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ANTI-TUMOR ACTIVITY OF FOUR AYURVEDIC HERBS IN DALTON LYMPHOMA ASCITES BEARING MICE AND THEIR SHORT-TERM *IN VITRO* CYTOTOXICITY ON DLA- CELL-LINE.

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Abstract

The anti-tumor activity and chemopreventive potential of four Ayurvedic herbs viz. *Curcuma longa* L., *Ocimum sanctum* L., *Tinospora cordifolia* (Wild) Miers ex Hook.f & Thomas and *Zizyphus mauritiana* Lam. were evaluated using Dalton Lymphoma ascites (DLA) tumor model in Swiss Albino mice. The outcome was assessed using survival time, peritoneal ascitic fluid (Tumor volume) and hematological indices as parameters. Animals were divided into five groups (n = 6) viz. one DLA control and four Herb + DLA treated groups. All the four herb + DLA groups were pre-treated with respective herbs for 7 days and hematological indices were measured for entire five groups. On day-8 animals were inoculated with $1x10^6$ DLA cells i.p., and Herb + DLA groups were continued with oral herbal treatment for 21-days. Hematological parameters and tumor volume were assessed to find the effects of herbs. Short term *in vitro* cytotoxicity was determined by Trypan Blue exclusion method and LDH leakage assay using different concentrations of herbal extracts and 5-FU as a positive control and IC_{50} for each herbal extract and 5-FU were determined. Oral administration of crude herb increased the survival time and decreased the peritoneal ascitic fluid content significantly. Hb, RBCs and total WBC which were altered by DLA inoculation were restored significantly by all the herbs except *O. sanctum*. All the four herbs showed *in vitro* cytotoxic activity against DLA cell-line. Moreover inter group comparison of all the four herbs for anti-tumor activity showed efficacy in the following order- *T. cordifolia* > *Z. mauritiana* ≥ *C. longa* > *O. sanctum* respectively.

Key Words: *Curcuma longa; Ocimum sanctum; Tinospora cordifolia; Zizyphus mauritiana;* DLA cell line; Cytotoxicity; Anti-tumor activity

Introduction

Cancer has been a leading cause of death in the age group 45-64 yrs in developed countries. With changing standard of living and food habits and also due to availability of curative treatment for many infectious diseases, cancer is surpassing other illnesses as a principle cause of morbidity and mortality even in developing countries (Noting, 2001). Surgery, radiotherapy and chemotherapy- the established treatment modalities for various cancers are costly, mutilating, having serious side effects and associated with residual morbidity as well as frequent relapses. Ayurveda- a science of health and longetivity has tried many herbal as well as Rasayana remedies with varying degree of success, but its main significance lies in its preventive approach. Hartwell has collected data, about 3000 plants, which possess anti-cancer properties and subsequently been used as potent anti-cancer drugs (Balachandran and Govindrajan, 2005). Among Indian Ayurvedic herbs, some 30 herbs have shown antitumor activities, and the number may rise as more and more herbs are studied (Ramakrishnan et.al., 1984). Searching the Ayurvedic arena for medicinal herbs with probable antitumor activity, there are multitudes of them having various pharmacological actions but many of them are difficult to identify, scarcely available, some have toxic side effects and are used infrequently; so we decided to select a few herbs that are well known, easily available, cheap, whose identity is non-controversial, free from any known toxicity and that are in vogue for centuries. *Curcuma longa* L., *Ocimum*

sanctum L., *Tinospora cordifolia* (Wild.) Miers ex Hook. f. & Thoms. and *Zizyphus mauritiana* Lam. having antioxidant, immunomodulatory and chemopreventive potential, were selected to evaluate their ability and relative efficacy to prevent or modify the course of DLA in Swiss albino mice.

Previous *in vivo* studies of above herbs for cancer cell lines had been carried out by using some specific extracts in high concentration, that is impossible to achieve in blood, via intraperitoneal root. In present study oral route for administration of herbs was preferred purposefully to directly establish the therapeutic relevance of the herbs tested. The results of *ex vivo* studies in which the inoculation of tumor cells and administration of herbal extracts both intraperitoneal may not be representative of the actual efficacy of the herbs because absorption, first-pass metabolism, protein binding, serum concentration of actual circulating active compounds and metabolites i.e. the pharmacokinetic effects for oral as well as parenteral route is bypassed. This experiment was designed to put the herbs to an acid test of efficacy to find out the truth about conventional claims and isolated studies of activities contributory to anticancer effects. Though conventionally these herbs have been consumed orally only, to the best of our knowledge nobody has made a study of anti-tumor activity of these herbs by using oral root of administration. Naturally the outcome of this study should be relevant to validate as well as improvise the conventional use.

Curcuma longa (CL) (family Zingiberaceae), known as Haldi in India and Turmeric in English, has demonstrated a wide spectrum of therapeutic effects such as anti inflammatory, antioxidant, anti mutagenic, antitumor, antifungal, antiviral, antibacterial, antispasmodic and hepatoprotective. Recently its potential utility in acquired immune deficiency syndrome (AIDS) was demonstrated (Kohli et.al., 2004).

No acute toxicity in mice was observed on administration of tumeric powder with dose as high as 10g/kg-bw (Sittisomwong et.al., 1990). The lowest published toxic oral dose for mouse is 13650mg/kg for 13-weeks the toxic effect being changes in liver weight (NTPTR., 1993).

Ocimum sanctum (OS) (family Labiatae), known as Tulsi in India and Holy Basil in English is known to have adaptogenic activity (Rege et.al., 1999). OS contains a volatile oil consisting of about 70% eugenol as well as methyl eugenol and caryophyllene. Other constituents with likely pharmacological activity include the triterpenoids ursolic acid, rosmarinic acid, oleanic acid; flavonoids apigenin and luteolin; alkaloids; saponins; phenylpropane glucosides and tannins. The seeds contain a fixed oil containing five fatty acids, including about 17% linolenic acid and just over 50% linoleic acid (Archana and Namasivayam, 2000). It has numerous pharmacological activities like hypoglycemic, antistress, immunomodulatory, analgesic, antipyretic, anti-inflammatory, antiulcerogenic, antihypertensive, CNS depressant, hepatoprotective, chemopreventive, radioprotective, antitumor and antibacterial properties (Samson et.al., 2006, Adhvaryu et. al., 2007).

OS ethanolic extract 200mg/kg-bw for 30-days in rats and 500mg/kg-bw for 15-days in mice did not produce any toxic side effects (Vats, et.al., 2004, Panda and Kar, 1998). Doses upto 4g/kg-bw for 14-days did not produce any toxicity or mortality in rats (Shetty, et.al., 2007). The LD_{50} of aqueous extract of OS in mice was found to be ≥5gm/kg-bw (Umadevi and Ganasoundari., 1995).

Tinospora cordifolia (TC) (family Menispermaceae); is a large climbing shrub, growing throughout tropical India; and popularly known as Giloya in Hindi and *Tinospora* in English. It contains tinosporine, tinosporide, tinosporaside, cordifolide, cordifol, heptacosanol, clerodane furano diterpene, diterpenoid furanolactone tinosporidine, columbin and β-sitosterol. The aqueous extract of guduchi stem has shown the presence of arabinogalactan that showed immunological activity. The bitter principle present shows adaptogenic, antispasmodic, anti-inflammatory, antipyretic, anti-neoplastic, hypolipidemic, hypoglycemic, antioxidant, immunopotentiating and hepatoprotective properties (Jagetia and Rao, 2006; Adhvaryu et al., 2007). It is used in general debility, digestive disturbances, loss of appetite and fever in children. It is also an effective immunostimulant (Kapil and Sharma 1997).

400mg/kg-bw of aqueous extract of TC given per oral (PO) to Swiss albino mice for 60 days did not produced any significant toxic effect (Grover et.al., 2000). LD_{50} for TC PO in Swiss albino mice was found to be 2650 (2209-3901) mg/kg (Atal et.al., 1986).

Zizyphus mauritiana (syn. *Zizyphus jujuba* L.) (ZM),(family Rhamnaceae) is a plant of very common occurrence. It grows wild in forests and also on wastelands throughout India. In India it is commonly known as 'Ber' and in English it is known as Indian berry. Pharmacologically active compounds from seeds include jujubojenin, jujubosides-A1 B and C, acetyljujuboside B1, protojujubosides A, B, & B1 (Yoshikawa et al., 1997; Matsuda et al., 1999). The seeds are traditionally used for insomnia and anxiety. The active compounds are reported to have a potent immunological adjuvant activity (Matsuda et al., 1999) and inhibitory effects on hippocampal formation *in vivo* and *in vitro* probably through its anti-calmodulin action (Zhang et al., 2003).

ZM is reported to have very low toxicity when taken orally, in mice and rats; a huge single dose of 50g/kg-bw produced no toxic symptoms and a daily dose of 20g/kg-bw for 30 days did not produced toxic reactions. No side effects were reported (Zhu, 1998).

Materials and Methods

Plant material and Drug

Different varieties of CL have been widely cultivated in different parts of India and they contain 1.5-4% of active principle curcumin. Variety *Selum* found to have ~ 4% of curcumin content was selected for present experiment. The dried rhizome of *Selum* was purchased from local market and ground to make fine powder. The leaves and inflorescence of dark variety of OS known as *Krishna Tulsi* cultivated in the Botanical garden of botany department of Veer Narmad South Gujarat University was collected and shade dried to make fine powder. The crude powder of TC known as *Giloya* or *Amrita* was purchased from local pharmacy (ASFA- GMP-ISO 9001-2000 certified). Fruits of *Randeri* variety of ZM cultivated in Dist. Surat were collected and seeds were ground to make a fine powder. CL, OS and ZM were identified and certified by Prof. Dr. M.H.Parabia (HOD) a taxonomist at Dept. Of Biosciences, Veer Narmad South Gujarat University, Surat. The voucher specimen for CL, OS and ZM – No. MRA/0501, MRA/0502 and MRA/0503 respectively were deposited in the herbarium of Bapalal Vaidya Botanical Research Centre of the same institute.

Standardization was done by HPTLC fingerprinting using curcumin (KONARK herbals Pvt. Ltd.), ursolic acid (ANCHROM India), tinocrdin (Wockhardt Pharmacy) and saponin (ANCHROM India) as standards for CL, OS, TC and ZM respectively for confirming the quality of crude powders.

5-Flurouracil (5-FU) 50 mg/ml available as Florac manufactured by Cadila-Oncocare was purchased from local pharmacist.

Preparation of extracts for in-vitro study

95% curcumin was a gift by KONARK herbals. HPTLC fingerprinting was done to verify curcumin content and three peaks of curcuminoids. Aqueous extract of OS, hydro-ethanolic (1:1) extract of TC and methanolic extract of ZM were prepared by Soxhlet method and the collected liquid extracts were spray-dried to give the final product which was stored in refrigerator. The yields for OS, TC and ZM extraction procedure were 9%, 10% and 7% (W/W) respectively. All the above extracts were standardized by HPTLC fingerprinting to confirm the peaks of active and/or known fractions.

Animals

Adult Swiss female albino mice (35-45g) were provided by SPAN Diagnostic Ltd, and whole experiment was carried out in animal house of SPAN research centre. The permission was obtained from animal ethical committee CPCSEA and its guidelines were followed through out the experimental procedure. Mice were housed in polypropylene boxes, in a controlled environment (temperature 23º ± 2º c and 12hr dark and light cycle) with standard laboratory diet and water *ad libitum* in the animal house of the same institution.

Dosage

For *in vivo* studies, crude herb was fed to the mice *ad libitum.* Mouse chow was fortified with crude herb powder in a way to administer ~200mg/kg-bw/day. Mouse food intake varied between 6-15g/day with an average of 10g/day. 25g chow was given per mouse. 200mg/kg-bw would be achieved by giving 7-9mg in 10g of mouse chow. So, 18-22 mg of crude herb powder was added in 25g of mouse chow for each mouse according to starting weight of the mouse. The minimum - maximum intake for each herb was calculated to be: CL: 185-212mg/kg-bw; OS: 178-218mg/kg-bw; TC: 195-210mg/kgbw and ZM: 190-215mg/kg-bw respectively.

DLA Cell line

DLA cell line was inoculated in peritoneal cavity of Swiss albino mice at Ayurvedic College; Jaipur, by courtesy of Dr Sunil Gupta who maintained the cell line obtained from Dr. Kuttan, Amala Cancer Research Centre, Thrissur. Weekly intraperitoneal inoculation of 10^6 cells in Swiss albino mice was done to maintain the cell-line supply.

Experimental Design

In Vivo Study

Mice were divided in to 5 groups (n = 6), one control and four herb treated groups. Control group was given standard mouse chow while treated groups were given mouse chow fortified with respective herbs and the daily intake was ensured to be in acceptable range by daily weighing of residual chow. On day-7 blood was drawn from tail for hematological parameter like Hb, RBCs, WBCs count, differential count and platelet count. On day eight, mice in all the five groups were inoculated with 10^6 DLA cells intraperitoneally and the oral herbal treatment was continued in the same manner for 21days after inoculation of the herb + DLA groups as described previously. Mice were observed daily for the development of ascites and general condition. Survival time for each group was observed for two months and tumor volume (ascitic fluid) was taken on day 28 for all the surviving mice. Mouse developing huge amount of ascites and becoming moribund was killed by deep anesthesia and that time was taken as survival time, and peritoneal fluid was aspirated and measured. The mean survival time (MST) of each group of 6 mice was noted. The MST of treated groups was compared with that of control group using the following calculation (Sur and Ganguly, 1994).

$$\text{Increase in Life Span} = \frac{T - C}{C} \times 100$$

Where T = MST for each, herb + DLA group.

C = MST for DLA control group.

On 21st day of inoculation, hematological parameters were observed among all the five groups to evaluate the effect of herbs. One mouse in DLA-control group became moribund on day 12 so all the parameters were taken before giving euthanasia.

In Vitro cytotoxicity study

Short term cytotoxicity was assessed by Trypan blue exclusion method and Lactate dehydrogenase (LDH) leakage assay (Gupta 2002; Decker and Lohman-Matthes 1988)

Trypan Blue exclusion method

$1x10^6$ DLA cells in phosphate buffer saline (PBS) with varying different concentrations of herbal standardized extracts and 5-FU as positive control prepared in 0.1% DMSO as solvent were incubated at 37º c for 3hrs in 5% CO_2 atmosphere in the filtered cap, flat bottom cell culture flasks. The viability of cells was determined by Trypan Blue exclusion method (Gupta, 2002). Depending upon the pilot study, different strength of CL, OS, TC and ZM were determined. For CL and TC: 10, 20, 30, 40, 50 µg/ml; OS: 25, 50, 100, 250, 500 µg/ml; ZM: 10, 25, 50, 100, 250 µg/ml and 5-FU: 10, 20, 30, 40, 50 µg/ml were tested to determine IC_{50}.

$$\% \text{ Cell Death} = \frac{\text{No. of Dead cells}}{\text{No. of viable cells + No. of dead cells}} \times 100$$

Lactate Dehydrogenase (LDH) leakage assay

LDH leakage assay was carried out using LDH cytotoxicity detection kit by Clontech Laboratories Inc., according to protocol in the user's manual. To determine $IC_{50,}$ different concentrations of herbal extracts as well as positive control 5-FU as stated above were incubated with 100 µl of DLA cell suspension having $1x10^6$ cell/ml in 96 well plate and incubated at 37°c for four hrs in 5% CO_2 atmosphere. All the control and tested substances were tested in triplicates and mean ± SEM of the absorbance value were taken to calculate cytotoxicity.

$$\text{Cytotoxicity \%} = \frac{\text{Triplicate absorbance – low control}}{\text{High control – low control}} \times 100$$

Statistical Analysis:

All the values were expressed as mean ± SEM. The data were statistically analyzed by One Way ANOVA followed by Dunnett's test for comparison with control. P values ≤0.05 were considered significant.

Results

The effects of CL, OS, TC and ZM on survival of DLA bearing mice are shown in Table 1. None of the control group survived beyond 30 days, while 100% survival was observed in CL TC and ZM group and 83.4% in OS group. The percentage increase in MST was 49.98% for CL (P ≤0.05), 22.71% for OS (ns), 90.22% for TC (P ≤0.001) and 56.45% for ZM (P≤0.05) as compared to control group.

Hematological parameters as investigated on day 7 of herb treatment did not reveal any significant change from that of control group (CT) (data not shown).

Hematological parameters were repeated after 21 days of inoculation of DLA cells to see the effects of DLA tumor in entire five-groups (Table 2). Hb (P≤ 0.01), RBC counts (P ≤0.01) and lymphocyte% (P ≤0.01) decreased while total WBC count (P ≤0.01), neutrophil% (P≤0.001) and platelet count (P ≤0.01) increased in DLA-CT as compared to CT. OS-DLA did not show any significant change when compared to DLA-CT. CL, TC and ZM restored Hb, RBC and WBC count significantly while platelet, lymphocyte and neutrophil counts were restored by CL only.

As shown in Table 3, there was marked reduction in ascitic fluid volume i.e. tumor volume in all herb treated groups. The tumor volume of DLA-CT was 11.67 ± 0.88 ml, where as it was found to be 1.67 ± 0.33 ml in CL-DLA, 5.42 ± 0.3ml in OS-DLA, 0.58 ± 0.33ml in TC-DLA and 1.5 ± 0.37 ml in ZM-DLA treated groups (P ≤0.001 for all four herb groups).

Figure 1 shows the % uptake of trypan blue dye at five different concentrations of four herbs and 5-FU as a positive control. IC_{50}s were found to be 29.7µg/ml for 5-FU, 29.46 µg/ml for CL (95% curcumin), 167.74 µg/ml for OS (aqueous extract), 24.11µg/ml for TC (Hydroethanolic extract) and 66.67 µg/ml for ZM.

Figure 2 shows % cytotoxicity by LDH leakage assay producing slightly different IC_{50} for 5-FU and entire four herbs viz. 29.9 µg/ml for 5-FU, 30.39µg/ml for CL, 175.71 µg/ml for OS, 25.76 µg/ml for TC and 79.72 µg/ml for ZM, confirming the cytotoxic activity of all the four herbs.

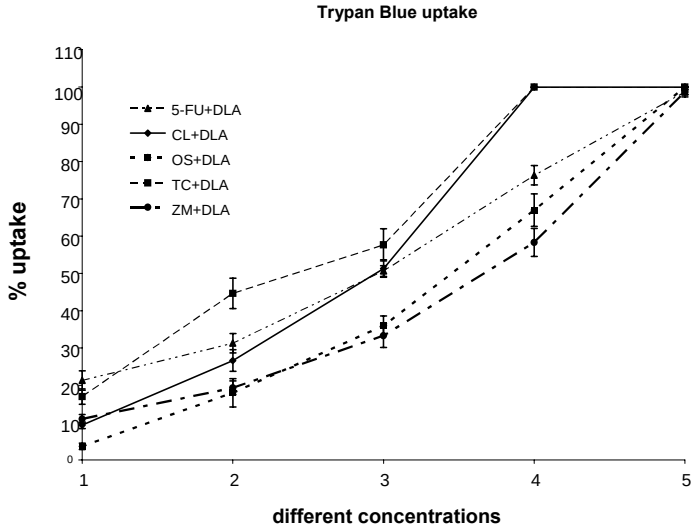

Figure 1: Trypan blue uptake
Values are expressed as mean ± SEM
Trypan blue uptake % in DLA cells after 3-hrs incubation with four herbs and 5-FU at five different concentrations (10, 20, 30, 40, 50 μg/ml for 5-FU, CL and TC; 10, 25, 50, 100, 250 μg/ml for ZM; 25, 50, 100, 250, 500 μg/ml for OS). IC_{50} for 5-FU- 29.7 μg/ml; CL- 29.46 μg/ml; OS- 167.74 μg/ml; TC- 24.11 μg/ml; ZM- 66.67 μg/ml.

Table 1 Effect of CL, OS, TC and ZM treatment on survival of tumor bearing mice.

Treatment	% Survival after 30 days	% Survival after 45 days	% Survival after 60 days	MST days	% increase in Life Span
DLA-CT	0	0	0	26 ± 2.8	-
CL-DLA	100	0	0	39 ± 1.1*	49.98
OS-DLA	83.4	0	0	32 ± 0.5	22.71
TC-DLA	100	50	33.3	49 ± 6.5***	90.22
ZM-DLA	100	33.3	0	40 ± 2.3 *	56.45

n = 6 animals in each group; days of treatment 21.
Values are expressed as mean ± SEM. *P ≤ 0.05 ***P ≤ 0.001

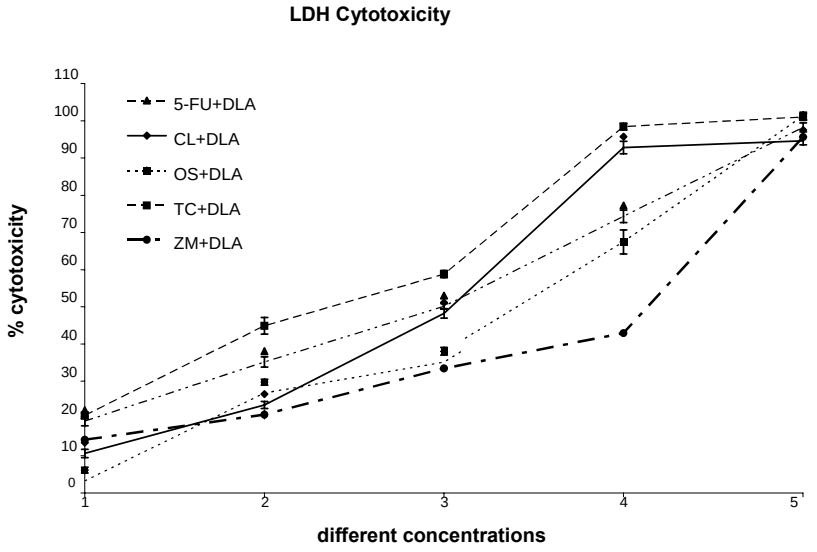

Figure 2: LDH Cytotoxicity
Values are expressed as mean ± SEM
LDH leakage assay shown as % cytotoxicity in DLA cells after 4-hrs incubation with four herbs and 5-FU at five different concentrations (10, 20, 30, 40, 50 μg/ml for 5-FU, CL and TC; 10, 25, 50, 100, 250 μg/ml for ZM; 25, 50, 100, 250, 500 μg/ml for OS). IC_{50} for 5-FU- 29.9 μg/ml; CL- 30.39 μg/ml; OS- 175.71 μg/ml; C- 25.76 μg/ml; ZM- 79.72 μg/ml.

Table 2 Effect of CL, OS, TC and ZM treatment on Hematological parameters.

	Hematological parameters						
Treatment	**Hb**	**RBCs**	**WBCs**	**Platelets**	**Differential Count**		
					Lymphocytes	**Neutrophils**	**Eosinophil + Monocytes**
	g %	10^6/ cmm	10^3x X	10^5 x X/cmm	%	%	%
CT	18.3±1.05	6.2±0.28	11.4±1.82	6.1±1.37	67±3.92	31±3.88	2±0.47
DLA-CT	13.3±0.68 [b]	4.7±0.34 [b]	22.5±2.57 [b]	12.95±0.73 [b]	40±4.41 [b]	56±3.99 [c]	4±0.61
CL-DLA	17.8±1.03 [d]	6.66±0.24 [e]	13.45±3.2 [f]	6.55±1.04 [e]	59±6.3 [d]	39±5.9 [d]	2±0.33
OS-DLA	14.9±0.92	5.1±0.32	26.1±3.88	13.5±0.85	31±3.9	66±3.6	3±0.49
TC-DLA	17.18±0.69 [d]	6.13±0.34 [d]	11.2±0.74 [f]	14.71±1.87	39±3.7	58±3.33	3±0.42
ZM-DLA	17.48±0.85 [d]	6.5±0.29 [e]	13.93±1.85 [f]	10.52±1.14	38±3.05	58±2.94	4±0.49

n = 6 animals in each group; values are expressed as mean ± SEM
CT- Blood sample drawn on Day-7. DLA-CT- Blood sample drawn before euthanasia or day 28.
herb + DLA - Blood sample drawn on day 28.
[b] $P \leq 0.01$, [c] $P \leq 0.001$- for CT vs. DLA-CT.
[d] $P \leq 0.05$, [e] $P \leq 0.01$, [f] $P \leq 0.001$- for DLA-CT vs. herb + DLA.

Table 3 Effect of CL, OS, TC and ZM treatment on tumor volume

	Tumor Volume on day 28 in ml
DLA – CT	11.67 ± 0.88
DLA – CL	1.67 ± 0.33 ***
DLA - OS	5.42 ± 0.3 ***
DLA – TC	0.6 ± 0.32 ***
DLA – ZM	1.5 ± 0.37 ***

One Way ANOVA

F = 86.12 P ≤ 3.03E- 014

n = 6 animals in each group; Values are expressed as mean ± SEM
***$P \leq 0.001$

Discussion

The results of present experiment clearly suggest the chemopreventive and cytotoxic activities of CL, OS, TC, and ZM. For *in vivo* study the doses for entire four herbs were extrapolated from human maximal dose prescribed in Ayurvedic literature for different purposes including treatment of "Gulma" (cancer) in case of CL, OS

and TC (Balachandran and Govindrajan, 2005). As crude herbs used are free of any toxic side effects, testing of a lower dosage was not imperative and higher dosage would not have therapeutic relevance. As no chemotherapeutic agent has ever been used to 'prevent cancer', no drug was taken as a positive control for in vivo study. α – tocopherol, selenium, α-lipoic acid and other vitamins and antioxidant agents have been employed for reversing pre-cancerous conditions like oral leukoplakia and dyskeratosis, but none of them are actual anti-cancer agents the way chemotherapeutic agents are. Herbal anti-cancer compounds are unique in their feature of having anti-oxidant and immunostimulant activity preventing cancer growth indirectly along with a direct cytotoxic effect towards malignant and/or other apoptotic cells.

Tumor volume is one of the important criteria for direct or indirect anticancer activity. The *in vivo* experiment revealed chemopreventive potential of all the four herbs as shown by the small size of tumor volume on 28^{th} day of DLA-inoculation in the herb treated groups compared to DLA-CT. Other important criteria for assessing the value of any antitumor drug are prolongation of life span and decrease of WBC count from blood (Rajkapoor et.al., 2004). TC, ZM and CL prolonged survival time significantly in decreasing order. OS failed to show significant increase in survival time. Other studies have revealed ethanolic OS extract to be more effective when given intra-peritoneally, however there are conflicting reports on efficacy for different extracts on different cell lines (Karthikeyan et.al., 1999b). Despite a higher IC_{50} of ZM in cytotoxicity study, its chemopreventive ability was comparable to CL. This could be due to indirect mechanisms involving immune system and ZM had been shown to have strong intrinsic immunostimulatory activity (Adhvaryu et.al., 2007).

The cytotoxicity of chemotherapeutic agents is effective on malignant as well as all the rapidly dividing cells. Hair loss, mucosal ulcerations and suppression of hemopoiesis occur due to same reason. The anemia encountered in tumor bearing mice is mainly due to reduction in RBCs or hemoglobin % and this may occur either due to iron deficiency or hemolytic or myelopathic condition (Gupta et.al., 2004). In this study, Hb, RBCs and total WBC counts were restored to near normal level by CL, TC and ZM suggesting their ability to counter the effect of DLA tumor in mice.

Immunostimulation, immunomodulation, effect on humoral immune response, antiangioneogenesis activity in addition to direct cytotoxicity towards inoculated malignant cells might be the probable mechanisms by which the inhibition of induced tumor growth was achieved. Anti-inflammatory and anti oxidant activities are also important in preventing proliferation of certain cancer cell lines (Rebeca et.al. 1998, Steele et.al. 1998). Curcumin and Ursolic acid in OS are known to have anti inflammatory, cyclo-oxygenase inhibitory and antioxidant activities (Balanehru and Nagarjan, 1992). Aruna and Sivaramakrishnan (1990, 1992) reported that administration of OS to mice significantly elevated glutathione and more than 78% glutathione-S-transferase activity. These effects could enhance survival though may not affect the tumor directly (Karthikeyan et.al., 1999a).

TC showed the highest efficacy in terms of survival as well as tumor volume control in present study, though the exact mechanism is not clear. Available evidences suggest decline in clonogenecity and glutathione-S-transferase activity; activation of tumor associated macrophage, increase in lipid peroxidation and LDH release to be probable mechanisms behind the cytotoxic activity. Detailed phytochemical screening indicated the cytotoxic compounds in TC to be alkaloids, diterpenoid lactones, glycosides, steroids, sesquiterpinoid, phenolics and polysaccharides. Most of these compounds work synergistically and hence the extract or whole TC may be a promising drug entity to enter the evidence based therapeutics for cancer (Jagetia and Rao, 2006).

ZM followed TC in efficacy. A review of literature shows that ZM is rich in biologically active compounds such as triterpenes, cyclopeptide alkaloids and flavonoids that have been shown to exhibit inhibitory effects on histamine release, COX-1&2, activation activity of choline acetyl transeferase, cytotoxic activity and immunological adjuvant activity (Lee et al., 2004). As a component of the Sho-saiko-to (TJ-9), a Kampo herbal formula, ZM showed a chemopreventive potential in a large series of patients with cirrhosis of liver where it prevented liver cancer and enhanced the activity of natural killer cells, hence may be called an immunopotentiator (Oka et.al., 1995).

CL followed ZM in efficacy but it was excellent in normalizing all the hematological parameters and potential to control tumor volume was equivalent to that of ZM. The anticancer potential of curcumin stems from its ability to suppress the proliferation of wide variety of tumor cells, down regulate transcription factors NF-kappa β, AP-1 and Egr-1; down regulate the expression of COX-2, LOX, NOS, MMP-9, uPA, TNF, chemokines, cell surface adhesion molecules and cyclidine 1; down regulate growth factor receptors EGFR and HER-2; and inhibit the activity of c-JUN, M-terminal kinase, protein tyrosine kinases and protein serine/threonine kinases (Aggrawal et al 2003). Curcumin has got anti inflammatory and anti oxidant activity and it can suppress tumor initiation, promotion and metastasis (Aggrawal et.al., 2003).

The IC_{50} determined by *in vitro* cytotoxicity study showed the cytotoxic activity of CL and TC to be comparable to 5-FU- an established chemotherapeutic agent. It should be noted that 5-FU has known side effects

like nausea, stomatitis, diarrhea, mucosal ulceration and anemia thrombocytopenia due to myelosuppression (Chabner et.al., 2006), while herbal agents were free from such toxicity. ZM and OS have much higher IC_{50} though ZM proved better than CL in *in vivo* studies taking MST and tumor volume as a parameter of efficacy suggesting indirect mechanisms to be important in preventing tumor growth and enhancing survival. This could be due to strong immunostimulant and anti-complementary activities of ZM. Though in vitro studies in general have limited therapeutic relevance, it could be very helpful for deciding optimal dose for local application for conditions like pre-malignant keratosis of the skin and multiple superficial basal cell carcinomas.

The indirect actions limiting tumor growth includes activation of tumor associated macrophages and induction of apoptosis in tumor cells. These effects should encourage a study of radiation sensitizing potential so that the dosage of radiation may be reduced with enhanced killing of tumor cells with diminished side effects on normal cells due to protective effects of these herbs on normal cells. It is important to note here that though 5-FU has radiosensitising property, it lacks any protective action for normal cells and is not free from toxic side effects (Chabner et.al., 2006). CL, OS, TC and ZM all four herbs have cytotoxic activity towards DLA cell line and chemopreventive potential against DLA induced tumor in Swiss albino mice. The highest efficacy of TC followed by ZM, CL and OS with differing profile on hematological parameters would suggest different mechanisms of action by various compounds and a study of formulations based on IC_{50} of each compound may reveal additive or synergistic effects. As these herbs are having excellent safety profile by negative toxicological studies as described earlier, they may be ideal candidate for a prospective trial for different human cancer cell lines and cancer patients as well.

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