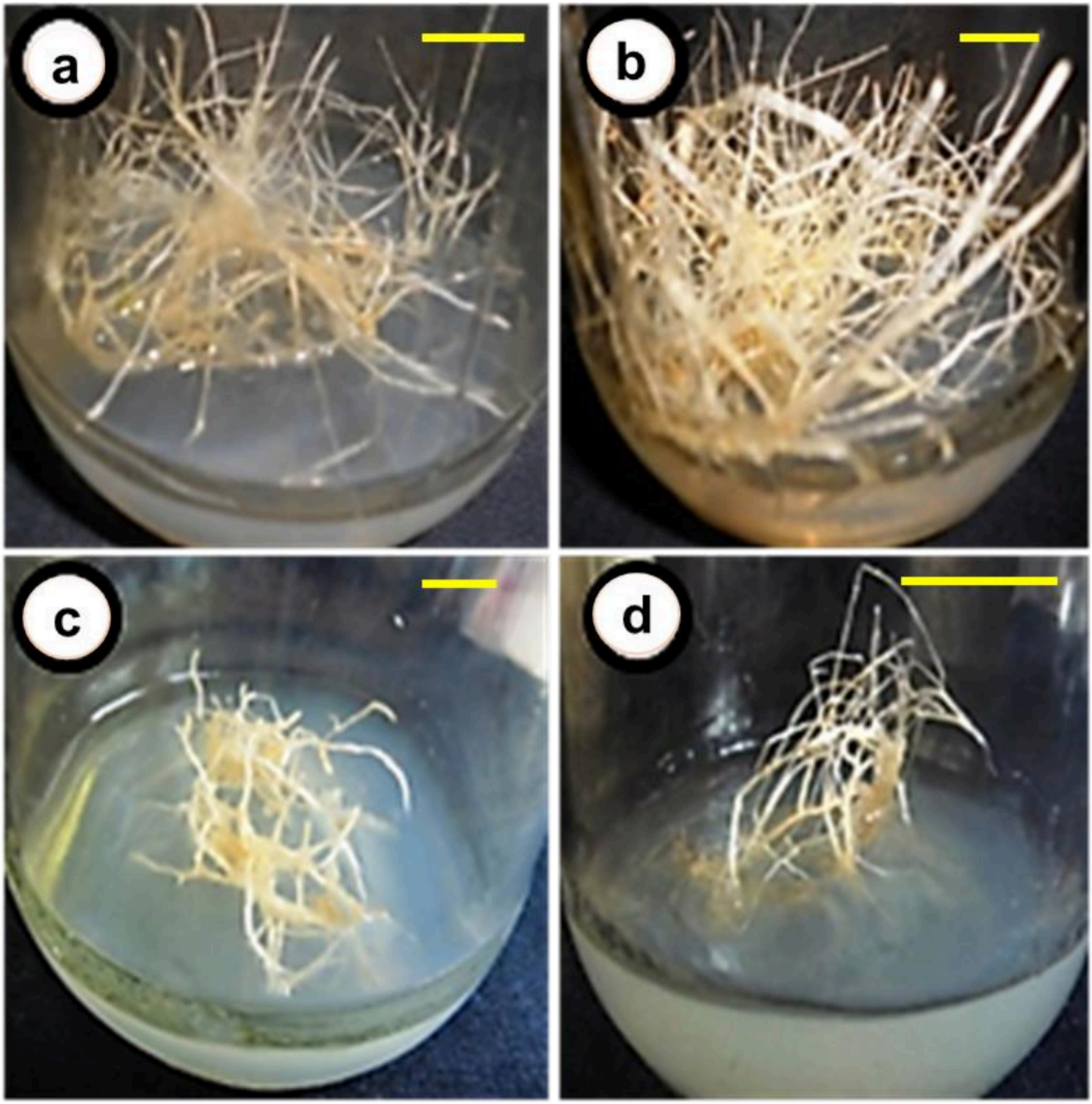

Figure 2

Evaluation of growth of a transformed rhizoclone (ST5F4) induced by *R. rhizogenes* A4T strain following *in vitro* culture in MS0 medium gelled with different agar-agar concentrations (mg l^{-1}) (a) 0.4 (b) 0.6 (c) 0.8 (d) 1.0 (*bar: 1 cm*)

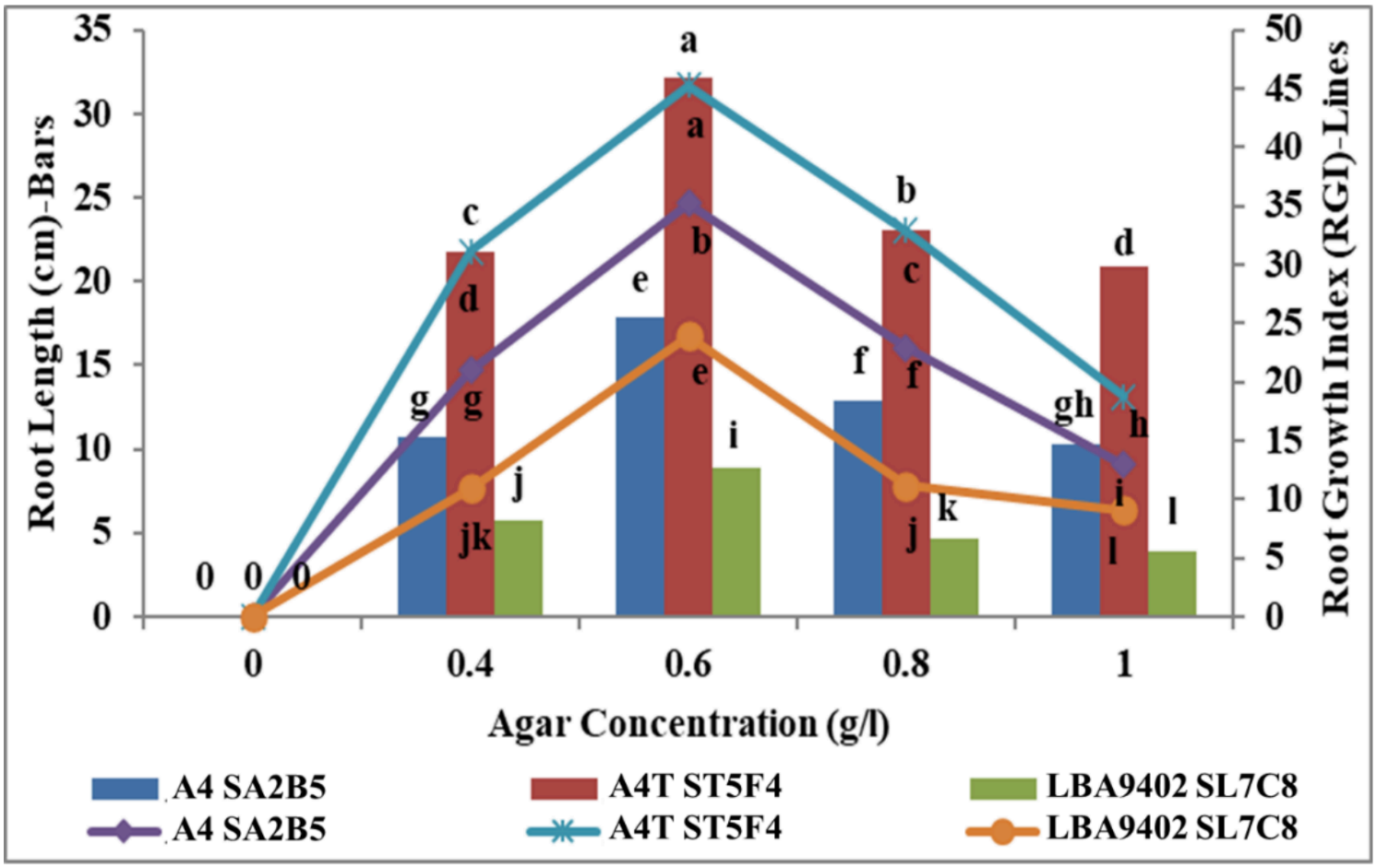

Figure 3

Effect of agar concentrations (mg l^{-1}) in MS0 culture medium on root length (cm) and root growth index (RGI) of different rhizoclones (*Column bars with the different letter are significantly different at p ≤ 0.05 as determined by Duncan's New Multiple Range Test*)

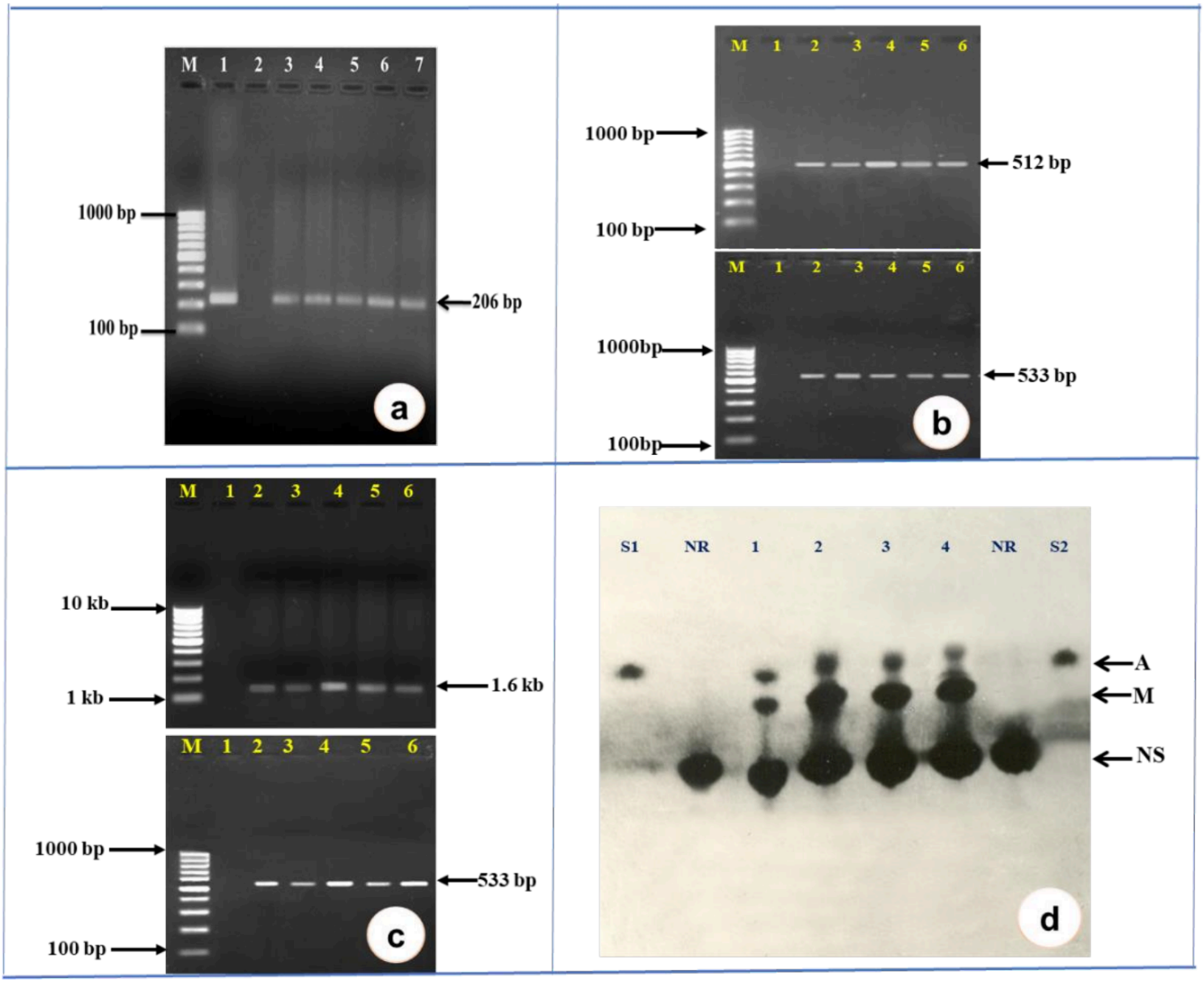

Figure 4

(a) Gel image depicting PCR amplification of *rol*B ORF (206 bp) in rhizoclones, incited by *R. rhizogenes* A4T strain. M: 100 bp DNA ladder (Bangalore Genei-Merck, India), *Lane 1*: Plasmid DNA from A4T (Positive control), *Lane 2*: Root DNA from the naturally grown plant (Negative control), *Lane 3-7*: DNA from selected transformed rhizoclones ST2D1, ST3E3, ST5F4, ST7G6 and ST8H7, respectively. (b) RT-PCR analysis of the expression of the *mas2* gene in selected transformed rhizoclones. M: 100 bp DNA ladder, *Lane 1* (NR): cDNA-PCR product from a natural root (root from a naturally occurring plant) (Negative Control), *Lanes 2, 3, 4, 5 and 6*: cDNA-PCR product from five randomly selected transformed rhizoclones, ST2D1, ST3E3, ST5F4, ST7G6 and ST8H7, respectively. Transformed rhizoclones show an amplified band of 512 bp. The lower panel shows the amplification (533 bp) of actin used as a loading control. (c) RT-PCR analysis of the expression of the *ags* gene in selected transformed rhizoclones. M: 1 kb DNA ladder (upper gel image), *Lane 1* (NR): cDNA-PCR product from a natural root (root from a naturally occurring plant) (Negative Control), *Lanes 2, 3, 4, 5 and 6*: cDNA-PCR product from five randomly selected transformed rhizoclones, ST2D1, ST3E3, ST5F4, ST7G6 and ST8H7, respectively., Transformed rhizoclones show an amplified band of 1.6 kb. The lower panel shows the amplified band (533 bp) of actin used as a loading control (M: 100 bp DNA ladder (lower gel image). (d) Paper electropherogram demonstrating opine production in transformed rhizoclones induced by *R. rhizogenes* A4T strain. NS: Neutral Sugar; *Lane S1 & S2*: Standard (Agropine); *Lane NR*: Natural roots (roots from a naturally occurring plant) (Negative Control); *Lanes 1–4*: Transformed rhizoclones (ST2D1, ST3E3, ST5F4 and ST7G6, respectively). (A: Agropine, M: Mannopine)

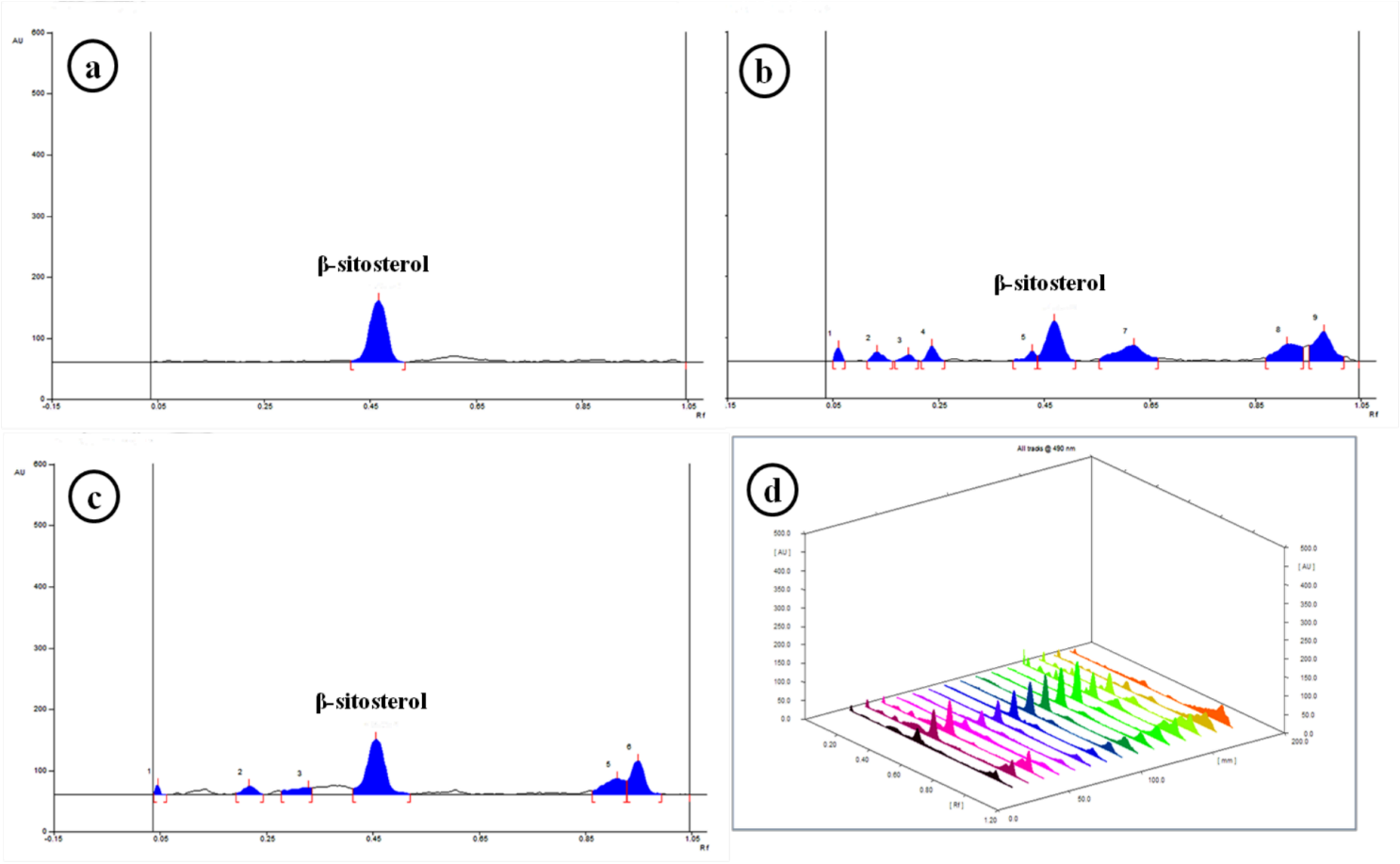

Figure 5

HPTLC chromatogram of transformed root (TR) *vis-à-vis* natural root (NR) samples confirming the production of β-sitosterol. (**a**) Chromatogram of the standard β-sitosterol. (**b**) Chromatogram of NR of *in vivo* plants. (**c**) Chromatogram of TR (ST5F4 rhizoclone). (**d**) Scanning of bands (R_f 0.46, individually at 490 nm) from A4T-elicited TR (ST5F4 rhizoclone) revealing super-imposable spectra with standard β-sitosterol suggesting homology in composition.

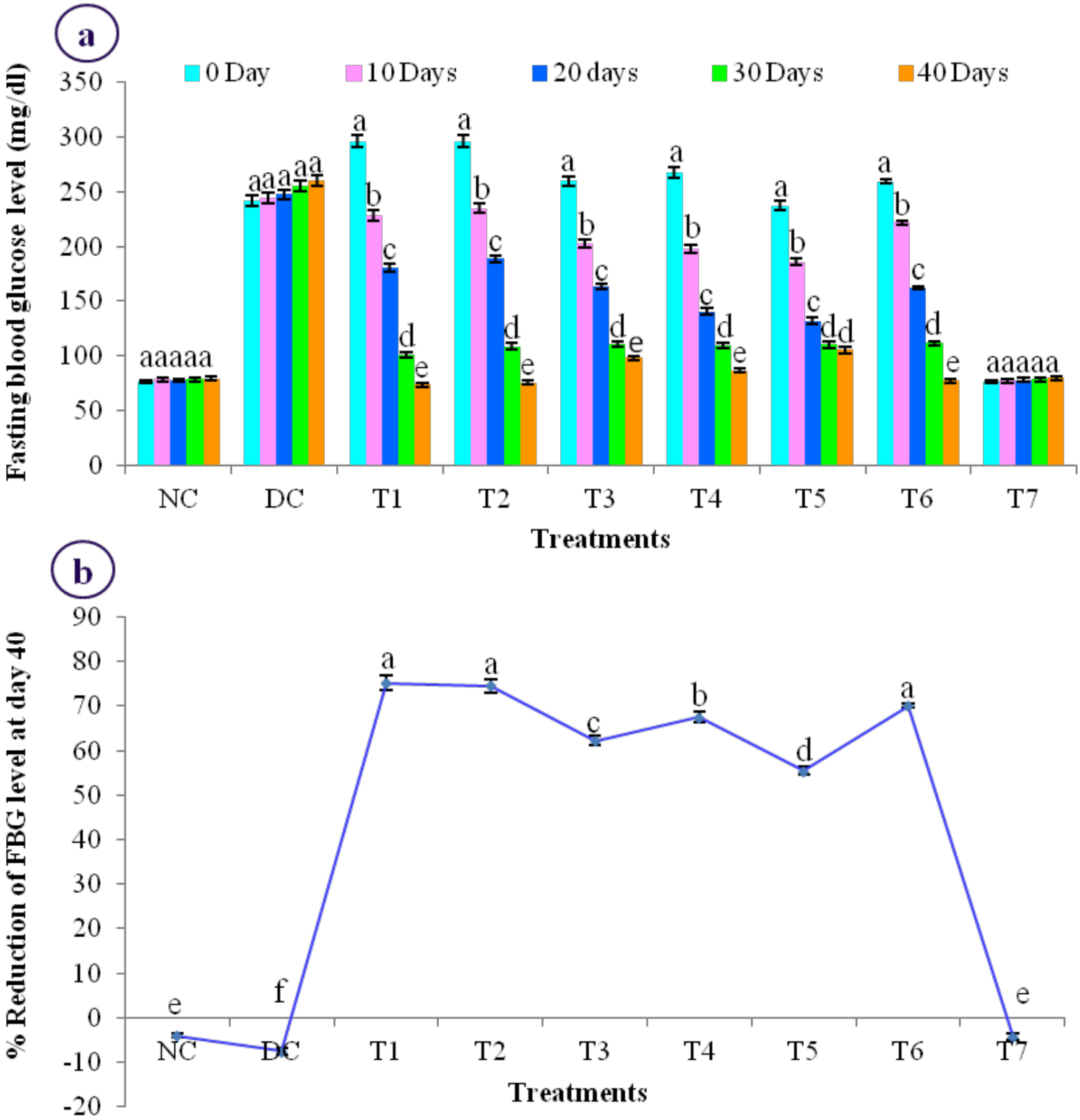

Figure 6

Effect of different TR/NR extracts and β-STL isolate on fasting blood glucose (FBG) level in STZ-induced diabetic rats (a) Changing levels of FBG during 0-40 day period (b) Percentage reduction in FBG on the 40th day

(NC: Normal Control; DC: Diabetic Control; T1: D + GLBC; T2: D + TRAEt; T3: D + NRAEt; T4: D + TRMEt; T5: D + NRMEt; T6: D + β-STL; T7: N + β-STL)

(D- Diabetic rats; N-Normal rats; GLBC: Glibenclamide; TRAEt: Transformed Root - Aqueous Extract [400 mg/kg b.wt.]; NRAEt: Natural Root - Aqueous Extract [400 mg/kg b.wt]; TRMEt: Transformed Root - Methanolic Extract [400 mg/kg b.wt.]; NRMEt: Natural Root - Methanolic Extract [400 mg/kg b.wt]; β-STL: β-sitosterol [20 mg/kg b.wt.])

Data pooled from 3 experiments each with five replicates as recorded on the 40th day after treatment. Each treatment group contained six animals. Within treatment group values (Mean ± SEM) plotted as bar/point followed by different alphabets in superscript are significantly different ($p \leq 0.05$; Duncan's New Multiple Range Test)

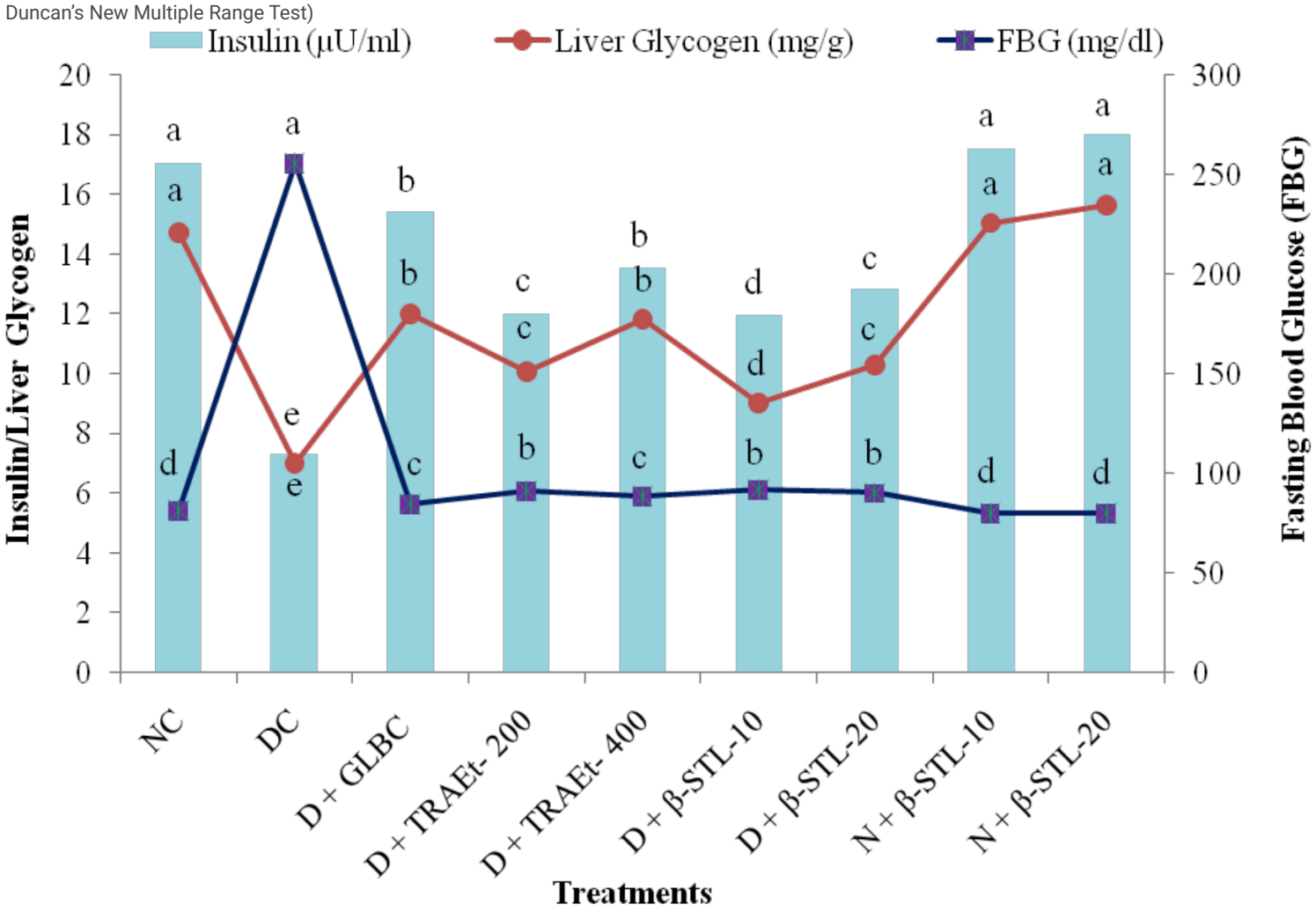

Figure 7

Effect of transformed root (TR) extract or β-sitosterol isolate of *S. acuta* on insulin, liver glycogen and fasting blood glucose levels on the 40th day in STZ induced diabetic rats.

(NC: Normal control; DC: Diabetic control; GLBC: Glibenclamide; TRAEt-200: Transformed Root - Aqueous Extract at 200 mg/kg b.wt.; TRAEt-400: Transformed Root - Aqueous Extract at 400 mg/kg b.wt.; β-STL-10: β-sitosterol at 10 mg/kg b.wt.; β-STL-20: β-sitosterol at 20 mg/kg b.wt.; N: Normal rats; D: Diabetic rats).

Data pooled from 3 experiments each with five replicates as recorded on the 40th day after treatment. Each treatment group contained six animals. Values (Mean ± SEM) plotted as bar/point followed by different alphabets in superscript are significantly different ($p \leq 0.05$; Duncan's New Multiple Range Test).

Supplementary Files

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