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Research Article

IN VITRO DERIVED CALLUS AND SHOOT OF A MEDICINAL HERB *SOLANUM TRILOBATUM* AND THEIR EFFECT ON HEPATOCELLULAR CARCINOMA

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ABSTRACT

Objective: To develop a protocol for culturing callus and regenerate shoots *invitro* from the leaf of *Solanum trilobatum* and study its anti-hepatocarcinogenic effect in an animal model.

Methods: Surface sterilized explants (0.5-1.0 cm) length were placed on the MS basal medium supplemented with various concentrations of 2, 4 D (0.45, 2.26, 4.52, 11.31 and 12.56 μM), NAA (0.54, 1.34, 2.69, 5.37, 13.43 and 26.85 μM), BA (0.44, 1.11, 2.22, 4.44, 8.88 and 13.32 μM) for callus induction. MS basal medium supplemented with 10% coconut milk and various concentrations of BAP (2.2 - 22 μM) dissolved in ethanol was used for shoot initiation. Rats divided into four groups were administered with DEN and DEN (200 mg/kgbwt) i.p along with methanol extracts of leaves, callus and shoot of S.T (250 mg/kgbwt) orally for 3 months.

Results: Rats administered with DEN showed significant deviation ($p < 0.05$) of marker enzymes ALT, AST, LDH and TB in the serum.

Conclusion: Administration of crude extracts of leaf, callus and shoot of S.T offers better protection against hepatocellular carcinoma.

Keywords: *Solanum trilobatum* extracts, Marker Enzymes, Callus induction, Hepatocellular carcinoma.

INTRODUCTION

Liver is the largest and most complex internal organ in the body. It plays an important role in metabolism, detoxification and excretion of xenobiotic compounds [1, 2]. The liver is normally involved in the metabolism of proteins, fats and carbohydrates. The treatment for liver diseases has become a challenging problem of the modern medicine [3]. A number of herbal preparations have been advocated for treating liver diseases. Research investigations conducted on several plant products as liver protective are well documented [4]. Additionally, recent studies have suggested that natural antioxidants in complex mixtures ingested with the diet are more efficacious than pure compounds in preventing oxidative stress-related pathologies due to particular interactions and synergism [5]. Among the various plants associated with the life of Indians, one such plant is ST. Linn [family: Solanacea] that is used traditionally in different parts of India for various ailments [6].

There are some reports of the active Principles isolated from plant parts having anti hepatotoxic effects against various toxicants. Curcuminods from curcumalonge rhizomes [7] well found to offer protection against CCl_4 and galactosamine poisoning. However, studies on the production of active principles using plant tissues culture techniques for liver disorders are scanty. Keeping in mind the lack of reports in this aspect and the advances and advantages of plant tissue culture techniques in producing secondary metabolites, the present study has been taken up. This work focuses on the liver protective effect of the leaf, callus and shoot regenerated *invitro* from the Medicinal herb against S.T against DEN toxicity in an animal model.

MATERIALS AND METHODS

Plant material

The healthy plants of *Solanum trilobatum* were collected from Madhavaram and plant were raised in pots containing soil and Farm Yard Manure (FYM) in 1:1 ratio under Green house condition at Poonga Biotech Research Centre, Chennai - 600 113, India.

Explants preparation

The explants were surface sterilized following the procedures as described by Janarthanam and Sumathi (2010) [8]. The *Solanum trilobatum* explants were collected from healthy potted plants kept in the green house. The explants namely node and juvenile leaf were washed thoroughly in running tap water and then washed with 70% ethanol. The explants were then immersed in 0.1% (w/v) mercuric chloride ($HgCl_2$) for 5 minutes for surface sterilization and then washed repeatedly in sterile double distilled water three to five times to remove traces of $HgCl_2$ adhering to the explants. The cut ends of the explants were again trimmed with the help of sterile blade to eliminate any possible residue of sterilant and the explants were used for culturing.

Preparation of tissue culture media

Murashige and Skoog (1962) [9] medium was used as sole basal medium. The composition of media is given in Table. 1. Four individual stock solutions of macro nutrient, micro nutrient, micro-iron and vitamins were prepared and stored. Stock solutions of salts were prepared using double distilled water and the required volume made up to with standard volumetric flask. Iron stock solution was stored in amber bottle to prevent photolysis. All the stock solutions were stored in at 4°C and meso-inositol; cytokinin and auxin stock solutions were freshly prepared and used every month. For preparation of medium, all four stock solutions were mixed thoroughly with required amounts of sterile distilled water. Sucrose 3% [30 g/L], 0.01% meso-inositol [100mg/L] and required amount of plant growth regulators [PGRs] were added and the pH was adjusted to 5.6 ± 0.2 with 1N HCL or 1N NaOH prior to autoclaving. The gelling agent agar [Himedia Grade 301] 0.9% [w/v] was added to the prepared media constituents and mixed well before dispensing into glassware. The contents were labeled and sterilized in an autoclave at 15 lb pressure for 15-20 minutes at 121°C. After inoculating with explants, all the culture vials were kept under 16/8h [light/dark] photoperiod at 25 ± 2 °C.

Culture conditions

The cultures were incubated in culture chamber at 25 ± 2°C for the light condition, the culture vials were placed on the rack at a distance of 25 cm from the light source. A 16/8 h [light / dark] photoperiod of cool white light was provided from 2000 Lux.

Callus induction

Healthy and disease free young green leaves were collected from four months old mother plants and they were used as explants for callus induction. Surface sterilized explants [0.5-1.0 cm] length were placed on the MS basal medium supplemented with various concentrations of 2, 4 D [0.45, 2.26, 4.52, 11.31 and 12.56 μM], NAA

[0.54, 1.34, 2.69, 5.37, 13.43 and 26.85 μM], BA [0.44, 1.11, 2.22, 4.44, 8.88 and 13.32 μM] for callus induction. The optimum callus were inoculated on combination of auxin and cytokinin for maximum callus induction and the culture incubated under dark at 25 ± 2°C for 5 days. These were then transferred into light condition.

Establishment of culture

Primary callus was established from the nodal explants. For secondary callus production, a small portion of primary callus was excised using sterile knife holder and was subcultured periodically once in three weeks. The secondary callus was used for all experimental studies Regenerated callus from the above was used for shoot initiation. MS basal medium supplemented with 10% coconut milk and various concentrations of BAP [2.2-22μM] dissolved in ethanol was used for shoot initiation. Shoots produced at least four roots of 2-3 cm length and 6-8 leaves were transferred to a plastic cup in a vermiculite and red soil [3:1] mix for two weeks before transferring to a potting mix under glass house conditions for further studies.

Animals

Male wistar rats weighing about 200 ± 20g were used for the study. They were housed in well conditioned room with 12h light/12h dark photoperiod. They were fed with standard animal feed [Lipton India, Bangalore, India] and water *ad Libitum*. Experiments were conducted in accordance with the Institutional ethical committee [Biotech SBU.001/10]

Experimental design

Animals were divided into 5 groups with six animals each.

Group I: received 0.2ml of DMSO intraperitoneally and treated as experimental control.

Group II: Administered with DEN [200 mg/kgbwt single i.p] in normal saline for the entire experimental period of 3 months

Group III: Administered with DEN [200 mg/kgbwt single i.p] and co-administered with methanol leaf extract [250 mg/kgbwt] orally for 3 months.

Group IV: Administered with DEN [200 mg/kgbwt single i.p] and co-administered with methanol callus extract [250 mg/kgbwt] orally for 3months.

Group V: Administered with DEN [200 mg/kgbwt single i.p] and co-administered with methanol shoot extract [250 mg/kgbwt] orally for 3 months.

After 3 months of extracts administration rats were anesthetized and blood samples were collected by Sino orbital puncture. Serum separated was used for all Biochemical estimations.

Biochemical Estimations

Marker enzymes such as aspartate amino transferase [AST] [10], Alanine amino transferase [ALT] [10], Alkaline Phosphatase [ALP] [11] Lactate Dehydrogenase [LDH] [12], Total Bilirubin [TB] [13] were assayed in Serum. All the biochemical assays were read at specific wavelength using Shiadzu spectrophotometer, UV-1601 Model.

Statistical analysis: Values reported are mean ± SE. The statistical analysis was carried out using analysis of variance [ANOVA] followed by Dunnet's 't' test. P values < 0.05 were considered as significant [14]

RESULTS

The response for the growth and development of calli varied with different explants. Callus was developed from leaf and node explants of *Solanum trilobatum* were cultured on MS media with individual concentration of 2, 4-D, NAA and BA.

Among 15 individual concentrations tried. Leaf explants showed better callus initiation than nodal explants, In *Solanum trilobatum*, the percentage of calli development was highest with the leaf [35.50 ± 5.0 %] followed by nodal explants [25.0 ± 5.0 %]. Maximum Callus Biomass production in leaf explants inoculated on MS media supplemented with 4.52 μM 2, 4-D [Table 1] [Fig: 1] whereas higher concentration of 2, 4-D [22.62 μM] did not show callus induction. Callus in the 2, 4- D [4.52μM] supplemented medium was found well developed, albino, spongy, and loosely arranged. The moisture content of callus was also high as compared to other auxins supplemented media. In NAA supplemented medium the callus was pale, yellowish green in color, more friable, hard and granular. Callus grown on medium supplemented with BA was green in color more compact, hard and granular.

Among three plant growth regulators were studied, the response of callus was good in 2, 4-D [4.52 μM] compared to others [Table.1]. The rate of callus induction and the percentage of callus response varied depending upon the 2, 4-D concentrations used. The response of callus was 32% on MS media supplemented with 2, 4 –D [4.52 μM] and the rate of proliferation was recorded from 12th day after inoculation up to 40 days with four day intervals.

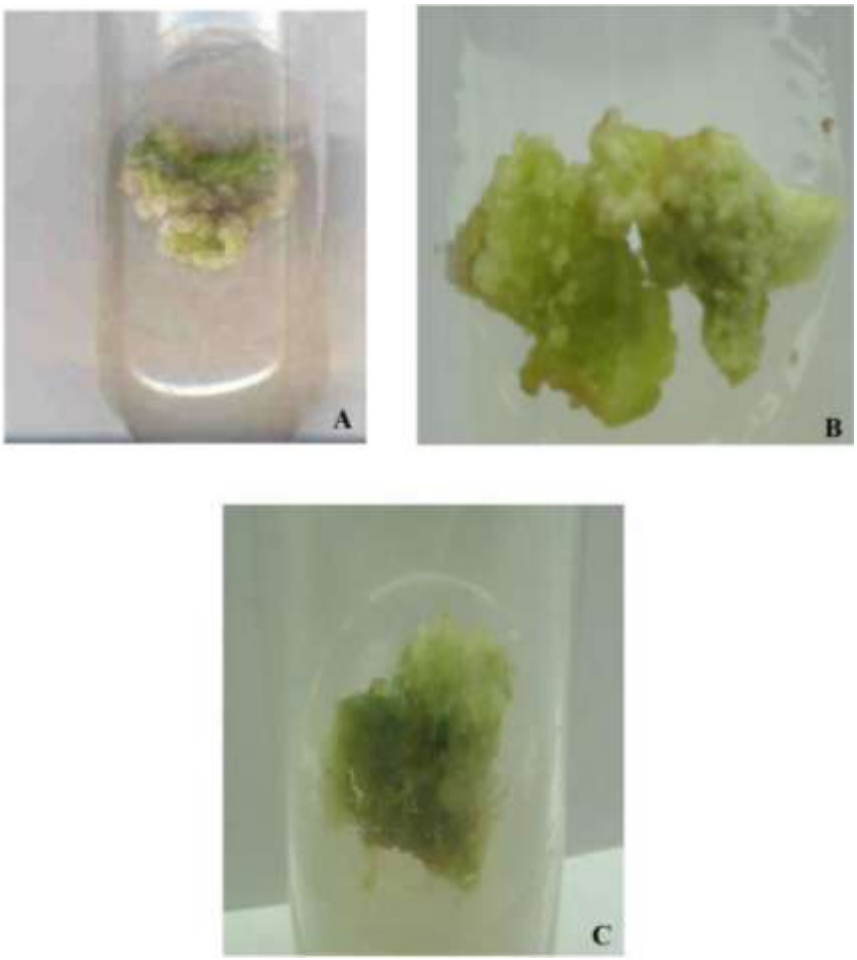
Serum Marker enzymes

Rats administered intraperitoneally with DEN [Group II] showed significant elevation [P<0.05] of marker enzymes ALT, AST, ALP and LDH in the serum .TB content also significantly increased when compared to the control group I. However in group III, IV, V which received 250 mg/kgbwt of S.T are didn't show drastic changes in the marker enzyme levels. The levels of AST, ALT, ALP and LDH in the serum of S.T extract treated groups were well within the range of control rats. Similarly TB Content was also not significantly altered in group III, IV & V administered with S.T extract when compared to control group I [Table II]

Table 1: Effect of plant growth hormones on callus induction from leaf and nodal explants

PGR Concentration (μM)			Node	Juvenile leaf
2,4-D	NAA	BA	Response %	Response%
0.45	-	-	11.67 ± 2.8	10.00 ± 5.0
2.26	-	-	21.67 ± 5.7	23.33 ± 7.6
4.52	-	-	18.33 ± 2.8	35.50 ± 5.0
11.31	-	-	11.67 ± 2.8	18.33 ± 5.8
22.62	-	-	-	-
-	1.34	-	-	-
-	2.69	-	10.00 ± 0.0	26.67 ± 2.9
-	5.37	-	20.00 ± 5.7	18.33 ± 5.7
-	13.43	-	16.67 ± 2.9	15.00 ± 5.0
-	26.85	-	-	-
-	-	1.11	10.0 ± 5.0	15.0 ± 5.0
-	-	2.22	25.0 ± 5.0	25.0 ± 5.0
-	-	4.44	-	-
-	-	8.88	-	-
-	-	13.32	-	-

Results represent mean ± SD of three replicated experiments Data were recorded after 40 days of culture



a. Induction and growth of Callus from nodal explants, b. Induction and growth of Callus from leaf explants, c. Secondary callus developed from primary callus of leaf explants

Fig. 1: Callus initiation from different explants of *Solanum Trilobatum* L

Table 2: Effect of *Solanum trilobatum* extract (STE) on liver marker enzymes in the serum of control and experimental animals

Experimental Groups	AST(U/L)	ALT(U/L)	ALP(KAU/L)	LDH(U/L)	TB(mg/dl)
GPI (Control)	85.58 ± 0.84	37.47 ± 0.36	77.78 ± 0.44	85.58 ± 0.84	85.58 ± 0.84
GPII (DEN i.p)	160.50 ± 2.66**	56.65 ± 0.28**	144.82 ± 0.46**	146.01 ± 1.32**	2.13 ± 0.15**
GPIII (DEN + Leaf)	88.12 ± 0.56	40.57 ± 0.26*	79.36 ± 0.12	116.62 ± 0.80	1.32 ± 0.11*
GPIV (DEN + Callus)	87.67 ± 0.50	39.33 ± 0.33	78.34 ± 0.45	114.13 ± 0.49	1.02 ± 0.94
GPV (DEN + Shoot)	86.50 ± 0.22	38.79 ± 0.29	77.94 ± 0.67	115.62 ± 0.80	0.916 ± 0.18

Values are mean ± S.E (n=5) significance

** (P<0.05); * (P<0.01): Group I Vs Group II, III, IV, V

DISCUSSION

Diethyl nitrosamine [DEN] is a hepatotoxin and a carcinogen [15]. An increase in AST, ALT, ALP activities in DEN induced animals correlate the hepatotoxicity and carcinogenesis with the development of preneoplastic changes, increased severity and advanced stage of liver carcinoma. An increase in the serum of LDH in malignant liver diseases depends upon the extent of metastasis. The lowering in the activities of AST, ALT, ALP &LDH when induced with S.T extracts clearly indicate inhibition of carcinogenesis.

In the present study, drastic alterations in the level of serum marker enzymes AST, ALT, ALP and LDH were noted, indicating DEN mediated hepatic damages. In addition, Bilirubin an endogenous substance and degradation product of hemoglobin was also found to be significantly elevated in DEN treated rats, which also measure of hepatotoxicity [16] and could be attributed to impaired hepatic clearance due to hepatic parenchymal damage a biliary obstruction [17]. The enzymes ALT and AST are localized in mitochondria and cytosol of hepatic cells, ALP largely found in sinusoidal and bile canaiculi membrane [18] & also cytosol, in membranes of golgiapparatuses, Endoplasmic reticulum & nucleus [19]. The enzymes ALP is involved in transport of metabolites across cell membrane, protein synthesis, secretary activities and glycogen metabolism.ALP is a membrane bound enzyme and its alteration and likely to affect the membrane permeability and produce derangement in the transport of metabolites. ALP was noticed the serum and liver of hepatoma bearing animals [20]. It was observed that the ALP activity was raised in the serum of cervical carcinoma patients. The rise in an activity of ALP in cancer bearing animal may be due to disturbance in the secretary activity or in transport of metabolites or may be due to altered synthesis of certain enzymes in these conditions. ALP used as a specific tumor marker during diagnosis in the early detection of cancer [21]. An increase in ALP activity on DEN administration may be due to altered synthesis of

enzymes as in other hepatotoxic condition [22]. Activities of ALP are increased in precancerous lesion in primary liver cell carcinoma and carcinoma of bile duct. The lowering of the activity of these enzymes indicates the inhibition of pre-cancerous transformation in the liver of S.T treatment S.T + DEN animals. LDH, an intracellular enzyme catalyses the readily reversible reaction involving oxidation of lactate to Pyruvate. High concentrations of LDH are found in the liver. Elevation of total serum LDH activity is used as diagnostic indices for organ dysfunction [23]. The elevated enzyme activity in the serum of patients with lung and ovarian cancer [24] depict the changes in permeability of cell membranes and leakage of soluble enzymes. The possible reason for the elevated activity of LDH in cancer bearing rats may be due to enhanced glycolysis using the growth of tumor [25]. The reduction of LDH activity on treatment with S.T [leaf, callus & shoot] controlled the glycolysis and renders protection to membrane integrity [26].

CONCLUSION

From the foregoing discussion, it is clear that the *invitro* derived callus and shoot have strong anti-hepatocarcinogenic effects identical to the leaf of the wild plants. However, when compared to the leaf effect, callus and shoot activity is less may be because of decreased secondary active production. It is suggested that by manipulating the culture condition, secondary metabolite can be enhanced which will provide an effect drug for hepatocellular carcinoma. Further researches on the fractionation of the extracts, isolation, purification and characterization of active constituents responsible for the anti-hepatocarcinogenic activity and their histopathology of liver are in progress.

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