In vitro and *in vivo* antitumor and immunomodulatory studies of *Microcosmus exasperatus* against DLA bearing mice

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ABSTRACT

In vitro, in vivo antitumor and immunomodulatory activity of the ethanolic extract of the simple ascidian Microcosmus exasperatus was assessed against Dalton's Lymphoma Ascites (DLA) cells. 100% toxicity was observed at a concentration of 0.80 mg/ml. The extract at 50, 100, 150 and standard drug Vincristin at 80 mg/kg body weight was administered to different groups of tumor induced mice. The results showed significant reduction in weight of body, relative organs, solid tumor volume, packed cell volume, viable cells, WBC, neutrophils, serum Gamma Glutamyl Transpeptidase (GGT), cellular Glutathione (GSH) and an increase in median survival time, life span, non viable cells, haemoglobin, RBC, lymphocyte, eosinophil, bone marrow cellularity, β-esterase positive cells, antibody titer and plaque forming cells in spleen. Restoration of NO levels was observed on the 15th day. The activity was comparatively greater than that observed for the standard drug in all the parameters tested. The above results indicate the presence of bioactive compounds with antitumor property in the ascidian studied.

Keywords: *Microcosmus exasperatus*, *in vitro*, *in vivo*, antitumor, immunomodulatory, DLA
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INTRODUCTION

Cancer is a class of disease in which a cell or a group of cells undergo uncontrolled growth, invasion and metastasis. It can affect any individual at any stage and develop in any organ of the body. Rapid development of resistance to multiple chemotherapeutic drugs and many disorders arising due to radiation therapy has made the search for novel drugs a priority goal for cancer treatment. The search for new metabolites from marine organisms has resulted in the isolation of more or less 10,000 metabolites [1], many of which are endowed with pharmacodynamic properties. So, scientists have turned their attention to develop safe drugs from marine natural products. In recent years, many bioactive compounds have been extracted from various animals like tunicates, sponges, soft corals, sea hares, nudibranchs, bryozoans, sea slugs and other marine organisms [2,3]. Over the last 100 years ascidians have been shown to be a prolific source of natural products with promising biochemical potential [4]. Ascidian-derived natural products have yielded promising drug leads, among which ET 743 from *Ecteinascidia turbinata* was approved as a drug with the trade name Yondelis against refractory soft-tissue sarcomas [5]. Didemnin B from the family Didemnidae is efficient in the treatment of pancreatic cancer in animal models [6]. Cytotoxicity of ascidians to various cell lines have been reported world wide. The crude methanolic extract of ascidian *Polyclinum indicum* and its column fraction PI-8 showed cytotoxcity against cervical cancer cells (HeLa) with an IC$_{50}$ of 77.5 µg/ml and 1.12 µg/ml [7]. Anticancer activity of the methanolic extract of *Polyclinum madrasensis* revealed significant anticancer effect against EAC bearing Swiss albino male mice [8]. The crude methylene chloride extract of *P. madrasensis* exhibits *in vitro* cytotoxic effect on the HeLa cell lines [9]. The ethanolic extract of *Phallusia nigra* showed significant antiproliferative, antitumour and immunomodulatory activity against DLA and EAC tumor bearing mice [10,11,12]. The present study evaluates the *in vitro* and *in vivo* antitumour and immunomodulatory activity of ethanolic extract of *Microcosmus exasperatus* a commonly occurring simple ascidian of Tuticorin coast against DLA tumor bearing mice.

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MATERIALS AND METHODS

Specimen collection and identification: Samples of *Microcosmus exasperatus* were collected from the Tuticorin harbour area. Identification up to the species level was carried out based on the key to identification of Indian ascidians [13]. A voucher specimen AS 2279 was deposited in the national collections of ascidians in the museum of the Department of Zoology, A.P.C, Mahalaxmi College for Women, Tuticorin.

Systematic position: *Microcosmus exasperatus* belongs to Phylum: Chordata, Subphylum: Urochordata, Class: Ascidiacea, Order: Pleurogona, Suborder: Stolidobranchia, Family: Pyuridae, Genus: *Microcosmus* and Species: *exasperates*

Experimental animals: Adult Swiss Albino mice weighing 20-25 g were obtained from the Breeding section, Central Animal House, Dr. Raja Muthiah Medical College, Annamalai University, Chidambaram, Tamilnadu. The animals were kept in air-controlled room, fed with normal mice chow and water ad libitum. The experiments were conducted according to the rules and regulations of Animal Ethical Committee, Government of India.

Preparation of powder and extract: Epibionts, coral, shell fragments adhering to the test of the animal was removed, washed several times with filtered sea water, dried at 45° C and powdered. Ten grams of the powder was soaked overnight in 100 ml of 70 percent ethanol and filtered. The filtrate was centrifuged at 10,000 rpm at 4° C for 10 minutes. The supernatant was collected and evaporated to get a residue, which was used for *in vitro* studies. For *in vivo* animal experiments it was re-suspended in 1% gum acacia blended with vanillin and administered orally at different concentrations.

***In vitro* cytotoxic activity:** DLA cells ($1x10^6$ cells) were incubated with various concentrations (0.05, 0.10, 0.20, 0.40, 0.60, 0.80 and 1.00 mg/ml) of extract in a final volume of 1 ml for 3hr at 37° C. The viability of the cells was confirmed by trypan blue dye exclusion method [14,15].

Experimental protocol: Healthy Adult Swiss Albino mice were weighed and divided into five groups of six each. Group I acted as tumor control, Group II, III and IV received 50, 100, 150 mg/kg of extract and Group V standard drug Vincristin 80 mg/kg body weight. DLA cells were maintained as ascites tumors in Swiss albino mice. The cells were aspirated, washed thrice in normal saline counted using a haemocytometer and cell suspension of one million cells/ml was prepared. One ml of this suspension was injected intraperitoneally for 9 days. On the 10th day, body weight of the animal was noted. 24 hours after the last dose of the drug, one set of the animals were sacrificed and the weight of the vital organs such as spleen, thymus, liver and kidney were recorded and expressed as relative organ weights.

Solid tumor volume: Tumor was induced by injecting DLA cells ($1x10^6$ cells/animal) subcutaneously to the right hind limb of the animals for five groups. The radii of the tumor were measured using Vernier Calipers at 5 days intervals for one month starting with 15th day. The volume of the tumor was calculated using the formula

$$V=4/3 \prod r1^2\ r^2$$

where 'r1' and 'r' represent the major and minor diameter respectively [16]. This was compared with untreated control (Group I).

Median survival time and Percentage increase in life span (% ILS): MST was assessed by recording the mortality daily for six weeks and percentage increase in lifespan (% ILS) was calculated [17].

MST% = $\dfrac{\text{Median survival time of treated group} - \text{Median survival time of control group}}{\text{Median survival time of control group}}$ $\times 100$

Median survival time (MST) = (Day of first death + Day of last death) ∕ 2

Increase in lifespan = $\dfrac{\text{T - C}}{\text{C}}$ $\times 100$

where T = Treated and C = Control

Packed cell volume: The mice were dissected and the ascitic fluid was collected from the peritoneal cavity. The volume was measured using a graduated centrifuge tube and packed cell volume determined by centrifuging at

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10,000 rpm for 5 minutes. The HCT was read at the top of the packed red cells to the nearest 0.5%. The reading obtained for each tube was recorded on the sheet.

Viable and non viable cell count: The cells were stained with trypan blue (0.4% in normal saline) dye. Those that did not take up the dye were viable and that which took the stain are non viable. The viable and non viable cells were counted using Neubauer chamber.

Hematological parameters: Blood was collected from caudal vein of the experimental mice after thirty days and parameters such as haemoglobin, RBC, WBC and differential count was recorded [18].

Bone marrow cellularity and β esterase activity: Experimental animals treated for 5 consecutive days were sacrificed 24 hours after drug treatment. The bone cells were collected from the femur, made into single cell suspension and the number determined by using haemocytometer [19]. Bone marrow cells from the above preparation was smeared on clear glass slide and stained with Harri's Hematoxylin to determine the non-specific β-Esterase activity by the azodye coupling method [20].

Circulating antibody titer: Swiss Albino mice were divided into five groups of 6 animals. Group I was immunized with SRBC (0.1 ml, 20%). Group II, III and IV were treated with different dose of extract and the last group with Vincristin along with 0.2 ml SRBC for 5 consecutive days. Blood was collected from caudal vein every 3rd day after drug administration and continued for a period of 30 days. Serum was separated, heat-inactivated at 56° C for 30 minutes and used for the estimation of antibody titer [21] using SRBC as antigen.

Antibody producing cells: To determine the effect of the extract on the antibody producing cells, half of the experimental animals from the above treatment were sacrificed on different days starting from the third day after immunization up to the 9th day. Spleen was processed to single cell suspension and the number of plaque forming cells (PFC) was determined by the Jerne's plaque assay [22].

Serum Gamma Glutamyl Transpeptidase (GGT) and Nitric Oxide (NO) levels: Blood was collected from experimental animals on the 5th, 10th, 15th day and the serum was used for the estimation of GGT and NO levels [23,24,25].

Cellular Glutathione (GSH) and Nitric Oxide (NO) levels: Blood was collected at different time points (5th, 10th, 15th day) and the cells (1×10^6 cells/ml) were sonicated for 30 seconds and used for the estimation of GSH and NO [24,25,26].

Statistical Analysis: Values are expressed as mean ± SEM. The statistical analysis was done by one-way analysis of variance (ANOVA) followed by Dunnett's test. P-values less than 0.05 were considered to be significant.

RESULTS AND DISCUSSION

Effect on *in vitro* cytotoxic activity to DLA cells: The extract was found to be toxic at different concentration to DLA cells. Concentration of 0.05, 0.10, 0.20, 0.40, 0.60 and 0.80 mg/ml showed 16, 39, 64, 79, 84 and 100 percentage cytotoxicity (Figure 1). A comparison of the cytotoxicity revealed that the extract is less toxic than that of the ethanolic extract of *Phallusia nigra* [10].

Effect on organ weight: There was a significant decrease in the body weight of treated groups compared to the control. A highly significant decrease was observed in group IV treated with 150 mg/kg of extract (Table 1). This observation is supported by studies using various plant extracts [27,28]. An increase in the relative organ weight of the immunologically important organ like spleen and thymus in the tumor control can be attributed to their increased activity and production of immunocompetent cells. Hepato and renomegaly noted may be due to accumulation of fluids. The administration of the extract indicated a dose dependent decrease in the weight of the lymphoid organs which may be because of the bioactive components present in the extract supporting the activity of the immune system. The reduction in weight of liver and kidney on treatment may be the result of removal of toxic fluids by the action of the extract. The relative organ weight of all the vital organs were restored to normal on treatment with 150 mg/kg body weight of extract. Similar reduction has also been observed on treatment with the standard drug indicating the same mechanisms of action as that of Vincristin with the extract.

Effect on tumor volume: A significant reduction in tumor volume was observed on 20th, 25th and 30th day in the treated mice compared to control (Figure 2). The extract administration reduced tumor volume indicating inhibition in the growth and multiplication of tumor cells which may be due to the decrease in the ascites fluid acting as a

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direct nutritional source or the presence of compounds inhibiting mitosis, DNA synthesis or replication via enzyme pathways [29].

Effect on median survival time, life span, packed cell volume, viable and non viable cell count: Median survival time and percentage life span of mice increased (27.14 days) in the treated groups. Life span increased by 90.58% when compared to normal control. An increase in the median survival time and life span indicates the activeness of the extract screened. Packed cell volume (0.91ml) and viable cell count (5.24×10^6 cells/ml) decreased. Non viable cell count (2.46×10^6 cells) increased significantly (Table 2). This may be due to cytotoxic effect on tumor cells or by leading to macrophage activation and inhibition of vascular permeability by the extract as has been evidenced in plants [30,31].

Effect on hematological parameters: Haemoglobin and RBC content increased in treated groups. There was a significant reduction in the total WBC content in Group IV treated with 150 mg/ kg body weight of the extract. In the differential count, the lymphocytes increased where as neutrophils and eosinophils showed a decrease in treated mice (Table 3) which indicates stimulation of the haemopoetic system. Suppression of the myeloid lineage and development of anaemia is a major problem observed during chemotherapy [32]. The anaemia encountered in tumor bearing mice is mainly due to reduction in RBC or hemoglobin percentage and this may occur either because of iron deficiency**,** hemolytic or myelopathic conditions [33]. Treatment with the extract of *Microcosmus exasperatus* brought back the haemoglobin content, RBC and WBC cell count near to normal values. Low Neutrophil and Eosinophil content indicated that they started fighting off tumor progression. Similar results have been reported on treatment with the extract of *Phallusia nigra* and *Polyclinum madrasensis* indicating a same mode of action [10,34].

Effect on Bone marrow cellularity and β-Esterase activity: Bone marrow is a primary lymphoid organ involved in the continued proliferation and turnover of blood cells and differentiation of stem cells. Extract treated animals showed significant increase in bone marrow cellularity (29.14×10^6 cells/femur) and β-Esterase positive cells (1298/4000 cells) (Table 4). This observation indicates the presence of bioactive compounds bringing about an enhancement of the differentiation of stem cells and immunological response [35].

Effect on circulating antibody titer: The total antibody production increased significantly by the administration of the extract (Table 5). The maximum antibody titer value of 224.17 was observed on 12th day in Group IV treated with 150 mg/kg body weight. In the control animals the antibody titer value was only 124.55 on the same day. The stimulatory effect of the extract on the humoral arm of the immune system is indicated by an increase in the circulating antibody titer [36].

Effect on antibody forming cells: In group IV the maximum number of plaque forming cells (197.73 PFC/10^6 spleen cells) was observed where as in the control the number was 135.61 PFC/10^6 spleen cells on the sixth day (Figure 3). A higher PFC may be due to an elevated antibody titer. The activation of humoral immune response by the extract might have increased the plaque forming cells in spleen [35,10].

Effect on serum GGT and NO levels: On the 15th day an enhanced level of GGT in the serum of control tumor bearing mice (89.23 nmol p-nitroaniline/ml) was observed. There was a significant reduction (44.54 nmol p-nitroaniline/ml) after the administration of the extract of *Microcosmus exasperatus* (Table 6). The intra and extra cellular Glutathione concentration is maintained by GGT and it has been reported to play important role in antioxidant defence, detoxification and inflammation [37]. An oxidative stress during tumor induction might have increased the GGT level as an adaptive mechanism. A dose dependent decrease of GGT observed on the 15th day may be because of the transfer of Gamma Glutamyl Transpeptidase (GGT), from glutathione to amino acids and dipeptides [38]. The serum NO level was found to be maximum (31.27µM) on the 15th day of tumor progression in the control, which was reduced to 20.37µM in group IV. Important physiological response and apoptosis is regulated by NO which is lipophilic, highly diffusible short lived messenger [39,40]. The reduction in NO may lead to an increase in cell death.

Effect on GSH and NO levels: The GSH content was maximum 18.43 ± 0.56 nmol/mg protein on the 10th day of tumor growth in the control whereas in the treated group the level decreased to 8.98 ± 0.33 and 6.11 ± 0.21 on the 15th day (Table 7). The major non-protein thiol, GSH is required for tumor cell proliferation and metabolism [36]. Cancer cells have higher GSH levels than the surrounding normal cells, which is characteristic of higher cell proliferation rate. A significant reduction of GSH in group IV is indicative of the antiproliferative nature of the extract. Moreover GSH has been proved to be the master antioxidant which strengthens the immune system by producing T cells and changing the level of reactive oxygen species in isolated cells grown in laboratory which may play a role in reducing cancer development [41,42]. In the control NO level increased gradually and was found to be highest (19.38 ± 0.27) on 15th day of tumor progression. In group IV the NO level was restored to normal 7.22 ± 0.15. NO is supposed to exert anti cancer activity by altering the oxidation reduction status in tumor cells [40].

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Table 1: Effect on relative organ weight of tumor induced mice

Group	Dose (mg/kg body weight)	Relative Organ Weight (g/100 g body weight) after 10 days				
		Body weight	Spleen	Thymus	Liver	Kidney
I	Tumor control	41.26±1.68	0.87±0.054	0.43±0.011	3.94±0.13	1.99±0.034
II	50	36.54±1.04	0.51±0.016	0.41±0.013	3.17±0.18	1.31±0.046
III	100	27.11±0.84*	0.47±0.024*	0.34±0.017	2.84±0.11	1.08±0.011
IV	150	23.94±0.71**	0.41±0.018**	0.26±0.034*	2.29±0.34*	0.87±0.024*
V	Vincristin (80)	20.18±0.34**	0.39±0.016**	0.24±0.018*	2.04±0.13*	0.78±0.017**

*Data represented as mean ±SEM, (n=6). Significance between DLA control and extract treated group. * P < 0.05; ** P < 0.01.*

Table 2: Effect on median survival time, percentage increase of life span, packed cell volume, viable and non viable cell count

Group	Dose (mg/kg body weight)	Median Survival time (Days)	Increase of life span (%)	Packed cell volume (ml)	Viable cells 1x10^6 cells/ml	Non viable cells 1x10^6 cells/ml
I	Control	14.24±0.24	-	3.14±0.017	13.34±0.74	0.87±0.017
II	50	21.54±0.38*	51.26*	1.84±0.024**	6.54±0.33*	0.99±0.020*
III	100	24.93±0.74**	75.07**	1.13±0.014**	5.61±0.23**	1.38±0.018**
IV	150	27.14±0.39***	90.58***	0.91±0.017***	5.24±0.18***	2.46±0.011***
V	Vincristin (80)	24.11±0.24**	69.31**	0.83±0.026***	5.73±0.34**	2.57±0.014***

*Data represented as mean ±SEM, (n=6). Significance between DLA control and extract treated group. * P < 0.05; ** P < 0.01; *** P < 0.001.*

Table 3: Effect on hematological parameters

Group	Dose (mg/kg body weight)	Hb (gm %)	RBC (million/mm^3)	WBC (10^3 cells/mm^3)	Differential Count (%)		
					Lymphocytes	Neutrophils	Eosinophils
I	Tumor control	8.33±0.11	3.05±0.17	13.66±0.84	35.34±0.14	65.41±0.24	9.25±0.11
II	50	8.94±0.16	3.46±0.14	11.17±0.18	41.16±0.21	56.13±0.18	2.71±0.07
III	100	9.79±0.21*	3.94±0.11*	9.39±0.31*	46.19±0.17*	50.11±0.14	3.70±0.03
IV	150	11.86±0.37**	4.21±0.27**	7.44±0.21**	50.16±0.14**	43.24±0.22**	6.60±0.12*
V	Vincristin (80)	11.94±0.31**	4.13±0.18**	8.05±0.36**	51.44±0.11**	40.26±0.14**	8.30±0.18*

*Data represented as mean ±SEM, (n=6). Significance between DLA control and extract treated group. * P < 0.05; ** P < 0.01.*

Table 4: Effect on Bone marrow cellularity and β-esterase activity

Group	Dose (mg/kg body weight)	Bone marrow cellularity (10^6 cells/femur)	β-esterase activity (β-esterase positive cells /4000 cells)
I	T. control	16.23±0.68	881±28
II	50	19.44±0.73	1004±39*
III	100	24.94±0.87**	1184±48**
IV	150	29.14±0.64***	1298±24***
V	Vincristin (80)	27.36±0.88**	1264±31***

*Data represented as mean ±SEM, (n=6). Significance between DLA control and extract treated group. * P < 0.05; ** P < 0.01; *** P < 0.001.*

Table 5: Effect on antibody titer

Days Treated	Antibody titer Dose (mg/kg body weight)				
	Group I T. Control	Group II 50	Group III 100	Group IV 150	Group V Vincristin (80)
3	14.36±0.13	18.27±0.14	20.36±0.24	22.14±0.18	20.33±0.24
6	29.33±0.84	55.60±0.74	79.16±0.14	89.16±0.24	87.14±0.13
9	68.68±0.39	81.17±0.23	99.17±0.27*	116.34±0.21**	109.15±0.18**
12	124.55±1.04	159.44±2.51	198.57±3.64**	224.17±6.31***	201.66±0.28***
15	103.68±0.84	118.36±0.28	124.66±0.84	133.68±2.04	120.11±1.93
18	73.26±0.80	81.36±0.34	84.67±0.49	76.17±0.23	83.17±0.94
21	41.33±0.17	48.57±0.94	32.61±0.17	45.40±0.27	41.66±0.84
24	26.16±0.13	22.66±0.21	18.16±0.21	19.17±0.13	21.99±0.67
27	20.11±0.14	16.13±0.11	13.61±0.14	14.21±0.17	10.44±0.13
30	12.16±0.24	11.14±0.13	10.11±0.12	9.16±0.14	8.94±0.16

*Data represented as mean ±SEM, (n=6). Significance between DLA control and extract treated group. * P < 0.05; ** P < 0.01; *** P < 0.001.*

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Table 6: Effect on the serum GGT and NO levels

Group	Dose (mg/kg body weight)	GGT (nmol p-nitroaniline/ml)			NO (µM)		
		5th day	10th day	15th day	5th day	10th day	15th day
Group I	T. Control	29.12±0.67	64.89±1.38	89.23±1.31	14.56±0.25	26.27±0.91	31.27±0.13
Group II	50	19.34±0.12	47.28±0.98	56.33±0.21	10.64±0.21	19.99±0.73	28.72±0.61
Group III	100	18.94±0.95	41.93±0.68*	49.92±0.63*	10.27±0.21*	19.92±0.17	21.42±0.73*
Group IV	150	16.82±0.28*	38.91±0.22**	44.54±0.91**	9.87±0.27**	18.48±0.84**	20.37±0.53**
Group V	Vincristin (80)	21.67±0.94	43.88±0.94*	51.09±0.25*	11.04±0.18	22.53±0.62	24.62±0.56

*Data represented as mean ±SEM, (n=6). Significance between DLA control and extract treated group. * P < 0.05; ** P < 0.01.*

Table 7: Effect on the cellular GSH and NO levels

Group	Dose (mg/kg body weight)	GSH (nmol/mg protein)			NO (µM)		
		5th day	10th day	15th day	5th day	10th day	15th day
Group I	T. Control	6.91±0.37	18.43±0.56	12.01±0.16	11.33±0.89	15.28±0.43	19.38±0.27
Group II	50	7.98±0.21	12.29±0.78	10.06±0.14	8.11±0.68*	10.43±0.36	14.69±0.23
Group III	100	7.27±0.63	9.78±0.23*	8.06±0.13*	6.91±0.14**	8.24±0.19*	11.45±0.27
Group IV	150	6.86±0.12	8.98±0.33*	6.11±0.21**	4.13±0.27**	4.98±0.21**	7.22±0.15*
Group V	Vincristin (80)	7.91±0.32	8.39±0.16*	5.26±0.62**	5.66±0.24**	5.69±0.42**	6.23±0.44**

*Data represented as mean ±SEM, (n=6). Significance between DLA control and extract treated group. * P < 0.05; ** P < 0.01.*
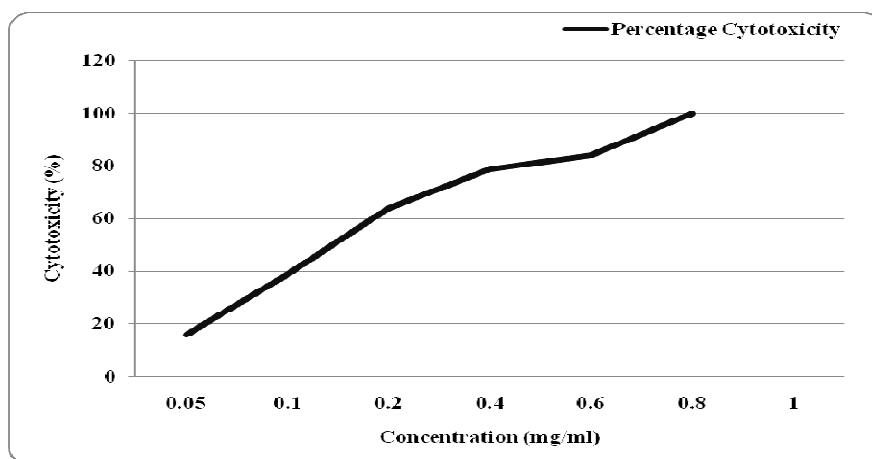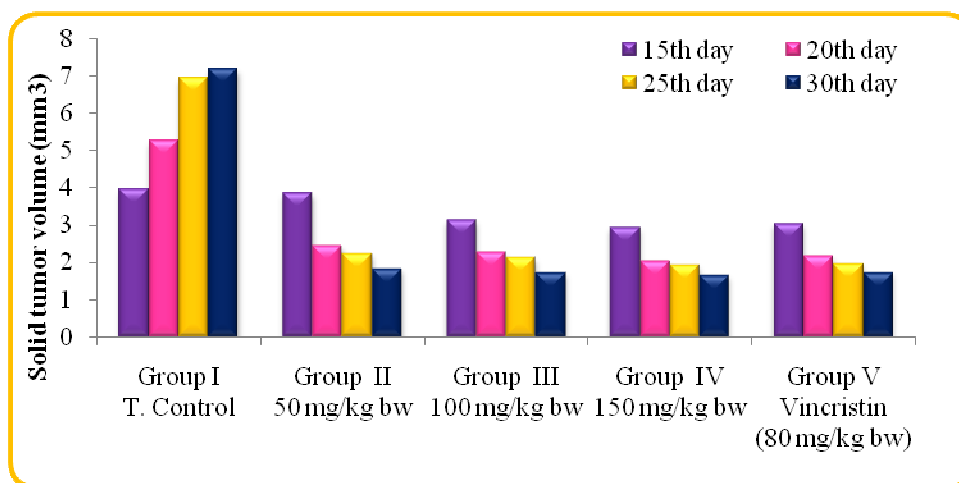

Figure 1: Cytotoxicity of ethanolic extract of *Microcosmus exasperatus* to DLA cells

Figure 2: Effect on solid tumor volume
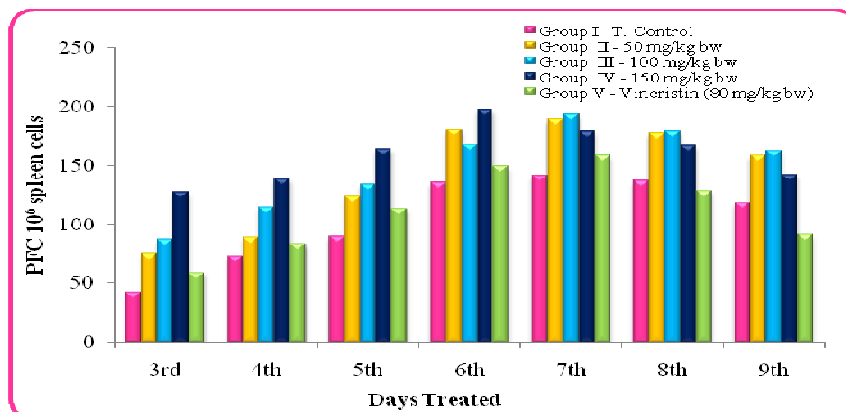
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Figure 3: Effect on antibody forming cells

CONCLUSION

A comparison of the results obtained for the ethanolic extract *Microcosmus exasperatus* at a dose of 150 mg/kg body weight with that of standard drug indicates a significant antitumor and immunomodulatory activity. A preliminary GC-MS studies of the ethanolic extract has shown the presence of compounds like Hexadecyl ester of trichloroacetic acid, Tetradecanoic acid, n-Hexadecanoic acid, Trifluoro- 2,2-dimethylpropyl ester of acetic acid, 26-Nor-5-cholesten-3á-ol-25-one, Cholestan-3-ol, 6,9,12-Octadecatrienoic acid, phenyl methyl ester, (Z,Z,Z)-, N-[4-bromo-n-butyl]-2-piperidinone, showing antioxidant, anticancer, Cancer preventive, antimicrobial, anti-inflammatory, hepatoprotective, hypoglycemic, antiasthmatic, antipyretic and antiandrogenic activities [43]. Further studies on the isolation of compound responsible for the activity and the mechanism involved are needed to come to a definite conclusion.

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