Phytochemical and therapeutic evaluation of leaf and *in vitro* derived callus and shoot of *Solanum trilobatum*

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Abstract: This study focuses on the phytochemical properties and the anti-hepatocarcinogenic effects of the leaf and *in vitro*-derived callus and shoot extracts of *Solanum trilobatum*. In the leaf, callus and shoot, the presence of sugar, proteins, alkaloids, flavonoids, saponins, tananins, cardiacglycoside, terpenoid and lipids was established by preliminary phytochemical screening. Surface-sterilized explants (0.5-1.0 cm) were placed on the MS basal medium supplemented with different concentrations of 2,4-dichlorophenoxyacetic acid (0.45, 2.26, 4.52, 11.31 and 12.56μM), naphthylacetic acid (NAA; 0.54, 1.34, 2.69, 5.37, 13.43 and 26.85μM) and 6-benzylaminopurine (BA; 0.44, 1.11, 2.22, 4.44, 8.88 and 13.32μM) for callus induction. Explants from node and callus culture were inoculated on the MS basal medium supplemented with varying concentrations of BA (0.44-22.20 μM) and NAA (0.54-10.74μM) for shoot multiplication. Rats were divided into five groups and administered with diethyl nitrosamine (DEN) and DEN (200mg/kg bwt) intraperitoneally along with methanol leaf and *in vitro*-derived callus and shoot extracts (250mg/kg bwt) orally for 3 months. A significant deviation (P<0.05) in marker enzymes such as alanine transaminase, aspartate transaminase, lactate dehydrogenase and total bilirubin was found in rats administered with DEN. The liver tissue was used for the analysis of glutathione reductase, lipid peroxidation, glutathione peroxidase, glutathione S-transferase, superoxide dismutase and catalase. DEN administration caused a significant elevation in serum enzymes and total bilirubin. Moreover, antioxidant enzymes were drastically inhibited with significant reduction in glutathione and increased lipid per oxidation. Increased glutathione level and reduced lipid peroxidation were also evident in *S. trilobatum*-treated rats. However, crude *S. trilobatum* and *in vitro*-derived callus and shoot extracts offered better protection against free radical toxicity induced by DEN.

Keywords: Diethyl nitrosamine, free radical, antioxidant, liver, *Solanum trilobatum.*

INTRODUCTION

Recently, there is a renewed interest worldwide in plants as pharmaceuticals. The discovery of numerous biologically active molecules by the pharmaceutical industry and the increasing use of crude extracts of plants for self-medication by the general public are clear evidences (Chellaram *et al.,* 2009a, 2011). Several of these herbal drugs are extracted from plants growing in wild strands. Nearly 95% of plants used as ingredients in traditional drugs are collected from forests and other natural reserves (Nadkarani, 1979). However, limited distribution, low availability, inaccessibility and anthropogenic pressure have caused a large decline in several of the wild populations, making them rare species. *Solanum trilobatum* Linn (family: Solanaceae) has been one of the common Indian medicinal plants used for many centuries to treat asthma, cough, dyspnoea, chronic febrile infections and parturition. It shows cardiac, tonic and carminative actions. Sobatum, β solamarine, solaine, solasodine, glycoalkaloid, disgenin and tomatidine are its chief constituents. This plant has been reported to possess a broad spectrum of antibiotic, antibacterial and anticancer activities (Mohanan, 1996). The active agent (sobatum) obtained by petroleum ether extract was demonstrated to have anticancer properties by *in vitro* and *in vivo* experiments. The existing variability in a breeding population may not be sufficient for modern plant-breeding purposes; thus an effort has to be made to broaden the existing gene pool of crops. Because of these complications and adverse ecological impacts plant cell culture have been used as an alternative source of the natural products and novel lead compounds with medicinal properties (Chellaram *et al*., 2009b and Prem Anand, 2011).

Primary liver cancer ranks fifth in frequency among all malignancies in the world. A vast majority of primary liver cancer is hepatocellular carcinoma (HCC). HCC frequently occurs in the form of a solid tumor. It is highly prevalent with 620,000 new cases reported per year worldwide, of which more than 80% of cases are being reported from China, Africa and South East Asia (Ribes *et al*., 2008). It is highly aggressive, with around 595,000 deaths per year (Parkin *et al*., 2000). HCC presents with limited therapeutic options. Therefore, more research focusing on the biological bases of this malignancy is anticipated in order to develop new strategies for treatment. An animal model of diethyl nitrosamine

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(DEN)-induced hepatocarcinogenesis is ideal to investigate liver tumor formation in stages, including preneoplastic foci, neoplastic nodules and HCC nodules, similar to the liver cancer in humans. DEN is one of the most potent environmental carcinogens that may primarily induce tumors of the liver because of its relatively simple metabolic pathway (Handa, 1986). Several herbal preparations have been used for treating liver diseases. Investigations into a few plant products with the potential to protect liver have been reported (Priya, 2011). Because free radicals and reactive oxygen species play a central role in liver disease progression, dietary antioxidants may be suggested as therapeutic agents (Chellaram, 2012).

Recent studies have suggested that dietary antioxidants are more efficacious than pure compounds in preventing oxidative stress-related pathologies (Vitaglione *et al*., 2004). The present investigation on the hepatoprotective properties of plants is important because hepatic cancers pose a serious threat to human beings (Priya, 2011, Chellaram 2009c) and that the hepatic system undergoes constant assault more than any other organ system in the human body. The present study attempts to assess the antioxidant and anticancer effects of the leaf and *in vitro*-derived callus extracts of a well-known medicinal herb, *S. trilobatum*, as well as their phytochemical composition.

MATERIALS AND METHODS

Plant material

Healthy *S. trilobatum* plants were collected (Madhavaram, Tamilnadu, India) and raised in pots containing soil and farmyard manure in the ratio of 1:1 under green house condition at Poonga Biotech Research Centre (Chennai, Tamilnadu, India).

Explant preparation

For surface sterilization of explants, the method described by Janarthanam (2009) was employed. *S. trilobatum* explants (node and juvenile leaf) were collected from healthy potted plants, washed thoroughly in running tap water and then with 70% ethanol, and then immersed in 0.1% (w/v) $HgCl_2$ for 5 minutes and then washed repeatedly in sterile double distilled water three to five times to remove traces of $HgCl_2$. The cut ends of explants were again trimmed with the help of a sterile blade to eliminate any residue of sterilant.

Preparation of tissue culture media

The preparation of the basal medium was as described by Murashige and Skoog (1962). For this purpose, four individual stock solutions of macronutrient, micro-nutrient, microiron and vitamins were prepared. Stock solutions of salts were prepared using double distilled water to the required volume in a standard volumetric flask. Iron stock solution was stored in an amber bottle to prevent photolysis. All stock solutions were stored at 4°C, and mesoinositol, cytokinin and auxin stock solutions were freshly prepared and used. To prepare the medium, all four stock solutions were mixed thoroughly with sterile distilled water. Sucrose 3% (30 g/L), 0.01% mesoinositol (100mg/L) and plant growth regulators (PGRs), as necessary, were added, and the pH was adjusted to 5.6±0.2 with 1N HCl or 1N NaOH prior to autoclaving. A gelling agent agar (grade 301; Himedia) was added (0.9% w/v) to the medium and mixed well before dispensing the medium into a glassware. The contents were sterilized in an autoclave at 15 lb for 15-20 minutes at 121°C. The cultures were incubated in a culture chamber at 25±2°C for the necessary light conditions. The culture vials were placed on the rack at 25 cm away from the light source. A 16/8h (light/dark) photoperiod of cool white light was provided by 2000 Lux.

Callus induction

Healthy and disease-free green leaves were collected from four-months-old plants. Surface-sterilized explants (0.5-1.0cm) were placed on the MS basal medium supplemented with various concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D; 0.45, 2.26, 4.52, 11.31 and 12.56µM), naphthylacetic acid (NAA; 0.54, 1.34, 2.69, 5.37, 13.43 and 26.85µM) and 6-benzylaminopurine (BA; 0.44, 1.11, 2.22, 4.44, 8.88 and 13.32µM) 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 nodal explants. For secondary callus production, a small portion of primary callus was excised using a sterile knife holder and was subcultured periodically once in three weeks. The secondary callus was used for all experimental studies; regenerated callus was used for shoot initiation. Shoots with at least four roots of length 2-3 cm and 6 to 8 leaves were transferred to plastic cups with a mixture of vermiculite and red soil (3:1) for two weeks before transferring to a potting mix under glass house conditions.

Age of callus and biomass yield in solid medium

Two grams of actively growing callus of *S. trilobatum* was inoculated in a 250mL Erlenmeyer flask containing 50mL of MS solid medium supplemented with 2,4-D (4.52µM), NAA (2.69µM) and BA (2.26µM). The cultures were incubated under 16h photoperiod at 25±1°C for 36 days. At every three days, the yield of callus biomass was measured in terms of fresh weight (FW, g/L) and dry weight (DW, g/L).

Phytochemical screening of S. trilobatum

All phytochemical tests were performed on *S. trilobatum* leaves and *in vitro*-derived callus. Hundred grams of dried

leaves and standardized callus of *S. trilobatum* was taken and deposited into different organic solvents such as chloroform, methanol, and petroleum ether solution at a concentration of 10%w/v and allowed to stand overnight (table 5 a,b,c for concentration details). The extracts were filtered through Whatmann no.1 filter paper to remove cellular materials and other insoluble components. The extracts were allowed to concentrate using a rotary flash evaporator under reduced pressure. Qualitative tests for alkaloids, flavonoids, carbohydrates, glycosides, saponins and tannins, terpenoids, proteins and anthraquinone were performed. The tests for alkaloids showed positive. Mayers test, Wagners test and Dragendorff test were carried out using standard procedures. The results are presented in table 2.

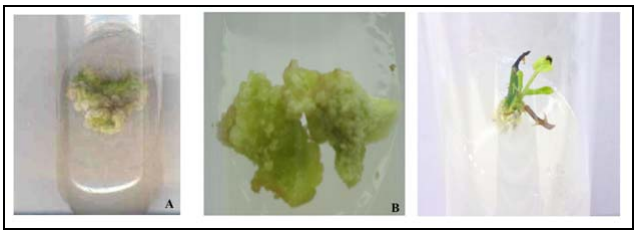

Fig. 1: Stages of callus grown in vitro: (A) emergence of callus from leaf explant; (B) callus growth *in vitro*; (C) shoot generation from the callus.

Animals

Male Wister rats weighing about 200±20g were used in the study. They were housed in a well-ventilated room with 12 h light/12h dark photoperiod. They were fed with standard animal feed (Lipton India, Ban galore, India) and water *ad Libitum*. Experiments were conducted in accordance with the institutional ethical committee guidelines (Biotech SBU.001/10)**.**

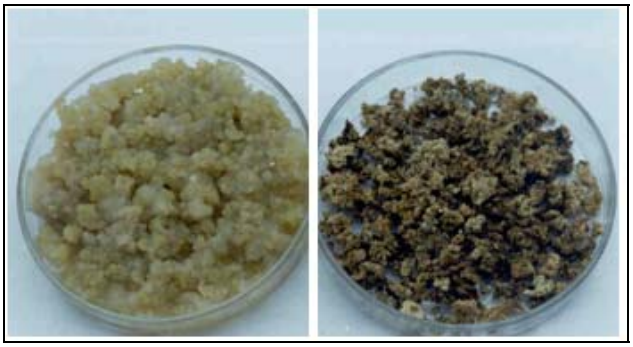

Fig. 2: Fresh weight and dry weight of callus of *S. trilobatum.*

Experimental design

Animals were divided into five groups, with six animals in each group. Group I received 0.2ml DMSO intraperitoneally and used as experimental control. Group II was administered with DEN (200mg/kg bwt single intraperitoneally) in normal saline for 3 months. Group III was administered with DEN (200mg/kg bwt single intraperitoneally) and co administered with methanol leaf extract (250mg/kg bwt) orally for 3 months. Group IV was administered with DEN (200mg/kg bwt single intraperitoneally) and co administered with methanol callus extract (250mg/kg bwt) orally for 3 months. Group V was administered with DEN (200mg/kg bwt single intraperitoneally) and co administered with methanol shoot extract (250mg/kg bwt) orally for 3 months.

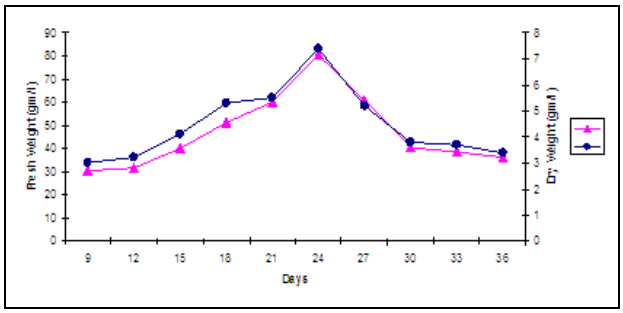

Fig. 3: Callus biomass in solid medium of a growing culture of *S. trilobatum*

After 3 months of treatment, the rats were anesthetized and blood samples collected by sino orbital puncture. The serum was used for all biochemical estimations.

Biochemical estimations

We assessed serum aspartate transferase (SAT), alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and total bilirubin (TB) from the serum. All biochemical assays were read at specific wavelengths using Shimadzu Spectrophotometer (UV-1601 Model).

Antioxidant enzyme estimations

The animals were euthanized and liver was excised and washed in saline. Tissue homogenate was prepared in 0.1 M Tris-HCl buffer (pH 7.4) and used for the estimation of lipid peroxidase (LPO), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST) and glutathione reductase (GSH).

STATISTICAL ANALYSIS

Values are reported as mean ±SE. Statistical analysis was carried out using ANOVA followed by Dunnet's *t*-test. *P* values <0.05 were considered significant.

RESULTS

The growth and development of calli varied among different explants. Among 15 individual concentrations tested, leaf explants showed better callus initiation compared with nodal explants. The percentage of calli development was high for the leaf (35.50±5.0%) than for the nodal explant (25.0±5.0%). The maximum callus yield was in leaf explants supplemented with 4.52 µM 2,4-D

Table 1: Effect of plant growth regulators (PGRs) on callus induction

PGR concentration (μ)			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	-	-

Table 2: Preliminary phytochemical screening of leaf and callus of *S. trilobatum*

S. No.	Test	Extract	Leaf extract	Callus extract	Shoot extract
1	Test for Alkaloids	a) Mayer	+	+	+
		b) Wagner's test	+	+	+
		c) Dragendorff's test	+	+	+
2	Test for Flavonoids	a) Shinoda's test	+	+	+
		b) Alkaline reagent test	+	+	+
3	Test for Carbohydrates	a) Benedict's test	+	+	+
		b) Molisch's test	+	+	+
4	Test for Glycosides	a) Borntrager's test	+	+	+
		b) Keller – Killani test	+	+	+
		c) Legal's test	+	+	+
5	Test for Proteins	a) Ninhydrin test	+	+	+
		b) Biuret test	+	+	+
6	Xanthoproteic test	Xanthoproteic test	+	+	+
7	Test for Saponins	a) Froth test	+	+	+
		b) Lead acetate test	+	+	+
8	Test for Tannins	a) Ferric chloride test	+	+	+
		b) Lead acetate test	+	+	+
		c) 10% K2Cr2O7	+	+	+
9	Test for Terpenoids	a) Salkowski test	+	+	+
10	Test for Anthraquinone	a) Ammonia test	+	+	+
11	Test for Phlobotannins	Test for Phlobotannins	+	+	+
12	Test for Phytosterol	a) Libermann – Burchard test	+	+	+
13	Test for Polyphenols	a) Test for Polyphenols	+	+	+

(table 1, fig. 1), wherein the callus was well developed, spongy and loosely arranged. Whereas, a very high concentration of 2,4-D (22.62μM) was not in favor of callus induction. The moisture content of callus was also high as compared to other auxin supplemented media. In NAA-supplemented medium, the callus was pale, yellowish green, more friable, hard and granular. In BA-supplemented medium, the callus was green, more compact, hard and granular. Fig. 2 shows that the maximum biomass yield was on day 24 in solid medium (82.3 and 7.8g DW/L), after which the biomass yield gradually decreased (fig. 2). The callus remained green up to 36 days and then started browning, being symptoms of aging. The plantlets were then transferred to paper cups (diameter 6cm) containing red soil, vermiculite and farmyard manure in the ratio of 1:1:1 and allowed to harden. The plantlets were covered with polythene bags to prevent transpiration and to maintain a relative humidity

Table 3: Marker Enzyme level in the serum of different treatment group rats

Experimental Gps	SAT (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 (diethyl nitrosamine ip)	160.50±2.66**	56.65±0.28**	144.82±0.46**	146.01±1.3**	2.13±0.15**
GPIII (diethyl nitrosamine +Leaf)	86.50±0.22	38.79±0.29	77.94±0.67	114.13±0.49	0.916±0.18
GPIV (diethyl nitrosamine +Callus)	87.67±0.50	39.33±0.33	78.34±0.45	115.62±0.80	1.02±0.94
GPV (diethyl nitrosamine +Shoot)	88.12±0.56	40.57±0.26*	79.36±0.12	116.62±0.80	1.32±0.11*

Values are mean ±SE (n=5) significance **(P<0.05); *(P<0.01): Group I Vs Group II, III, IV, V

Table 4: Antioxidant enzymes, lipid peroxidation and glutathione levels in the liver of experimental rats

Experimental groups	GPx	GST	SOD	CAT	GSH	LPO
Group I (control)	115.12±0.77	13.79±0.38	12.11±0.37	115.21±0.79	5.22±1.65	4.67±1.12
Group II (diethyl nitrosamine ip)	81.08±0.59**	8.07±0.16**	6.64±0.24**	84.60±0.68**	2.22±0.13**	8.28±0.29**
Group III (diethyl nitrosamine +Leaf)	111.22±0.71	12.39±0.25	11.67±0.35	105.51±1.55	4.87±0.83	5.22±0.27
Group IV (diethyl nitrosamine plus callus)	112.32±0.78	13.15±0.48	12.84±0.33	110.22±0.88	5.12±0.88	6.26±0.22
Group V (diethyl nitrosamine plus shoot)	113.35±0.88	13.35±0.35	13.84±0.38	112.28±0.08	5.14±0.89	6.28±0.28

Values are expressed as GPx (nmol GSH oxidized/min/mg), GST (U/min/mg Protein), SOD (U/g Protein), CAT (nmol/min/mg Protein), GSH (nmol/g tissue) and LPO (nmol/mg) Values are mean ±S.E (n=5). **(P<0.05): GroupI Vs Group II, III, IV, V

Table 5(a): Percentage yield of the sample extracts of *Solanum trilobatum* leaves.

Fraction(s)	Weight of Powdered Samples (g)	Weight of the Sample Extract (g)	% Yield
Chloroform	100	2.25	2.25
Methonal	100	8.03	8.03
P.E	100	4.56	4.56

% Yield = (Weight of the Sample Extract *100) /Weight of Powdered Sample used (g) Overall Yield percentage = (2.25+4.56 + 8.03) =14.84%

Table 5(b): Percentage yield of the sample extracts of *Solanum trilobatum* callus.

Fraction(s)	Weight of Powdered Samples (g)	Weight of the Sample Extract (g)	% Yield
Chloroform	100	2.56	2.56
Methonal	100	9.00	9.00
P.E	100	4.86	4.86

% Yield = (Weight of the Sample Extract * 100) / Weight of Powdered Sample used (g) Overall Yield percentage = (2.56 +9.00+ 4.86) =16.82%

of 80%. They were maintained at 25±2°C in 16/8h photoperiod for two weeks. The plantlets in paper cups were fertigated with half-strength MS salt solution every 3 days. Once hardened, the plantlets were transferred to earthen pots and kept in field conditions. All the plant parts studied showed the presence of most phytochemicals, but mostly concentrated in shoot followed by callus and leaf.

Serum marker enzymes

Rats administered with DEN (group II) showed a significant elevation (P<0.05) in ALT, SAT, ALP and LDH in the serum. TB was significantly higher compared to the control (group I). However, groups III, IV and V that received 250mg/kg bwt of *S. trilobatum* showed no significant changes in marker enzyme levels. The levels of SAT, ALT, ALP and LDH in *S. trilobatum*-treated groups were well within the range of control rats. Similarly, TB was also not significantly altered in groups III, IV and V compared to the control (group I; table 3).

Antioxidant enzymes

Oxidative stress in the liver caused by DEN was assessed by measuring the levels of LPO, GSH, GPx, GST, CAT and SOD. DEN-administered rats (group II) exhibited significant elevation (P<0.05) in LPO and reduction in GSH. Similarly, the levels of GPx, GST, CAT and SOD were also reduced compared to control rats. While the

Table 5(c): Percentage yield of the sample extracts of *Solanum trilobatum* shoot.

Fraction(s)	Weight of Powdered Samples (g)	Weight of the Sample Extract (g)	% Yield
Chloroform	100	2.02	2.02
Methonal	100	9.03	9.03
P.E	100	5.56	5.56

% Yield = (Weight of the Sample Extract * 100) / Weight of Powdered Sample used (g) Overall Yield percentage = (2.02 + 9.03 + 5.56) = 18.86%

administration of leaf extract showed no significant change, rats treated with callus and shoot extracts showed a marginal, non-significant difference in oxidative stress indicators and antioxidant enzymes level. The results are presented in table 4.

DISCUSSION

Hepatic damage can be assessed by estimating the levels of serum enzymes such as AST, ALP, and ASP, which are originally present in the cytoplasm. During hepatopathy, these enzymes leak into the blood stream in conformity of the extent of liver damage induced by toxins. In addition, bilirubin, an endogenous substance and degradation product of hemoglobin, was also significantly elevated in DEN-treated rats, which is a measure of hepatotoxicity and could be attributed to impaired hepatic clearance due to hepatic parenchymal damage and biliary obstruction. The elevation of LDH in total serum is considered a diagnostic index for organ dysfunction. The increased concentrations of these enzymes in the serum were due to the result of leakage from damaged cells into the circulation. In the cancer state, the transport function of cell organelles can get disrupted, resulting in enzyme leakage due to altered permeability of the plasma membrane and thereby decreased levels of marker enzymes in the cell and increased levels in the serum (Shajahan, 2005). LPO has been thought to be responsible for several deleterious effects in biological systems. Once initialized, it progresses by a free radical reaction mechanism, causing cellular damage (Ester Bauer, 1990). The obstruction of a hydrogen atom from the side chain of polyunsaturated fatty acids in membrane lipids is a case. Increased LPO will alter membrane fluidity and potential and thereby lead to a loss of cellular function and cell death. However, *S. trilobatum* administration decreased LPO levels in the study animals, due to the free radical scavenging activity of *S. trilobatum*. SOD acts as a first line of defense against superoxide radicals generated as a byproduct of oxidative phosphorylation (Zimmer, 2001). Furthermore, CAT or GPx converts H_2O_2 to H_2O. Depletion in the activities of these antioxidant enzymes can be owed to enhanced radical protection during DEN metabolism. Glutathione is needed to maintain the normal state of cells and to counteract the deleterious effects of oxidative stress. GSH is involved in many cellular processes, including detoxification of endogenous and exogenous compounds (Sallie, 1991). Thus, the decline in the activities of SOD, CAT, GPx, GR, GSH and GST in tumor-bearing animals was due to increased tumor growth. The increase in antioxidant enzymes after *S. trilobatum* administration suggests the free radical scavenging activity of *S. trilobatum* (Floyd, 2000). The levels of antioxidant enzymes were higher in callus and shoot extracts compared to leaf extract, and thus callus and shoot extracts would significantly reduce DEN toxicity. Most importantly, the present results are borne out the fact that *S. trilobatum* is rich in phytochemicals such as phenolics, flavones and tannins. Thus, the study well demonstrated that the extracts of leaf, callus and regenerated shoot of *S. trilobatum* posses anti-hepatocarcinogenic effects, which deserve to be explored therapeutically in larger animals.

CONCLUSION

It has been clearly demonstrated that the approach utilized in this study is successful in finding the effect of leaf, callus and shoot extract on HCC and this scientific investigation may be utilized for the development of novel innovative drugs in future for the management of HCC.

FUTURE WORK

The global changing scenario is showing a tendency towards use of nontoxic plant products having good traditional medicinal background. 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 and testing the efficacy of secondary metabolites through animal cell lines and exploring market potential are in progress. It is evident from the present study that biotechnogically derived Tissue culture plant part can also be effective in curing HCC. This plant can be used safely for longer duration as a cheap source of active therapeutics for alleviation of commonly occurring ailments by the poor and under privileged people of various countries.

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