2014 4th International Conference on Biotechnology and Environment Management
IPCBEE vol.75 (2014) © (2014) IACSIT Press, Singapore
DOI: 10.7763/IPCBEE. 2014. V75. 14

Optimization of *Gymnemic acid* production with anti-diabetic studies and regeneration of Langerhans cells from *Gymnema sylvestre*

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Abstract. Gymnemic acid (GA), a well-known anti-diabetic compound has been detected in methanol extracts of intact leaves and *in vitro* callus cultures derived from leaf explants of *Gymnema sylvestre* using HPTLC and HPLC. The present study, the leaf explants of *G. sylvestre* induced in MS medium supplemented with 2,4-D (1.5 mg L^{-1}) and KN (0.5 mg L^{-1}) induced the maximum callus biomass, green compact callus at 45 days of culture determined by growth curve analysis. The plant growth regulators of 2,4-D and KN induced the formation gymnemic acid under all light conditions studied except dark conditions. Analysis of the callus growth phase under all treatments revealed that gymnemic acid accumulation was maximum (12.22 mg/g d.w) in the callus during stationary phase of 45 days. Green–white coloured callus cell groups contains gymnemic acid and formed on the surface of the callus culture under blue light respectively. In addition, the callus culture of *G. sylvestre* was cultivated under different light conditions (white, red, blue, green light) stress. The plant growth regulators kinetins enhance the gymnemic acid formation in blue light, but were unable to induce gymnemic acid in darkness. Factors such as light, temperature, sucrose and photoperiod were studied to observe their effect on GA production. Temperature conditions completely inhibited GA production. Out of the different sucrose concentrations tested, the highest yield (35.4 mg/g d.w) was found at 5% sucrose followed by 12 h photoperiod (26/86 mg.g d.w). Maximum GA production (58.28 mg/g d.w) was observed in blue light. Blue light enhanced by gymnemic acid accumulation up to 4.4 fold of that found under white fluorescent light and 2.8 fold of that found in intact leaves. Gymnemic acid detection and quantification were carried out using thin-layer chromatography (TLC), high performance thin-layer chromatography (HPTLC), High-performance liquid chromatography (HPLC) and gravimetric techniques. GA detection peak area and their absorption spectra were evaluated through HPTLC and HPLC with the standard GA. Quantification of GA had showed the linearity, accuracy, robustness and precision by HPLC. GA content was significantly higher in gravimetric method than HPLC. In addition, we have characterized the gymnemic acid role in anti-diabetic experiment; 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 obesity related mechanism and the determinants implicated.

Keywords: *gymnema sylvestre*, callus culture, HPTLC, HPLC, Gymnemic acid, Anti-diabetic studies

1. Introduction

Gymnema sylvestre is an important medicinal plant belonging to the family Asclepiadacae. This climber plant is extensively used in almost all the Indian systems of medicine as a remedy for rheumatism, cough, ulcer, and pain in the eyes [1]. The root of this plant has been reported as a remedy for snakebite [2]. The plant occurs mainly in the Deccan peninsula of western India, Tropical Africa, Vietnam, Malaysia, Sri Lanka and is widely available in Japan, Germany, and the USA as a health food. In Japan, there are Gymnema Teas and Gymnema chewing gum made from *G. sylvestre* leaves which are promoted as a natural method for

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controlling obesity, insulin secretion, diabetes and inhibiting the taste of sweetness [3]. Several products are marketed under brand names such as Body Slatto Tea® (18.95 $), Gymnema® (7.00 $), Gymnema Tea® (15.50 $), Gymnema Diet® (29.0 $), Sugar Off® (49.20 $), Glucoset™ (12.50 $), Cinndrome X™ (41.90 $) and Pilisoft™ (17.85 $) respectively.

In vitro techniques are very useful in ensuring sustainable, optimized sources of plant-derived natural products. However, *ex situ* cultivation should be preceded by proper evaluation of the plants for their ability to produce the required bioactive constituents before commencing cultivation or introducing the technology to potential growers. The ability of the plants to produce certain bioactive substances is largely influenced by the physical and chemical environments in which they grow. Plants also produce certain chemicals to overcome abiotic stresses. Plant use light not only as an energy source for photosynthesis, but also as an important environmental signal. Light can affect morphogenesis and the formation of plant metabolites as a signal and stress factor from phytohormones. In most plant cell cultures, secondary metabolism, including the production of phenolic terpenoid and alkaloid compounds, is stimulated by light.

Gymnema sylvestre is an important medicinal climber belongs to the family Asclepiadaceae.This climber is extensively used in almost all the Indian systems of medicines a remedy for rheumatism, cough, ulcer, and pain in the eyes. It is also useful for inflammations, cough, ulcer, and pain in the eyes. It is also useful for inflammations, dyspepsia, constipation, jaundice, and so forth. The roots of this plant have been reported as a remedy for snakebite. The plants occur mainly in the Deccan peninsula of western India, Tropical Africa, Vietnam, Malaysia, Sri Lanka and is widely available in Japan, Germany and the USA as a health food. Previously reported that the extracts of G. sylvestre plays a major role in blood glucose homeostasis through increased serum insulin level through regeneration of the endocrine pancreas. The present report is an advancement over the earlier protocol because it describes the establishment of in vitro callus from the leaf explants of *G. sylvestre* and the enhancement of GA using various types of abiotic stress factors and quantified gymnemic acid via TLC, HPTLC and HPLC. In addition, to identy the isolated G. sylvestre callus extract molecules that contribute to or promote beta cell regeneration, maintained the lipid profile such as Triglyceride, cholesterol, HDL, LDL, VLDL; it was found that pancreas weight and glycogen content were increased in the liver of alloxan-induced diabetic Wistar rats.

2. Results and Discussion

2.1. Callus induction under abiotic 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). Callus induction was obtained in MS medium supplemented with auxins and cytokinins in leaf explants of *G. sylvestre.* MS with 2,4-D (1.5 mg L^{-1}) and KN (0.5 mg L^{-1}) had induced the green compact callus, and maximum biomass was determined by growth curve analysis between 35-45 days of stationary phase, whereas other auxins and cytokinins combinations had induced the green friable, brown friable, white friable and white watery callus. Adenine sulphate was added to the optimum plant growth regulators, concentration induced the green compact callus and biomass drastically reduced in leaf explants of *G. sylvestre.* The analysis of G. sylvestre callus growth in batch culture under blue light conditions showed the green compact callus yield higher biomass at stationary phase of 35-45 days of the culture (Fig. 1). Influence of temperature has many effects on the mechanisms of metabolic regulation, permeability, nutritional needs, and the rate of intracellular reactions in plant cell cultures. In our experiment 30℃ increased the callus biomass and gymnemic acid content than control treatment.

2.2. TLC, HPTLC, HPLC analysis

Gymnema sylvestre in vivo leaf and *in vitro* callus extracted with methanol supernatants were screened using TLC, HPTLC and HPLC. In TLC and HPTLC the 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 gymnemic acid standard (0.43) (Fig. 2). In HPTLC analysis, methanol solvent was run upto 80 mm and scan chromatogramsat 200 nm under UV reflectance mode.

Gymnemic acid content leaf and callus extract data were compared with standard gymnemic acid. For HPLC analysis, the leaf and callus methanol extracts (20 µl) were upload in HPLC system to quantify gymnemic acid under retention time (Fig. 3 and 4). Imoto et al., 1991 repored that a methanol extract contains gymnemic acid through HPLC. Green friable *in vitro* callus was induced MS medium supplemented with NAA (1.0 mg L^{-1}) and 2,4-D (1.5 mg L^{-1}), and the GA content was drastically reduced than cytokinins and auxins concentrations in 35-45 days of stationary phase. The above mentioned optimum growth regulators sample kept under abiotic stress conditions and the developed callus methanol extracts were further analyzed. Blue light with optimum plant growth regulators was induced the maximum GA (53.94 mg g^{-1}) than 5% sucrose treatment (33.39 mg g^{-1}) followed by 3mM NH_4NO_3. Gymnemic acid content was reduced in this order 12 h photoperiod, red light, green light and 30 ℃ respectively. In case of dark treatment, GA content was absent.

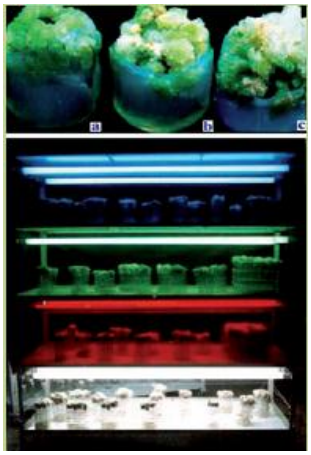

Fig. 1: *In vitro* callus induction under abiotic stress conditions.

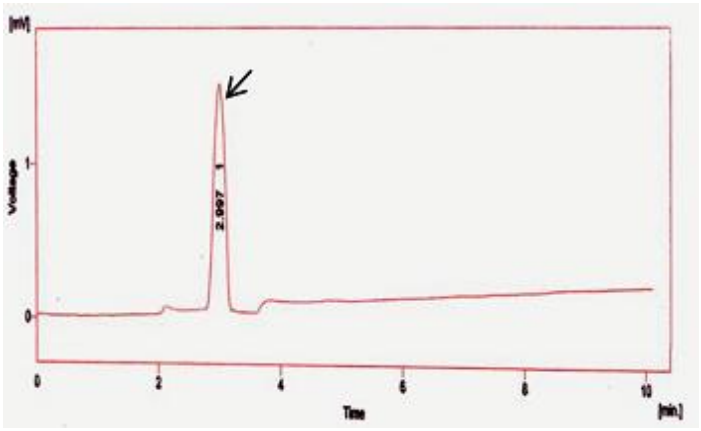

Fig. 2: Standard gymnemic acid

The present study, the leaf explants of *G. sylvestre* induced in MS medium supplemented with 2,4-D (1.5 mg/l) and KN (0.5 mg/l) induced the maximum callus biomass, green compact callus at 45 days of culture determined by growth curve analysis. The plant growth regulators of 2,4-D and KN induced the formation gymnemic acid under all light conditions studied except dark conditions [4]. Analysis of the callus growth phase under all treatments revealed that gymnemic acid accumulation was maximum (12.22 mg/g d.w) in the callus during stationary phase of 45 days. Green–white coloured callus cell groups contains gymnemic acid and formed on the surface of the callus culture under blue light respectively. In addition, the callus culture of *G. sylvestre* was cultivated under different light conditions (white, red, blue, green light) stress. The plant growth regulators kinetins enhance the gymnemic acid formation in blue light, but were unable to induce gymnemic acid in darkness. Factors such as light, temperature, sucrose and photoperiod were studied to observe their effect on GA production. Temperature conditions completely inhibited GA production. Out of the different sucrose concentrations tested, the highest yield (35.4 mg/g d.w) was found at 5% sucrose followed by 12 h photoperiod (26/86 mg.g d.w). Maximum GA production (58.28 mg/g d.w) was observed in blue light. Blue light enhanced by gymnemic acid accumulation up to 4.4 fold of that found under white fluorescent light and 2.8 fold of that found in intact leaves. Gymnemic acid detection and quantification were carried out using thin-layer chromatography (TLC), high performance thin-layer chromatography (HPTLC), High-performance liquid chromatography (HPLC). GA detection peak area and their absorption spectra were evaluated through HPTLC and HPLC with the standard GA. Quantification of GA showed the linearity, accuracy, robustness and precision by HPLC. GA content was significantly higher in HPLC.

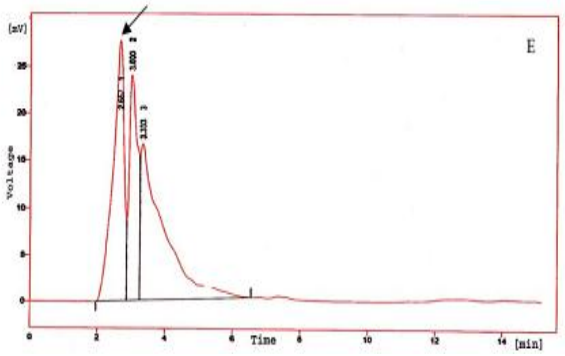

Fig. 3: *In vivo* leaf extract content gymnemic acid

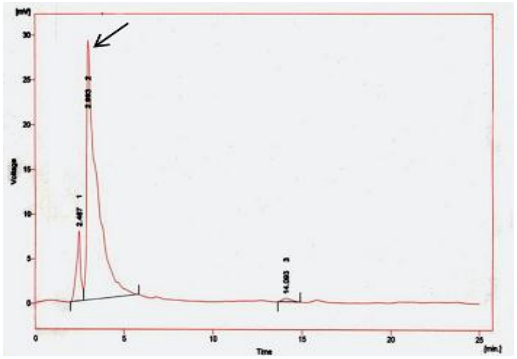

Fig. 4: Blue light callus extracts content

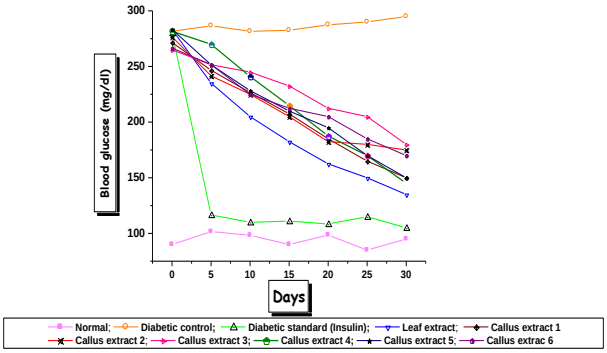

Fig. 5: Blood glucose treatment in Wistar rats

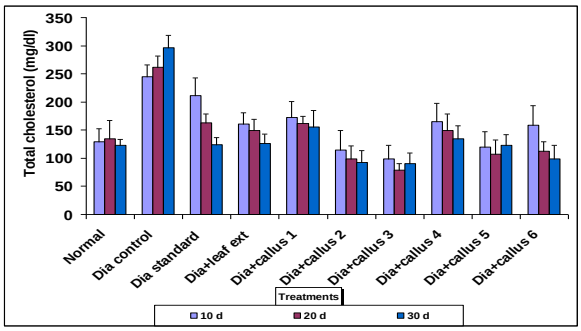

Fig. 6: Total cholesterol level in Wistar rats

Table 1**:** Regeneration of pancreatic beta – cells in diabetic rats treated with methanol extracts of *Gymnema sylvestre*

Treatment group	Beta-cell regeneration frequency (%)		
	10^{th} day	20^{th} day	30^{th} day
I - Normal	81.0^{a}	83.6^{a}	80.0^{a}
II -Diabetic control	16.0^{ij}	12.3^{j}	8.6^{j}
III - Insulin	51.0^{d}	53.0^{d}	62.5^{bc}
IV – Leaf extract	49.0^{de}	58.6^{b}	66.6^{b}
V - Callus extract 1	53.3^{b}	50.0^{de}	59.6^{d}
VI - Callus extract 2	42.3^{f}	47.0^{f}	54.3^{f}
VII - Callus extract 3	51.3^{bc}	55.0^{bc}	57.6^{de}
VIII - Callus extract 4	30.3^{g}	32.6^{gh}	39.0^{gh}
IX - Callus extract 5	28.0^{gh}	31.6^{i}	45.0^{g}
X - Callus extract 6	27.0^{i}	34.0^{g}	37.6^{i}

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

2.3. Anti-diabetic studies

The chronic administration of *G. sylvestre* leaf and callus extracts (200 mg/kg/day, P.O) resulted significant reduction in plasma glucose level at different periods in the experimental duration of 30 days in diabetic Wistar rats [5]. However, the standard drug insulin (4U/Kg/day I.V) exhibited significant and oral potent antidiabetic activity with maximum percent reduction of plasma glucose (72.4%) on 30 day compared to the diabetic control group (Fig. 5). Alloxan treatment caused a significantly increase of blood glucose

level i.e., above 290 mg/dl. Treatment of crude methanol extract of the leaf and callus exracts decreased blood sugar significantly (Fig. 5). All callus extracts proved to be anti-diabetic the esult was follows: leaf extract 283.3 to 135.0 mg dl^{-1}; callus extract 1: 271.6 to 130 mg dl^{-1}; callus extract 4: 281.6 to 145.0 mg dl^{-1}; callus extract 5: 283.3 to 150 mg dl^{-1}; and callus extract 6: 266.6 to 170.0 mg dl^{-1} (Figure 5).This study aims to assess whether or not a methanol extract of *Gymnema sylvestre* is able to normalize blood glucose and lipid profile levels in experimentally-induced diabetic Wistar rats. Same doses of the extracts were administrated orally for 40 days. The rats were bled at the beginning of the experiment and at 10 days intervals. Blood glucose, cholesterol, triglyceride, very-low density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoproteins (HDL) were estimated. Results showed that *G. sylvestre* leaf and blue light-induced *in vitro* callus extracts normalized blood glucose level (72.4%) on 30^{th} as compared to the diabetic control group, reduced triglyceride, VLDL, LDL levels and increased HDL level (Fig. 6-11). The research reported here deals with leaf and callus extracts of *Gymnema sylvestre*, which significantly increase the weight of the whole body, liver, pancreas and liver glycogen content in alloxan-induced diabetic rats (Wistar rats) (data not shown). The gymnemic acid of leaf and callus extracts significantly increases the regeneration of β-cells in treating rats, when compared with the standard diabetic rats (Figure 12; Table 1). The Wistar rats animal studies reverted to a diabetic state once the *G. sylvestre* leaf and callus extracts were discontinued. In addition, we have characterized the gymnemic acid role in anti-diabetic experiment; 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 obesity related mechanism and the determinants implicated.

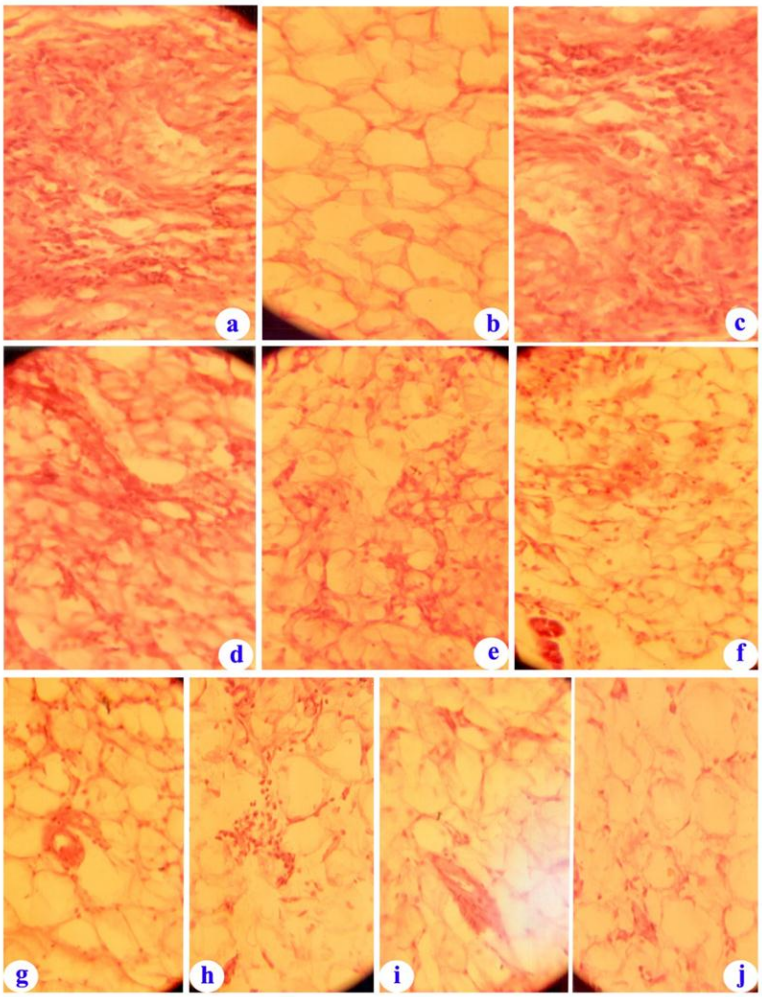

Fig. 7: Histological examination of pancreatic β-cells in treated animals

a-Normal rats; b-Diabetic control; c-Diabetic standard (insulin); d-*G. sylvestre* leaf extract; e-*G. sylvestre* callus 1; f-callus extract 2; g-callus extract 3; h-callus extract 4; i-callus extract 5; j-callus extract 6.

3. Acknowledgements

This work has been supported by the University Malaya Research Grant from University of Malaya, Kuala Lumpur, Malaysia and Bharathidasan University, Tiruchirappalli, Tamil Nadu, India.

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