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Antineoplastic Activity of Crude Saponin Mixture from the Roots of *Luffa tuberosa* (Roxb.) in Ehrlich Ascites Carcinoma Bearing Mice

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Abstract – The antitumor activity of crude saponin mixture obtained from *Luffa tuberosa* (Roxb.) (Fam; Cucurbitaceae) hairy roots (CSLT) in mice transplanted with Ehrlich ascites carcinoma (EAC) was investigated. The EAC-bearing mice receiving 150 and 300 μg/kg body weight, (i.p) of CSLT have shown a dose dependent elevation in tumor-free survival and a highest number of survivors were observed after administration of CSLT (300 μg/kg), which was considered as an optimum dose for its antineoplastic action. The mean survival time (MST) for this dose was approximately 47.1 ± 0.74 d, when compared with 19.0 ± 0.36 d of untreated control. Administration of 300 μg/kg CSLT resulted in 130% long-term increased survival time. The measurement of body weight, tumor volume, packed cell volume, viable and non-viable count indicated the efficacy of CSLT in tumor-bearing mice, there was a significant recovery in hematological profiles, and there was depletion in lipid peroxidation levels, and the antioxidant enzyme activities such as GSH, SOD and CAT were restored to near the normal levels. The CSLT was found to be devoid of conspicuous short-term toxicity in the mice when animals were intraperitoneally injected with 250, 500, 750 and 1000 μg/kg bodyweight. The treated mice showed conspicuous toxic symptoms only at a dose of 1500 μg/kg. Mortality of the animals was monitored up to 14 d post drug treatment, $1/7^{th}$ of the LD_{50} dose has been considered for the optimal antineoplastic activity.
Keywords – *Luffa tuberosa* (Roxb.) Ehrlich ascites carcinoma, hematological profiles, antioxidant, antineoplastic activity

Introduction

Natural products have been the mainstay of chemotherapy of cancer for the past 30 years. Most of them are obtained from plants or microorganisms, as the plant-derived drugs, vinblastine, vincristine, irinotecan, topotecan, etoposide, paclitaxel and other natural antibiotics, dactinomycin, bleomycin and doxorubicin are now in clinical use (Roberts J 1997; Mann J 2002). As a result of the National Cancer Institute's program to screen plants as a source of anticancer agents, one of the most widely used drugs for ovarian and breast cancer, paclitaxel was discovered. According to Cragg *et al.*, (1999) and Mann J (2002), more than 50% of anticancer drugs in use today are derived from natural sources.

Luffa tuberosa (Roxb.) or *Momordica tuberosa* (Roxb.) Cogn (fam-Cucurbitaceae) is perennial monocious trailing plant, with large turnip shaped tuberous rootstock and synonymously called *Momordica cymbalaria* Hook.F (Fenzl) (The wealth of India.1962). *L. tuberosa* is found in the south Indian states of Karnataka, Andra pradesh, Madhya pradesh, Maharastra and Tamil Nadu. The fruits are traditionally used for treatment of diabetes mellitus and associated hyperlipidemia (Kameshwar Rao *et al.*, 1999). The leaf of the plant has also been reported to be antibacterial (Alamagboul *et al.*, 1985). Moreover, the roots of *L.tuberosa* attribute variety of pharmacological properties such as smooth muscle relaxant (Govinda Das *et al.*, 1981A), antischistosomal (Sulaiman *et al.*, 1985), antiovulatory and abortificient (Hemadri and Sashibhushan Rao. 1983). The plant contains secondary metabolites like steroids, triterpenoids, flavonoids alkaloids, glycosides, carbohydrates, amino acids and minerals. The hairy roots comprises about 20% of steroidal and triterpenoidal saponins.

Saponins are steroid or triterpenoid glycosides, common in a large number of plants and plant products that are currently or potentially important in human and animal nutrition. Several biological effects have been

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ascribed to saponins. These structurally diverse compounds have also been observed to inhibit the growth of malignant cells, to kill molluscs, to be antioxidants, to impair the digestion of protein and the uptake of vitamins and minerals in the gut, to cause hypoglycemia, to scavenge free radicals and to act as anti-fungal and antiviral agents (George Francis *et al.*, 2002).

The present aim of this experiment is to explore the scientific basis for the utility of crude saponin mixture from *L. tuberosa* (Roxb.) hairy roots for protection of Swiss albino mice-bearing the Ehrlich Ascites Carcinoma.

Experimental

Chemicals – Thiobarbituric acid (TBA), Nitro blue tetrazolium (NBT), Nicotinamide adenine dinucleotide (NADH) (Loba Chemie, Mumbai, India), 5,5-dithio *bis*-2-nitro benzoic acid (DTNB), Folin-Ciocalteau phenol and reduced glutathione (GSH) (SISCO Research Lab, Bombay, India). All other chemicals and reagents used were of analytical grade and were obtained from the above indicated commercial sources.

Plant material – Whole plants of *Luffa tuberosa* (Roxb.) were collected from Gadag district of North Karnataka, India and surrounding areas in November and identified by taxonomist of Central National Herbarium (CAL), Botanical Survey of India (B.S.I.), Botanical Garden, Sibpur, Howrah. (No. CNH/1-1 (52)/2004-Tech II/706). Dated 17-06-2004. A voucher specimen of the whole plant is deposited in the herbarium of the department for future references,

Seperation of crude saponin mixture – The hairy roots of *L. tuberosa* were separated from the plant, sliced and dried under shade followed by an incubator for 2 d at 40 °C, crushed in an electrical grinder and then powdered. The powdered material (1.5 kg) was subjected for extraction with various solvent in soxhlet apparatus based on increasing polarity in order of petroleum ether, chloroform, methanol and hydro alcoholic mixture of methanol and water, (50 : 50). All the extracts were concentrated using Rota evaporator, the methanol and hydro alcoholic extracts were combined together and diluted with water and fractionated successively with ethyl acetate and n-butanol. The resulting n-butanol layer after concentrating was subjected for D 101 polymer resin and eluted with water in order to eliminate polysaccharides, which yielded crude saponin (33 g) as white amorphous powder. A portion of crude saponin after drying was used to ascertain the antineoplastic activity.

Animals – Male Swiss albino mice of about 6 to 8 weeks of age with an average body weight of 20 ± 2 g were used for the experiment. They were procured from the animal house, Indian Institute of Chemical Biology (IICB), Kolkata, India. The animals were grouped and housed in polyacrylic cages and maintained under standard laboratory conditions at a temperature 24 ± 2 °C. with 12 h dark and light cycle. They were allowed free access to standard pellet diet (Hindustan Lever, Kolkata, India) and water *ad-libitum*. The rats were acclimatized to laboratory condition for 7 d before commencement of experiment. All procedures described were reviewed and approved by the University Animals Ethical Committee.

Tumor cells – Ehrlich ascites carcinoma (EAC) cells were obtained from Chittaranjan National Cancer Institute (CNCI) Kolkata, India. The EAC cells were maintained *in vivo* in Swiss albino mice, by intraperitoneal (i.p.) transplantation of 2×10^6 cells/mouse after every 10 days, and 9 d old EAC cells were used for the screening of the CSLT.

Experimental protocol – Male Swiss albino mice were divided in to five groups of twelve animals (n = 12) each. The CSLT was dissolved in isotonic saline (0.9% NaCl w/v.) solution and used directly in the assay. EAC cells were collected from the donor mouse and were suspended in sterile isotonic saline. The viable EAC cells were counted (Trypan blue indicator) under the microscope and were adjusted at 2×10^6 cells/ml. 0.1 ml of EAC cells per 10 g body weight of the animals was injected (i.p.) on day zero (d 0). A day of incubation (24 h) was allowed for multiplication of the cells. Fourteen doses of the CSLT (150 and 300 μg/kg, 0.1 ml/10 g body weight) and 5-fluorouracil (20 mg/kg body weight) as standard (Kavimani *et al.*, 2000) were then injected intraperitonially from the first day up to the 14^{th} day with 24 h time intervals. Control animals received only vehicle (isotonic saline solution). Food and water were withheld 18 h before sacrificing the animals. On day 15, half of the animals (n = 6) from each cage were sacrificed and remaining animals kept for the observation of life span of the hosts.

Tumor growth response – The antineoplastic effect of CSLT was assessed by change in the body weight, ascites tumor volume, packed cell volume, viable and nonviable tumor cell count, mean survival time (MST) and percentage increase in life span (% ILS). The mean survival time of each group of 6 mice was monitored by recording the mortality daily for 6 weeks and % ILS was calculated using equations (Mazumder *et al.*, 1997; Gupta *et al.*, 2002).

MST = (Day of first death + Day of last death)/2

ILS (%) = [(Mean survival time of treated group/mean Survival time of control group) − 1] × 100.

Hematological parameters – Hemoglobin content, red blood cells (RBC), and white blood cells (WBC) counts were measured from freely flowing tail vein blood (D'Armour *et al.*, 1965; Wintrobe *et al.*, 1961). Differential leukocyte count of WBC was carried out using Lieshman stained blood smears (Dacie *et al.*, 1958) for all the groups of mice.

Biochemical parameters:

***In vivo* antioxidants assay** – After collection the blood samples, the mice were sacrificed by cervical dislocation. Then their liver was excised, rinsed in ice-cold normal saline solution followed by cold 0.15 M Tris-HCl (pH 7.4), blotted dried and weighed. A 10% w/v homogenate was prepared in 0.15 M Tris-HCl buffer and was used for the estimation of lipid peroxidation (LPO)(Ohkawa *et al.*, 1979)) and reduced glutathione (GSH)(Ellman GL.1979) (after precipitating proteins with TCA). The rest of the homogenate was centrifuged at 1500 rpm for 15 min at 4 °C. The supernatant thus obtained was used for the estimation of superoxide dismutase (SOD) and catalase (CAT) activities (Kakkar *et al.*, 1984; Aebi 1983).

Acute toxicity studies – The acute toxicity of CSLT was determined according to Prieur *et al.* (1973) and Ghosh (1984). The animals were fasted for 18 h. Fasted animals were divided in to 5 groups of 8 animals each. Each group of animals were intraperitoneally injected with 250, 500, 750, 1000 and 1500 µg/kg bodyweight of freshly prepared CSLT in isotonic saline (0.9% NaCl w/v.) solution intraperitoneally. The animals were provided food and water immediately after drug administration. Mortality of the animals was monitored up to 14 d post drug treatment, $1/7^{th}$ of the LD_{50} dose has considered for the antineoplastic activity.

Selection of optimum dose – A preliminary experiment was carried out to in order to ascertain the dose at which maximum anti-tumor activity of CSLT observed. For this the EAC-bearing mice were administered CSLT at the doses of 100, 200, 300, 400 and 500 µg/kg body weight once daily for a period of 6 consecutive days to the, the other group of mice received the normal saline for 6 day period and the mice were observed for the toxic behaviors and life span monitoring.

Statistical analysis – Data were expressed as the Mean ± S.E.M. The data were analysed statistically using S.P.S.S version 10. softwere using ANOVA, followed by Dunnett's multiple comparison test (DMRT). The minimum level of significance was fixed at $P < 0.05$.

Results and Discussion

The present study enlights that the crude saponin fraction of *L. tuberosa* (CSLT) has a remarkable antineoplastic and antioxidant activity in EAC-bearing mice at a concentration as low as 150 and 300 µg/kg bodyweight. These doses selected for the antitumor activity are found to be optimum by treatment of 24 h old tumors with various doses of CSLT which inhibited the body weight gain, indicating the arrest of cell proliferation and growth, however administration of 400 and 500 µg/kg of CSLT was accompanied by some toxic side effects and the reduction in life span was observed. Based on this observation the doses of CSLT were selected to 150 and 300 µg/kg body weights of tumor-baring mice.

The hairy tuberous root of *L. tuberosa* contains the higher content of steroidal and triterpenoidal saponins that are notified by the water solubility, stable foaming character and hemolytic properties. A survey of experimental results indicates that the saponin mixtures present in plants and plant products possess diverse biological effects when present in the animal body. Such as cell membrane permeabilization, antidiabetic, anticholesteromic, cytostatic, antioxidant, antifungal etc. as well the saponins are widely examined as possible tumor therapeutics because they results in anticarcinogenic effects. (Kim *et al.*, 2005; Friedman *et al.*, 2005). Most of the experiments to study the biological effects of saponins have been done using crude saponin extracts from plant sources rather than purified compounds. Because of the presence of a large number of structurally different saponins in plant extracts, it is difficult to pinpoint the individual saponins responsible for the observed effects (George Francis *et al.*, 2002).

The administration of crude saponin mixture of *L. tuberosa* at the dose of 150 and 300 µg/kg body weight caused significant dose-dependent retardation of tumor development and inhibition in the proliferation of carcinoma in Swiss albino mice, which was evident by the inhibition in the body weight gain, increase in life span (MST) of the experimental animals to 39.0 ± 0.73 d (150 µg/kg) and 47.1 ± 0.74 d (300 µg/kg) respectively. The group treated with the intraperitonial injection of standard 5-fluoruracil (20 mg/kg) shown 41.8 ± 0.47 days of survival time. Reduction in tumor volume, packed cell volume and viable cell count was observed in a dose-dependent manner in comparison to those of EAC control group ($P < 0.001$). Further, nonviable tumor cell counts at different doses of CSLT were increased when compared with the EAC control (Table 1).

Table 1. Effect of crude saponin mixture from *Luffa tuberosa* (Roxb.) hairy roots (CSLT) on body weight, mean survival time, % ILS, tumor volume, packed cell volume, viable and nonviable tumor cell count of EAC-bearing mice

parameters	EAC control (2×10^6 cells/ml per mice)	EAC + CSLT (150 μg/kg)	EAC + CSLT (300 μg/kg)	EAC + standard 5-Flurouracil (20 mg/kg)
body weight (g)	30.0 ± 0.81	24.5 ± 0.34[b]	21.3 ± 0.42[a]	20.5 ± 0.34[a]
mean survival time (Days)	19.0 ± 0.36	39.0 ± 0.73[a]	47.1 ± 0.74[a]	41.8 ± 0.47[a]
increase in life span (%)	---	105.2	130.0	115.7
tumor volume (ml)	4.60 ± 0.02	0.25 ± 0.03[a]	0.18 ± 0.50[a]	0.23 ± 0.11[a]
packed Cell Volume (ml)	2.38 ± 0.13	0.14±0.02[a]	0.10 ± 0.02[a]	0.13±0.06[a]
viable cell count ($\times 10^7$ cells/ml)	12.03 ± 0.14	2.33 ± 0.07[a]	1.56 ± 0.05[a]	1.81 ± 0.04[a]
nonviable cell count ($\times 10^7$ cells/ml)	0.36 ± 0.02	0.55 ± 0.01[a]	0.59 ± 0.01[a]	0.64 ± 0.02[a]

Data are expressed as the mean ± S.E.M, n = 6. ANOVA followed by Dunnett's multiple comparison test. Statistically significant when CSLT treated groups compared to EAC Control. [a]P < 0.001,[b]P < 0.01, [c]P < 0.05.

Table 2. Effect of crude saponin fraction from *Luffa tuberosa* (Roxb.) hairy roots on (CSLT) on hematological parameters of EAC-bearing mice

parameters	normal control (0.9% NaCl 5 ml/mice)	EAC control (2×10^6 cells/ml per mice)	EAC + CSLT (150 μg/kg)	EAC + CSLT (300 μg/kg)
heamoglobin (g %)	13.5 ± 0.17	9.41 ± 0.06[a]	11.6 ± 0.11[a]	12.33 ± 0.05[a]
RBC ($\times 10^9$/μl)	6.36 ± 0.04	3.51 ± 0.19[a]	5.53 ± 0.08[a]	6.11 ± 0.04[a]
WBC ($\times 10^9$/μl)	4.81 ± 0.11	16.05 ± 0.12[a]	7.13 ± 0.11[a]	5.61 ± 0.07[a]
monocyte (%)	1.68 ± 0.04	1.15 ± 0.04[a]	1.53 ± 0.03[a]	1.71 ± 0.03[a]
neutrophil (%)	15.38 ± 0.17	67.78 ± 0.26[a]	41.38 ± 0.6[a]	28.33 ± 0.60[a]
lymphocyte (%)	82.93 ± 0.18	31.06 ± 0.28[a]	7.08 ± 0.70[a]	69.95 ± 0.59[a]

Data are expressed as the mean ± S.E.M, n = 6. ANOVA followed by Dunnett's multiple comparison test. Statistically significant when EAC control compared with normal mice, and treated groups compared to EAC Control. [a]P < 0.001, [b]P < 0.01, [c]P < 0.05.

Other cucurbitaceous plants like *Momordica charantia* (Lee Hung *et al.*, 1995), *Luffa cylindrica* (Poma *et al.*, 1997), *Trichosanthes kirlowii* (Chang *et al.*, 2004), *Brayonia alba* (Beak *et al.*, 1995) have been reported to possess antineoplastic activity in different tumor models *in vivo* and *in vitro*. In EAC-bearing mice a regular rapid increase in ascites tumor volume was observed. Ascites fluid is considered to be direct nutritional source for tumor cells and a rapid increase in ascites fluid with tumor growth would be a means to meet the nutritional requirement of tumor cells (Prasad and Giri. 1994)

Myelosuppression and anemia are the two major problems encountered in cancer chemotherapy (Price *et al.*, 1958; Hogland HC. 1982). The anemia encountered in the tumor-bearing mice is mainly due to the reduction in the RBC and heamoglobin percentage and this may occur either due to iron deficiency or due to hemolytic or myelophathic condition (Feninger and Mider. 1954) The RBC counts and hemoglobin content in the EAC control group was decreased when compared to normal group. Treatment with CSLT has increased the RBC count and hemoglobin content close to the normal levels (P < 0.001). The total WBC counts were found to be increased in EAC control group when compared with normal group. Administration of CSLT in EAC- bearing mice reduced WBC count significantly as compared with EAC control (P < 0.001). In the differential count of WBC, the neutrophil count increased where as the lymphocyte count decreased to a greater extent in EAC control group. Treatment with CSLT at different doses recovered these altered parameters almost to the normal values (Table 2).

Malondialdehyde (MDA) is formed during oxidative degeneration as a product of free oxygen radicals (Valenzuela A.1990) which is accepted as an indicator of lipid peroxidation (Neilsen *et al.*, 1997). MDA, the end product of lipid peroxidation, was reported to be higher in cancer tissues than in non-diseased organ (Yagi K. 1987). Our findings indicated that the thiobarbuturic acid reactive substances (TBARS) levels in the tumorous tissues are higher than those in normal tissues. Treatment with CSLT reduced the level of MDA in tumor-bearing mice. Fig. 1 depict the levels of TBARS in liver tissue of experimental animals. In the present study, the levels of MDA were significantly (P < 0.001) increased in EAC control animals

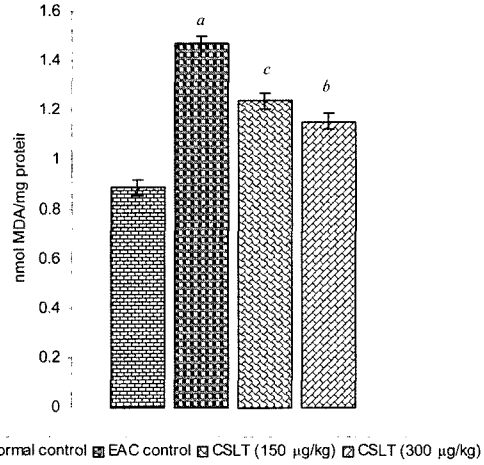

Fig. 1. Effect of crude saponins mixture of *L. tuberosa* roots (CSLT) on hepatic lipid peroxidation levels in EAC-bearing mice.
Data are expressed as the mean ± S.E.M, n = 6. ANOVA followed by Dunnett's multiple comparison test. Statistically significant when EAC control compared with normal mice, and treated groups compared to EAC Control. $^{a}P < 0.001$, $^{b}P < 0.01$, $^{c}P < 0.05$.

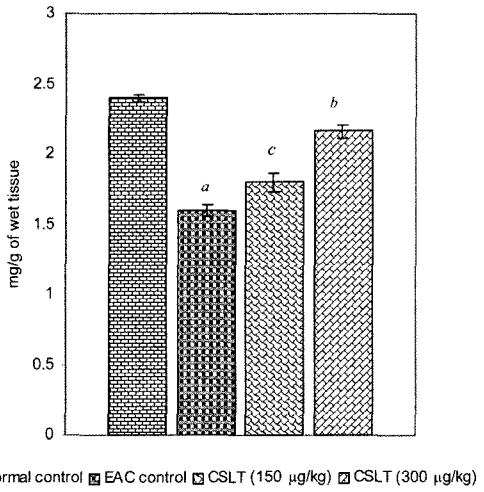

Fig. 2. Effect of crude saponins mixture of *L. tuberosa* roots (CSLT) on hepatic glutathione content in EAC-bearing mice.
Data are expressed as the mean ± S.E.M, n = 6. ANOVA followed by Dunnett's multiple comparison test. Statistically significant when EAC control compared with normal mice, and treated groups compared to EAC Control. $^{a}P < 0.001$,$^{b}P < 0.01$, $^{c}P < 0.05$.

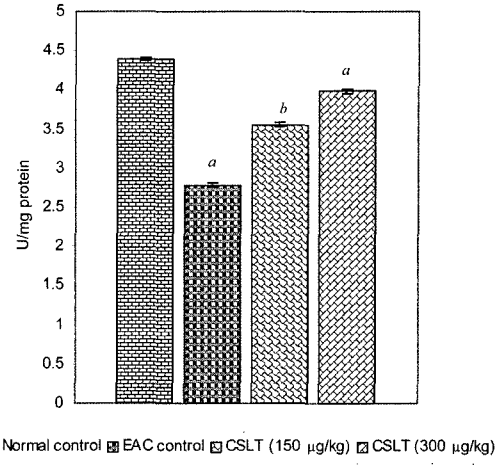

Fig. 3. Effect of crude saponins mixture of *L. tuberosa* roots (CSLT) on hepatic superoxide dismutase activity in EAC-bearing mice.
Data are expressed as the mean ± S.E.M, n = 6. ANOVA followed by Dunnett's multiple comparison test. Statistically significant when EAC control compared with normal mice, and treated groups compared to EAC Control. $^{a}P < 0.001$, $^{b}P < 0.01$, $^{c}P < 0.05$.

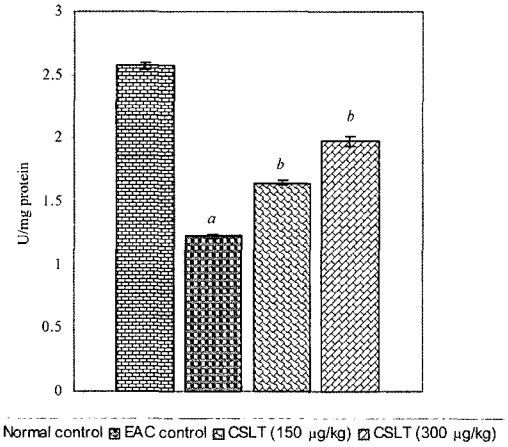

Fig. 4. Effect of crude saponins mixture of *L. tuberosa* roots (CSLT) on hepatic catalase activity in EAC-bearing mice.
Data are expressed as the mean ± S.E.M, n = 6. ANOVA followed by Dunnett's multiple comparison test. Statistically significant when EAC control compared with normal mice, and treated groups compared to EAC Control. $^{a}P < 0.001$, $^{b}P < 0.01$, $^{c}P < 0.05$.

when compared with normal control animals. After administration with CSLT significantly reduced the MDA levels when compared with EAC control animals. Results were expressed as nmoles MDA/mg proteins/ml.

Glutathione is known to protect the cellular system against the toxic effects of lipid peroxidation (Nicotera P and Orrenius S.1986) and Fig. 2 illustrates the level of reduced GSH in experimental groups. The levels of reduced GSH were significantly ($P < 0.001$) decreased in EAC control group when compared with normal control group. The level of reduced GSH was found to be increased after administration of CSLT at dose of 150 μg/kg and 300 μg/kg when compared with EAC control group.

SOD and CAT forms a part of the crucial process involved in cellular antioxidant defence mechanisms whereby peroxides and superoxides are inactivated (Vang *et al.*, 1997), The present study demonstrated that the

CSLT in varying doses activate these enzymes, the activities of which were depleted as a result of cancer. Fig. 3 shows the activity of SOD in liver tissue of experimental groups. There was a significant ($P < 0.001$) reduction in the activity of liver SOD in EAC control animals. Administration of CSLT increased the levels significantly as compared with EAC control animals. Fig. 4 shows that the activity of catalase (CAT) in liver tissue of experimental mice. It was also observed that a significant reduction in the activity of catalase in EAC control groups. Treatment with CSLT at a dose of 150 μg/kg and 300 μg/kg increased the levels significantly as compared with EAC control mice.

The possible mechanism of anticarcinogenic activity of crude saponin mixture of *L. tuberosa* (CSLT) considered to be selective action against malignant cells might be due to a destructive interaction between the altered cell membrane structure of tumor cells and saponins. The tumor-specificity of the cytotoxic action seems to be influenced by the structure of the sugar portion of the saponins (Kuroda *et al.*, 2001) after the hydrophobic aglycon core allowed saponins to traverse the mitochondrial membrane and thereby arresting cell cycle of tumor cells. In addition the saponins are also able to scavenge superoxides by forming hydroperoxide intermediates, thus preventing bio-molecular damage by free radicals (Yoshiki and Okubo, 1995). Hence the crude saponin mixture obtained from hairy roots of *L. tuberosa* has a great potential as antineoplastic drug.

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