Short communication

In vitro callus and in vivo leaf extract of Gymnema sylvestre stimulate β-cells regeneration and anti-diabetic activity in Wistar rats


A methanol extract of Gymnema sylvestre leaf and callus showed anti-diabetic activities through regenerating β-cells. Optimum callus was developed under stress conditions of blue light with 2,4-D (1.5 mg/l) and KN (0.5 mg/l), which induced maximum biomass of green compact callus at 45 days, as determined by growth curve analysis. Leaf and optimum callus extracts contain gymnemic acid, which was analyzed using TLC, HPTLC and HPLC methods. The research reported here deals with leaf and callus extracts of G. sylvestre, which significantly increase the weight of the whole body, liver, pancreas and liver glycogen content in alloxan-induced diabetic rats (Wistar rats). The gymnemic acid of leaf and callus extracts significantly increases the regeneration of β-cells in treated rats, when compared with the standard diabetic rats. It could have potential as a pharmaceutical drug for insulin-dependent diabetes mellitus (IDDM).

Keywords: Gymnema sylvestre, Gymnemic acid, Growth curve, Organ weight, Liver glycogen, β-Cell regeneration

Introduction

Type 1 diabetes, or insulin-dependent diabetes mellitus (IDDM), is a common pediatric chronic disease, affecting an increasing number of children every year. IDDM occurs due to autoimmune destruction of insulin-producing β-cells in the pancreas, resulting in low or no production of insulin, a hormone necessary for survival (International Expert Committee, 2009).

Gymnema sylvestre (syn. Periploca sylvestris Retz) is a traditional medicinal plant, with reported use as a remedy for diabetes mellitus, stomachic and diuretic systems of medicine (Mitra, 1995). The extract of G. sylvestre plays a major role in blood glucose homeostasis through increased serum insulin level through regeneration of the endocrine pancreas (Shanmugasundaram et al., 1983; Shanmugasundaram et al., 1990). G. sylvestre occurs mainly in the Deccan peninsula of western India, Tropical Africa, Vietnam, Malaysia, Srilanka and is widely available in Japan, Germany and the USA as a health food (Ye et al., 2000). Within the last 10 years, a number of Gymnema products, including Gymnema capsules, Gymnema tea, Bioshape®, and Dianxido® have appeared on the world market.

In the past few decades, secondary metabolite production from plant tissue culture has been identified as a tremendous resource for new drug development and clinical research in the fields of pharmacology and medicine. Plant cell culture extracts have also been used widely in the form of fractions and isolated compounds as potential bioactive molecules (Sokmen et al., 1999). In vitro developed callus tends to produce various active compounds, including gymnemic acid and gymmenagon (Kanetkar et al., 2006). However, external factors like phytohormone, shaking speeds, pH, and medium play an important role in gymnemic acid production in suspension cultures (Devi et al., 2006). In addition, sucrose, inoculum density, auxins, and aeration also play a very crucial role in the production of gymnemic acid through bioreactor-dependent cell growth (Lee et al., 2006). The present studies were undertaken to identify the G. sylvestre leaf and callus extract molecules that contribute to or promote β-cell regeneration and anti-diabetic effects. In addition, we have characterized the gymnemic acid role in anti-diabetic experiments; it was found that pancreas weight and glycogen content were increased in the liver of alloxan-induced diabetic Wistar rats. Furthermore, an emphasis is laid on β-cell regeneration and the determinants implicated.

Materials and methods

Plant material and sterilization

G. sylvestre plants (GS) were collected from the Pachamalai hills, and maintained in the plant science garden of the Bharathidasan University, Tiruchirappalli, Tamil Nadu, India. Leaf explants
were washed with tap water, Tripeol solution, then 70% ethanol for 30 min and 0.1% HgCl₂ for 2 min. Prior to inoculation, explants were washed several times in sterile distilled water.

**Callus induction**

Leaf explants of *G. sylvestre* were grown in MS medium (*Murashige and Skoog, 1962*) supplemented with 2,4-D (1.5 mg/l) and KN (0.5 mg/l). Callus culture was maintained at 25 ± 2 °C, 16 h/8 h (light/dark) photoperiod with 25 μmol m⁻² s⁻¹ of light intensity (Ahmed et al., 2009a).

**Callus developed under stress condition**

Callus cultures were maintained under different stress conditions for gymnemic acid production (Ahmed et al., 2009a). The protocol was as follows: different color light (blue, red, green and white fluorescent tubes); temperature (20 °C, 25 ± 2 °C, 30 °C and 35 °C); photoperiod (4 h/20 h; 8 h/16 h, 12 h/12 h, 20 h/4 h light/dark); sucrose (2%, 4%, 5%, 6%) and ammonium nitrate (1 mM, 2 mM, 3 mM, and 4 mM). Optimum callus biomass was determined using growth curve analysis, in all treatments.

**In vitro callus growth curve**

Plant growth regulators (PGRs) with stress treatment callus fresh and dry weight were determined at 15, 25, 35, 45 and 55 days. Maximum biomass (fresh, dry weight) and green compact callus were noted for all treatments.

**Phytochemical studies on leaf and in vitro callus**

*G. sylvestre* dried leaves and in *vitro* callus were extracted with methanol 5 times (Rehman et al., 2003). The collected methanol extract was centrifuged at 5000 × g for 10 min at room temperature, then the methanol supernatant carefully pipetted out into fresh eppendorf tubes without disturbing the interphase residues. Green-color supernatant (20 μl) was screened using TLC and HPTLC with standard gymnemic acid (Prof. Kazuko Yoshikawa, Kyoto Pharmaceutical University, Japan) for gymnemic acid quantification (Ahmed et al., 2009a). HPTLC was performed using a Camag HPTLC system (CAMAG, Switzerland) equipped with a sample applicator Linomat IV, TLC scanner III and integration software CAT 4.0. The mobile phase of Isopropyl alcohol:chloroform:methanol:acetic acid (5:3:1:0.5) was suitable for separation of gymnemic acid and scanned at 200 nm, respectively.

**HPLC studies in callus and leaf extracts**

Gymnemic acid was screened in leaf and callus (1 g dry wt.) extracts by above procedure. After centrifugation, an aliquot of the methanol supernatant (4 ml) was evaporated and dried. The residue (ca. 6 mg) was dissolved in MeOH (5 ml), and injected into an HPLC column (20 μl). For gymnemic acid separation, the following systems and protocols were used: water HPLC system (Shimadzu model, Japan), 510 pump, 7725 Rheodyne injector, C18 (ODS) reversed-phase column, water 486 UV detector, with mobile phase-water/methanol (35:65, HPLC grade), 0.1% acetic acid, sample applied: 20 μl, flow rate: 1 ml/min, read at 230 nm, on UV detector.

**Preparation of GS leaf and callus extracts**

In *vivo* Gymnema leaves extract (GLE) and in *vitro* callus extracts GCE₁–GCE₆ were prepared by the method reported previously (Ahmed et al., 2008). The extracted sample was evaporated and dissolved in saline water (Sterile NS saline, Baxter Pvt. Ltd., India) (200 mg/kg body wt./day/rat) given (P.O.) daily (Shanmugasundaram et al., 1990; Ahmed et al., 2008).

**Experimental animal**

All experiments were approved by Institutional Animal Ethics Committee (IAEC) procedure guidelines of CPCSEA. Male and female (8–9-month old) Wistar rats (body weight 200 ± 25 g each) were maintained at Bharathidasan University, Tiruchirappalli, on standard pellet diet (Hindustan Lever Ltd., Bangalore, India) with free access to tap water.

**Chemical preparation and induction of diabetes**

Alloxan monohydrate (Otta kemi, Mumbai, India) (100 mg/kg body wt./rat) was dissolved in distilled water and injected intraperitoneally (i.p.). The experimental rats entered a diabetic state after 72 h, then the Wistar rats were divided into groups for treatment with *G. sylvestre* leaf and callus extracts. Therapeutic human insulin (Torrent Pharmaceuticals Ltd., Mehsana, India) mixture was shaken well and injected daily (4U/kg body wt.) subcutaneously as a diabetic standard (Chattopadhyay 1998).

**Instruments and methods**

The body weight, liver and pancreas weights of the experimental Wistar rats were checked in fundamental weight instruments. Liver glycogen (*Raghiramalu et al., 1983*) and pancreas were removed for further analysis.

The removed pancreas was fixed in dewaxed and hydrated paraffin for micro section (6 μm thickness), then stained with hematocytin and eosin. In addition, stained pancreas was washed with Gomori’s aldehyde fuchsin for determination of β-cells in Langerhans islets (*Gomori, 1950*) as described below. For β-cells determination, the 0.5 g basic fuchsin was dissolved in 70% alcohol, to which were added 1.0 ml paraformaldehyde with 1.5 ml conc. HCl. Embedding hydrated paraffin sections were treated with Lugol iodine solution for 30 min, then bleached using 5% sodium thiosulfate for 2 min followed by washing of the section for 5 min in running tap water for oxidation. The β-cells were rinsed with 95% and 70% alcohol for 10 min, and then washed with distilled water followed by Ehrlich’s hematoxylin for 4 min and counter stain for 45 s, respectively. Finally, the stain coupling jar was treated with aldehyde fuchsin for 2–10 min. The end point of β-cells stood out clearly in dark purple against a colorless background.

**Treatment groups**

Group I (control): free access to tap water.
Groups II–X: diabetes-induced animals were divided into 9 groups administered saline, 4U/kg body wt. of insulin/day, 200 mg/kg body wt. (rat)/day GLE and GCE₁–GCE₆, respectively. The experimental Wistar rats were sacrificed at 10, 20 and 30 days.

The following treatment groups were used in this experiment: Group II – diabetic control (Alloxan); Group III – Diabetic standard (insulin); Group IV – diabetic rats + *G. sylvestre* leaf extract; Group V (GCE₁) – blue light with MS + 2,4-D (1.5 mg/l) + KN (0.5 mg/l); Group VI (GCE₂) – 5% sucrose with MS + 2,4-D (1.5 mg/l) + KN (0.5 mg/l); Group VII (GCE₃) – 12 h photoperiod MS + with 2,4-D (1.5 mg/l) + KN (0.5 mg/l); Group VIII (GCE₄) – 3 mM NH₄NO₃ with MS + 2,4-D (1.5 mg/l) + KN (0.5 mg/l), Group IX (GCE₅) – MS + 2,4-D (1.5 mg/l) + KN (0.5 mg/l) and Group X (GCE₆) – MS + NAA (1.0 mg/l) + KN (1.5 mg/l), respectively.
Statistical analysis

Only data which showed an advantageous effect were included in tables and presented in mean ± SE (replicated 3 times). Experimental design was completely random and factorial with control, alloxan alone, alloxan with insulin, alloxan with leaf and alloxan with callus extracts. The data were subjected to analysis of variance and mean separation was carried out using Duncan’s multiple-range test (DMRT) at 5% level significance (Gomez and Gomez, 1976).

Results and discussion

Callus initiation under stress conditions

In vitro callus failed without PGRs in MS medium, while different media (MS, B5, SH, WPM) with PGRs affected the callus initiation in leaf explants (data not shown). Trial of auxins alone, and auxins with cytokinins combinations showed callus induction, among which 2,4-D (1.5 mg/l) with KN (0.5 mg/l) induced green compact callus with maximum dry weight at 45 days.
Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g) of Wistar rats with leaf and callus (methanol) extracts (GSC1–GSC6) a</th>
<th>Liver weight (g) of Wistar rats with leaf and callus (methanol) extracts (GSC1–GSC6) a</th>
<th>Liver glycogen (mg/g) of Wistar rats with leaf and callus (methanol) extracts (GSC1–GSC6) a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group IV</td>
<td>BMI weight (g/animal)</td>
<td>Liver weight (g/animal)</td>
<td>Liver glycogen (mg/g)</td>
</tr>
<tr>
<td>Days</td>
<td>10th</td>
<td>20th</td>
<td>30th</td>
</tr>
<tr>
<td>Group I</td>
<td>213.0f</td>
<td>212.0f</td>
<td>211.0f</td>
</tr>
<tr>
<td>Group II</td>
<td>215.0g</td>
<td>214.0g</td>
<td>213.0g</td>
</tr>
<tr>
<td>Group III</td>
<td>217.0e</td>
<td>216.0e</td>
<td>215.0e</td>
</tr>
<tr>
<td>Group IV</td>
<td>220.0d</td>
<td>219.0d</td>
<td>218.0d</td>
</tr>
<tr>
<td>Group V</td>
<td>223.0c</td>
<td>222.0c</td>
<td>221.0c</td>
</tr>
<tr>
<td>Group VI</td>
<td>225.0b</td>
<td>224.0b</td>
<td>223.0b</td>
</tr>
<tr>
<td>Group VII</td>
<td>228.0a</td>
<td>227.0a</td>
<td>226.0a</td>
</tr>
<tr>
<td>Group VIII</td>
<td>231.0</td>
<td>230.0</td>
<td>229.0</td>
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<tr>
<td>Group IX</td>
<td>234.0</td>
<td>233.0</td>
<td>232.0</td>
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</table>

a Values are mean of 3 replicates per treatment and repeated thrice. Values with the same letters are not significantly different at 5% probability level according to DMRT.

Kumar et al. (2002) reported MS medium with PGRs were suitable for callus proliferation of G. sylvestre, but callus proliferation in other auxins with cytokinins concentration was not pursued, because of their poor quality and production (data not shown). Recently we have reported on callus production in different media such as MS, SH, WPM and BS media, among which MS media with auxins and cytokinins were suitable for callus production (Ahmed et al., 2009a). Stress treatment and callus growth curve were screened at different days (15, 25, 35, 45 and 55 days). Lag phase (15–25 days), callus initiation and proliferation were observed. 25–35 days (exponential phase), biomass and green compact callus increased significantly. However, stationary phase at 45 days, maximum biomass and green compact callus were shown. Decline phase, the nature and biomass of callus were significantly reduced as compared to other phases. In Asclepiadaceae species, the stationary phase was observed at 22 days; at this phase cryptosin was isolated from Cryptolepis buchanani (Venkateswara et al., 1987) and gagaminine from Cynanchum wilfordii (Shin et al., 2003).

Optimized concentration was maintained under stress conditions; leaf explants under blue light, 5% sucrose induced the maximum biomass with green compact callus, then photoperiod, temperature and ammonium nitrate concentrations (Fig. 1A–E). Photoperiod and temperature affected physical appearance, producing white watery and white friable callus (data not shown). These calluses were stored for a long time and the media turned brown in color. Blue light revealed green compact callus with maximum biomass at 45 days. Cryptochrome showed a top peak at 450 nm, a wavelength close to the blue light; under these conditions, more Pr transformed into Pfr for phenyleanumeric acid. Standard gymnemic acid showed as a single brown band; however, the callus extract displayed additional brown bands under these conditions, more Pr transformed into Pfr for phenyleanumeric acid. Standard gymnemic acid showed as a single brown band; however, the callus extract displayed additional brown bands under these conditions, more Pr transformed into Pfr for phenyleanumeric acid. Standard gymnemic acid showed as a single brown band; however, the callus extract displayed additional brown bands under these conditions, more Pr transformed into Pfr for phenyleanumeric acid. Standard gymnemic acid showed as a single brown band; however, the callus extract displayed additional brown bands under these conditions. Various with the same letters are not significantly different at 5% probability level according to DMRT.

TLC, HPTLC, HPLC studies

Methanol supernatants of G. sylvestre leaf and in vitro calluses were screened using TLC and HPTLC. After chromatogram, samples were dried and sprayed with specific reagent (vanillin sulfuric acid reagent) at room temperature for detection of gymnemic acid. Standard gymnemic acid showed as a single brown band; however, the callus extract displayed additional brown bands with Rf value (0.44) greater than that of gymnemic acid (0.43). In HPTLC analysis, methanol solvent was run up to 80 mm and scan chromatograms at 200 nm under UV reflectance mode. Gymnemic acid content leaf and callus extract data were compared with standard gymnemic acid. The calibration curve was linear; the correlation coefficient indicated good linearity between concentration and area. To ascertain peak purity of test sample, we compared in vivo and in vitro methanol extract reflectance spectra with standard, which provides test sample purity (data not shown). Gymnestrogenin (triterpenoids) determine in G. sylvestre through HPTLC at 293 nm (Puratchimani and Jha, 2004). For HPLC analysis, leaf and callus methanol extracts (20 µl) were uploaded in HPLC system to quantify gymnemic acid under retention time. UV spectrophotometer peak area data were compared with standard gymnemic acid. The gymnemic acid content was increased in leaf, GSC1 and GSC2 over other callus extracts (data not shown). Imnoo et al., 1991 reported that a methanol extract contains gymnemic acid through HPLC. Many authors had isolated and identified gymnemic acid earlier. In 1989, Yoshikawa and co-workers isolated gymnemic acids from a hot water extract of G. sylvestre, which they named gymnemic acids I, II, III, IV, V, VI and VII, respectively, and evaluated using HPLC (Sugihara et al., 2000).
Histologic examination of pancreatic β-cells in alloxan-treated animals. (A) Pancreas of normal rats. Note abundant patches of β-cells (arrows) in purple color (200×); (B) pancreas of alloxan-treated rats, hardly and β-cells noticed, all islets degenerated (200×); (C) pancreas of alloxan-treated rats, after insulin treatments showed abundant patches of β-cells (arrows) in purple color (200×); (D) pancreas treated rats, after G. sylvestre leaf extract (200×); (E) pancreas treated rats, after G. sylvestre callus extract 1 (200×); (F) G. sylvestre callus extract 2 (200×); (G) G. sylvestre callus extract 3 (200×); (H) G. sylvestre callus extract 4 (200×); (I) G. sylvestre callus extract 5 (200×); (J) G. sylvestre callus extract 6 (200×). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Body, liver, pancreas weight and liver glycogen

As shown in Table 1, the long-term antihyperglycemic effect was examined for GS leaf and callus extracts. The whole body, liver and pancreas weights were reduced in the diabetic control group after 30 days, although oral administration of 200 mg/kg body wt. GS leaf and callus extracts significantly increased and maintained the body liver and pancreas weights in the 4 U/kg body wt. insulin group Wistar rats. We have recently published a review of the pharmacological activities, a phytochemical investigation and in vitro studies of G. sylvestre (Ahmed et al., 2009b).

Liver glycogen was significantly decreased in diabetic control rats, whereas with leaf and callus extracts of GS, liver glycogen significantly increased (Table 1). Restoration of hepatic glycogen by GS leaf and callus could improve the insulin secretion or inhibition of glucose-6-phosphatase in liver, and has prevented the conversion of glucose 6-phosphate to glucose (Shanmugasundaram et al., 1990). Shanmugasundaram et al. (1983) reported that G. sylvestre restored glycogen and enzymes in diabetic rabbit liver after 24 weeks of treatment. We earlier reported that leaf and callus extracts of G. sylvestre reduced blood sugar and lipid profiles such as cholesterol, triglyceride, HDL, LDL, VLDL in alloxan-induced diabetic Wistar rats (Ahmed et al., 2008). Shanmugasundaram et al. (1990) reported aqueous extract of G. sylvestre leaf (200 mg/kg body wt.) normalized blood sugar level in STZ-induced diabetic rats. However, gymnemic acid from G. sylvestre leaf regulated hyperglycemia (Gholap and Kar 2005) and an aqueous extract maintained the blood glucose level in normal Wistar rats (Rafiullah et al., 2006).

Histological examinations of pancreas

Fig. 2A–J depicts the histological examination (200×) of pancreatic β-cells in control, diabetic control, diabetic standard, GS leaf and callus extracts groups. Histological pancreas islets were checked separately in hematoxylin and eosin with aldehyde fuchsin section. In hematoxylin and eosin sections, the degree of atrophy, hydropic degeneration, necrosis, hyalinization, or fibrosis was checked (data not shown), while in aldehyde fuchsin sections, the β-cells percentage was recorded based on atrophy cells, and the β-granules were increased or decreased at the 10th, 20th and 30th
days (Table 2). In electron microscopic analysis, the pancreatic β-cells islets showed no pathological alteration in control Wistar rats. The nucleus, nuclear envelope, mitochondria, endoplasmic reticulum and Golgi complex of β-cells were normal (Fig. 2A). However, 80% of β-cells contained a number of secretory granules, which had a space between core and membranes diffusely distributed, no cytoplasm at end of experiment. The secretory granules showed moderate homogenous (or) slightly heterogenous electron density and single layered membrane (Fig. 2A).

Electron microscope analysis of β-cells in diabetic standard rats (alloxan alone) displayed the loss of nuclear envelope, mitochondrial vacuolization, swelling and dilatation of endoplasmic reticulum (Fig. 2B). During alloxan treatment, β-cytotoxin causes a massive destruction of β-cells in islets of Langerhans, and reduces the β-cells and insulin release (Colca et al., 1983). Diabetic standard (insulin), GS leaf and callus extracts treated groups showed significant increase in the secretory granule of Oβ-cells and reached to control level (Fig. 2D–J). The β-cells were apparently normal in the architecture of the nucleus, which revealed that gymnemic acid prevented the β-cells damage as compared to control rats. But low gymnemic acid content callus extracts decreased the secretory granules and losses of nuclear envelope, mitochondrial vacuolization, swelling and dilatation of the endoplasmic reticulum in diabetic rats.

Degenerative changes in pancreatic β-cells were minimized and normal morphology maintained in diabetic rats by administration of gymnemic acid content extracts as evidenced in histopathological examination. Pancreas hematoxylin/eosin with aldehyde fuchsin stains displayed the ruptured islets and decreased β-cells in diabetic control rats, whereas leaf and callus extracts regenerated the β-cells frequency in diabetic standard (Fig. 2C; 45.5%), GS leaf extract (Fig. 2D; 66.6%), leaf callus extract 1 (Fig. 2E; 59.6%), callus extract 4 (Fig. 2H; 39.0%), callus extract 5 (Fig. 2I; 45.0%) and callus extract 6 (Fig. 2J; 37.6%) (Table 2). In treatment pancreatic β-cells, AMP was activated and the protein kinases showed a unique function or connecting cellular energy status to the ability of insulin synthesis and secretion of β-cells (Da Silva Xavier et al., 2000). Alcohol extract of Gymnema sylvestre stimulated the insulin secretion in islets of Langerhans and pancreatic β-cell lines (Persaud et al., 1999). Srivastava et al. (1986) reported that pancreatic tissue was completely destroyed in alloxan treatment, after G. sylvestre leaf extract regenerated the prolonged survival and adaptogenic activity. However, a smaller dose of STZ (45 mg/kg body wt.) led to damage of the pancreatic acinar cells and reversed by Aegle marmelos (Arumugam et al., 2008). Successful β-cell regeneration was achieved using diabetic medicinal plants such as Momordica charantia, Beta vulgaris, Catharanthus roseus, and Eugenia jambolana (Das et al., 1996; Sharma et al., 2006).

In conclusion, the leaf and callus extracts of G. sylvestre have antihyperglycemic effects as determined using alloxan-induced diabetic Wistar rats. Our experimental data suggested that alloxan administered to Wistar rats through intraperitoneal route exhibits diabetic activity. We conclude that the effect of G. sylvestre leaf and callus extracts as strong herbal remedies is confirmed in this study, and suggest that they may be capable of fully restoring pancreatic β-cells function and thus curing type I diabetes. Studies of G. sylvestre callus extract have revealed how β-cells may be formed and regenerated in vitro and could provide further direction for potential drug development for IDDM treatment.

References

Table 2
Leaf and callus extracts of Gymnema sylvestre regenerate the pancreatic β-cells in alloxan-induced diabetic Wistar rats.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>β-Cell regeneration frequency (%)</th>
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<tbody>
<tr>
<td></td>
<td>10th day</td>
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<tr>
<td>Group I (normal)</td>
<td></td>
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<tr>
<td>Group II (diabetic control)</td>
<td></td>
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<td>Group III (insulin)</td>
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<td>Group IV (leaf)</td>
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<td>Group V (callus extract 1)</td>
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<td>Group VI (callus extract 2)</td>
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<tr>
<td>Group VII (callus extract 3)</td>
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<tr>
<td>Group VIII (callus extract 4)</td>
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<tr>
<td>Group IX (callus extract 5)</td>
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<tr>
<td>Group X (callus extract 6)</td>
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</table>

Values are mean of 3 replicates per treatment and repeated thrice. Values with the same letters are not significantly different at 5% probability level according to DMRT.


