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Evaluation of the Antineoplastic Activity of Guduchi (*Tinospora cordifolia*) in Ehrlich Ascites Carcinoma Bearing Mice

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The anticancer activity of dichloromethane extract of guduchi [*Tinospora cordifolia* (WILLD.) MIERS ex HOOK. F. & THOMS. Family: Menispermaceae (TCE)] in the mice transplanted with Ehrlich ascites carcinoma (EAC) was investigated. The EAC mice receiving 25, 30, 40, 50 and 100 mg/kg, TCE showed a dose dependent elevation in tumor-free survival and a highest number of survivors were observed at 50 mg/kg TCE, which was considered as an optimum dose for its neoplastic action. The average survival time (AST) and median survival time (MST) for this dose were approximately 56 and 55 d, respectively when compared with 19 d of non-drug treated controls. Administration of 50 mg/kg TCE resulted in 100% long-term survivors (up to 90 d). An attempt was also made to evaluate the effectiveness of TCE in the various stages of tumor development, where 50 mg/kg TCE was administered intraperitoneally after 1, 3, 6, 9, 12 or 15 d of tumor inoculation and these days have been arbitrarily designated as stage I, II, III, IV or V, respectively for reasons of clarity. The greatest anticancer activity was recorded for stage I, II and III where number of long term survivors (LTS) was approximately 33, 25 and 17%, respectively. However, treatment of mice at stage IV and V did not increase LTS, despite an increase in AST and MST. The EAC mice receiving 50 mg/kg TCE showed a time dependent depletion in the glutathione (GSH) activity up to 12 h post-treatment and marginal elevation thereafter. This depletion in GSH was accompanied by a drastic elevation in lipid peroxidation (LPx) and a maximum elevation in LPx was observed at 6 h that declined gradually thereafter. TCE exerted cytotoxic effect on tumor cells by reducing the GSH concentration and increase in LPx simultaneously.

Key words guduchi; Ehrlich ascites carcinoma; mice; survival; glutathione; lipid peroxidation

Chemotherapy is a major treatment modality for cancer and some of the plants like *Catharanthus roseus*, *Podophyllum peltatum*, *P. emodii*, *Taxus brevifolia*, *Ochrosia elliptica* and *Campototheca acuminata*, have provided active principles which are in clinical use for controlling advanced stages of malignancies.[1] However, most of these chemotherapeutic agents exhibit severe normal toxicity, resulting in undesirable side effects. Moreover, many of the active molecules sold for the treatment of cancer, are highly expensive, mutagenic, carcinogenic and teratogenic. Hence, there is a need to find alternative drugs, which are highly effective at non-toxic doses, inexpensive and accessible to common man. A need is therefore felt to search newer remedies, which are cheaper economically and do not have severe side effects of the pure compounds.

Medicines derived from plants have played a pivotal role in health care of ancient and modern cultures. Ayurveda, the Indian system of medicine mainly uses plant based drugs or formulations to treat various ailments including cancer. Recent surveys suggest that one in three Americans uses dietary supplements daily and the rate of usage is much higher in cancer patients, which may be up to 50% of patients treated in cancer centers.[2]

Tinospora cordifolia (WILLD.) MIERS ex HOOK. F. & THOMS is a large, glabrous, deciduous climbing shrub belonging to the family Menispermaceae.[3,4] It is distributed throughout tropical Indian subcontinent and China, ascending to an altitude of 300 m. The plant is commonly known as Guduchi, Giloy or Amritha, which are Hindu mythological terms that refer to the heavenly elixir that have saved celestial beings from old age and kept them eternally young. Guduchi is widely used in veterinary folk medicine and Ayurvedic system of medicine for its general tonic, antiperiodic, anti-spasmodic, anti-inflammatory, antiarthritic, anti-allergic and antidiabetic properties.[5—7] The whole plant is used in Ayurvedic "Rasayanas" to improve the immune system and the body resistance against infections and root is known for its antistress, anti-leprotic and anti-malarial activities.[7,8] Guduchi has been reported to be active against throat cancer in man and it has been reported to be non-toxic in acute toxicity studies *in vivo*, with almost no side effects.[9—11]

A variety of constituents have been isolated from *T. cordifolia* and their structures elucidated. They belong to different classes such as alkaloids, diterpenoid lactones, glycosides, steroids, sesquiterpenoid, phenolics, aliphatic compounds and polysaccharides. Leaves of *T. cordifolia* are rich in protein (11.2%) and are fairly rich in calcium and phosphorus.[7,12] Alkaloids like berberine, palmatine, tembetarine and magnoflorine have been isolated from the stem of *T. cordifolia*. The roots of *T. cordifolia* are also reported to contain other alkaloids like choline, tinosporin, isocolumbin, palmatine, tetrahydropalmatine and magnoflorine.[13—18] Our preliminary studies on the stem extracts of *T. cordifolia* have shown promising response in cultured human cancer cells, where various extracts of guduchi were found to reduce cell survival in a dose dependent manner. However, dichloromethane extract was found to be the most promising one and has been found to be non-toxic *in vivo* up to 1.2 g/b. wt.[11,19] The studies on the antineoplastic action of dichloromethane extract of *T. cordifolia in vivo* are lacking. This stimulated us to investigate the antineoplastic activity of dichloromethane extract of *T. cordifolia* in mice transplanted with Ehrlich ascites carcinoma.

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MATERIALS AND METHODS

Chemicals Petroleum ether (60—80 °C), chloroform, dichloromethane, hydrochloric acid (HCl), sodium chloride and sodium phosphate were procured from Ranbaxy Ltd., India; carboxymethyl cellulose (CMC), glutathione, thiobarbituric acid (TBA), trichloroacetic acid (TCA) and 5,5′-dithiobis-(2)-nitrobenzoic acid (DTNB) were procured from Sigma Chemical Co., Illinois, U.S.A., while cyclophosphamide (CPA) was procured from Khandelwal Laboratories Pvt. Ltd., Mumbai, India.

Collection and Extraction of Plant Material The identification of the plant *Tinospora cordifolia* (Willd.) Miers ex Hook. F. & Thoms. (Family: Menispermaceae) was done by Prof. U. Rajagopal (a well known taxonomist of this area), Department of Botany, Mahatma Gandhi Memorial College, Udupi, India and the herbarium specimen (RB-TC 02) has been stored with us. The stems were collected in the month of March locally, shade dried and coarsely powdered with the help of a ball mill. Guduchi stem powder (20 kg) was sequentially extracted with petroleum ether (60—80 °C) to remove the waxes and fatty substances, dried, chloroform to remove the chlorophyll content of the stems, dried and finally with dichloromethane using a Soxhlet continuous extraction apparatus for 1 week. The final dichloromethane extracts (henceforth TCE) were concentrated *in vacuo* and dried under reduced pressure. An approximate yield of 1.2% w/w was obtained.

Animal Care and Handling The animal care and handling was done according to the guidelines set by the World Health Organization, Geneva, Switzerland, INSA (Indian National Science Academy, New Delhi, India) and the "Guide for the care and use of Laboratory Animals" (NIH publication #86-23, revised in 1985). Ten to twelve weeks old female Swiss albino mice weighing 30 to 36 g were selected from an inbred colony maintained under the controlled conditions of temperature (23±2 °C), humidity (50±5%) and light (14 and 10 h of light and dark, respectively). The animals had free access to the sterile food and water. Four animals were housed in a polypropylene cage containing sterile paddy husk (procured locally) as bedding throughout the experiment. The study was approved by the Institutional Animal Ethical Committee of Kasturba Medical College, Manipal, India.

Tumor Model Ehrlich ascites carcinoma (EAC) procured from the Cancer Research Institute (ACTREC), Mumbai, India, was maintained and propagated by serial transplantation intraperitoneally in an aseptic environment. 10^6 viable EAC cells were injected intraperitoneally into each animal in an aseptic condition and the day of tumor inoculation was considered as day 0.

Preparation of Drug and Mode of Administration The TCE was dissolved in 100 μl of ethanol and diluted with sterile physiological saline (SPS) containing 1.0% CMC. CPA was dissolved in sterile saline containing 0.5% CMC. The SPS, CPA or TCE were administered intraperitoneally unless otherwise stated. All the drugs were prepared afresh immediately before use.

Selection of Optimum Dose The dose of TCE was selected following the standard protocol recommended by the Drug Evaluation Branch, Drug Research and Development, NIH, U.S.A.[20] Twenty-four hours after tumor inoculation, the animals were divided into the following groups:

SPS Control: The animals of this group received 0.3 to 0.36 ml of SPS containing 1% CMC once daily, consecutively for 9 d.

CPA Control: This group of animals was injected with 25 mg/kg b. wt. of CPA[21] once daily, consecutively for 9 d and served as the positive control.

TCE Group: The animals of this group were administered with 25, 30, 40, 50 and 100 mg/kg TCE once daily, consecutively for 9 d.

Stage Specific Evaluation A separate experiment was carried out to evaluate the antineoplastic action of TCE during various stages of tumor development, where tumors were inoculated and allowed to grow for 1, 3, 6, 9, 12 and 15 d and for reasons of clarity these days have been arbitrarily designated as stage I, II, III, IV, V and VI, respectively. The animals in the above stages of development of tumor, were divided into the following groups:

CPA Group: This group of animals received 25 mg/kg b. wt. of CPA, once daily for nine consecutive days at stage I, II, III, IV, V or VI of tumor development and served as concurrent positive control.

TCE Group: The animals of this group were administered with a single dose of 50 mg/kg TCE (the optimum dose) once daily, for nine consecutive days at stage I, II, III, IV, V or VI of tumor development.

The animals of all the experiments were monitored regularly for alteration in body weight, signs of toxicity and mortality. The weight of animals was recorded every third day up to 30 d after tumor inoculation in all the groups. A 33% of drug related deaths or a weight loss of 5 g per mouse was considered as an index of toxicity.[20] The animal survival was monitored daily up to 120 d, since the survival of animals up to 120 d is roughly equivalent to 5 years survival in man.[22] The tumor response was assessed on the basis of median survival time and tumor free survival. The MST and the AST were calculated from the animals dying within 120 d and those surviving 120 d were excluded from it. The MST and AST was calculated as follows

$$\mathrm{MST} = \frac{\text{first death} + \text{last death in the group}}{2}$$

$$\mathrm{AST} = \frac{\text{sum of animal death on different days}}{\text{number of animals}}$$

The increase in median life span (% IMLS) and increase in average life span (% IALS) were also calculated using the following formulae:

$$\mathrm{IMLS} = \frac{\text{MST of treated mice} - \text{MST of control}}{\text{MST of control}} \times 100$$

$$\mathrm{IALS} = \frac{\text{AST of treated mice} - \text{AST of control}}{\text{AST of control}} \times 100$$

Biochemical Analyses A separate experiment was carried out to estimate GSH and LPx in the tumor cells. The animals were inoculated with tumor cells as described above and the tumor was allowed to grow for 6 d so as to get a reasonable volume for aspiration of cells. On seventh day, the tumor bearing animals were divided into the following

groups:

SPS Control: The animals of this group received 0.3 to 0.36 ml of SPS containing 1 % CMC on day 7 post-tumor inoculation.

CPA Control: This group of animals was injected once with 25 mg/kg b. wt. of CPA on day 7 post-tumor inoculation.

TCE Group: The animals of this group were administered with a single injection of 50 mg/kg b. wt. of TCE on day 7 post-tumor inoculation.

Four animals from each group were sacrificed at 1.5, 3, 6, 9, 12, 18 or 24 h after the SPS, CPA or TCE administration. The tumor cells were aspirated in aseptic condition and were washed with SPS thrice. The cells were counted under an inverted microscope (Labovert microscope, Ernst, Leitz GmbH, Wetzlar, Germany) and 1×10^6 cells were disrupted using an ultrasonicator (Virsonic, Virtis, NY, U.S.A.) and processed for the estimation of glutathione and lipid peroxidation assays as follows:

Glutathione Glutathione (GSH) concentration of EAC cells was measured by the method of Moron *et al.*[23] Briefly, proteins were precipitated by 25% TCA, centrifuged and the supernatant was collected. The supernatant was mixed with 0.2 M sodium phosphate buffer pH 8.0 and 0.06 mM DTNB and incubated for 10 min at room temperature. The absorbance of the sample/s was read against the blank at 412 nm in a UV–Visible double beam spectrophotometer (Shimadzu UV-260, Shimadzu Corp, Tokyo, Japan) and the GSH concentration was calculated from the standard curve.

Lipid Peroxidation Lipid peroxidation (LPx) in EAC cells was measured by the method of Buege and Aust.[24] Briefly, the homogenate was mixed with TCA–TBA–HCl and heated for 15 min in a boiling water bath. After centrifugation the absorbance was recorded at 535 nm using a UV–Visible double beam spectrophotometer. The lipid peroxidation has been expressed as thiobarbituric acid reactive substances (TBARS) in nmol per 10^6 cells. The concentration of LPx in the sample was determined against the standard curve of Malonaldehyde.

Statistical Analysis The statistical analyses were performed using GraphPad Prism 2.01 statistical software (GraphPad Software, San Diego, CA, U.S.A.) in our laboratory. The statistical significance between the treatments was determined using the "*Z*" test for the survival studies,[25] whereas Student's '*t*' test was used for the biochemical estimations. A p value of <0.05 was considered statistically significant. All the data are expressed as mean±S.E.M. (standard error of the mean).

RESULTS

Selection of Optimum Dose There was no spontaneous regression of tumor in EAC transplanted mice of SPS group. The tumor bearing mice showed a constant weight gain and increase in the tumor volume due to cell multiplication and growth of EAC (Fig. 1). The MST was found to be approximately 19 d, whereas the AST was 18 d for the SPS group (Table 1). Treatment of mice 24 h after EAC inoculation with 25 mg/kg CPA increased the MST and AST up to 29 and 28 d, respectively. This was accompanied by an increase in the IMLS and the IALS, which were found to be approximately 58% and 56%, respectively (Table 1).

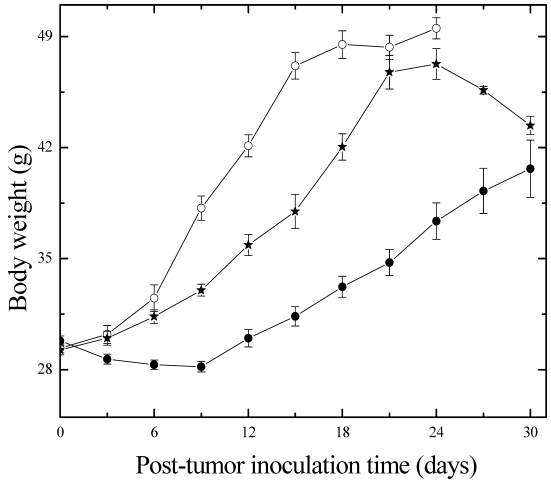

Fig. 1. Effect of 50 mg/kg of Dichloromethane Extract of *Tinospora cordifolia* (TCE) in Comparison with 25 mg/kg Cyclophosphamide (CPA) on the Body Weight of Mice Bearing Ehrlich Ascites Carcinoma

Open circles: SPS alone, closed circles: CPA alone and stars: TCE alone. SPS: sterile physiological saline.

Treatment of tumor bearing mice with TCE resulted in a dose dependent increase in the MST and AST. The greatest effect was observed for the animals treated with 30, 40 or 50 mg/kg TCE, where the MST increased approximately up to 26, 35 and 53 d, respectively when compared with the SPS treated group (19 d). The AST was also elevated approximately up to 26, 35 and 56 for 30, 40 and 50 mg/kg TCE, respectively, whereas the least effect was observed for 25 mg/kg TCE where MST and AST was found to be approximately 21 and 20 d respectively. This increase in AST and MST was non-significant when compared with SPS group. The MST increased approximately by 6 and 26 d in 40 and 50 mg/kg treated animals, respectively than CPA treatment (Table 1). Of all the TCE doses tested, the highest anticancer activity was observed for 50 mg/kg, where 100% healthy survivors were observed at the end of 110 d, whereas 33% survivors were observed at the end of 120 d (Table 1). Treatment of EAC mice with 50 mg/kg TCE increased MST approximately by 34, 29, 20, 29 and 26 when compared to 25, 30, 40 and 100 mg/kg of TCE and25 mg/kg CPA respectively, whereas the AST elevated approximately by 36, 31, 21, 31 and 28 respectively. The treatment of mice 24 h after EAC inoculation with 25, 30, 40, 50 or 100 mg/kg TCE arrested the weight gain at all TCE doses indicating inhibition of tumor cell proliferation and growth (Fig. 1). The administration of 100 mg/kg TCE was accompanied by toxic side effects like ruffling of hair, sluggishness and lacrimation in the recipients and none of the animals survived beyond day 6 post-tumor inoculation (Fig. 1). Therefore, 50 mg/kg TCE was considered as an optimum dose, which resulted in the highest number of long-term survivors. It also did not induce any toxic effects in the form of debility, loss of body weight and death and further studies were carried out using this dose.

Stage Specific Evaluation The evaluation of the anticancer activity of TCE was carried out in tumor bearing animals at stage I, II, III, IV, V and VI, respectively by administering a single dose of 50 mg/kg TCE once daily or 25 mg/kg CPA for nine consecutive days, at stage I, II, III, IV, V or VI. The administration of 25 mg/kg CPA exerted a significant an-

Table 1. Effect of Various Doses of Dichloromethane Extract of Guduchi (TCE) on Ehrlich Ascites Carcinoma in Mice

Treatment	Survival				Percent survival (d)			
	MST	IMLS	AST	IALS	30	60	90	120
SPS	19	—	18	—	0	0	0	0
CPA 25	29	57.9	28	55.8	16.7	0	0	0
TCE 25	21.5	13.1	20.5	14	0	0	0	0
TCE 30	26	36.8	25.7	43.3	0	0	0	0
TCE 40	35.5	86.8	34.9	94.3	91.7*	66.7**	0	0
TCE 50	55.5	192.1	56.4	213.3	100*	100*	100*	33.3**
TCE 100	26	36.8	25.7	43.3	0	0	0	0

$*p<0.0001$; $**p<0.05$ (when compared with SPS). SPS: sterile physiological saline; CPA: cyclophosphamide; MST: median survival time; AST: average survival time; IMLS: increase in median life span; IALS: increase in average life span. TCE 25: 25 mg/kg TCE; TCE 30: 30 mg/kg TCE; TCE 40: 40 mg/kg TCE; TCE 50: 50 mg/kg TCE; TCE 100: 100 mg/kg TCE. CPA 25: 25 mg/kg CPA.

Table 2. Effect of 50 mg/kg Dichloromethane Extract of Guduchi (TCE) on the Various Stages of Tumor Development in Mice Transplanted with Ehrlich Ascites Carcinoma

Treatment	Survival				Percent survival (d)			
	MST	IMLS	AST	IALS	30	60	90	120
SPS	19	—	18	—	0	0	0	0
TCE I	55.5	192.1	56.4	213.3	100*	50**	33.3†	33.3†
CPA I	29	57.9	28	55.8	16.7	0	0	0
TCE II	48	152.6	42.7	137.2	91.7*	66.7***	25†	16.7
CPA II	26.5	39.5	26.3	46.6	8.3	0	0	0
TCE III	43.5	128.9	39	116.7	66.7***	33.3†	16.7	0
CPA III	24	26.3	23.1	28.5	0	0	0	0
TCE IV	35	84.2	30.6	70	25	0	0	0
CPA IV	21.5	13	21.1	17.4	0	0	0	0
TCE V	23.5	23.7	23.9	32.8	8.3	0	0	0
CPA V	19	—	18.9	5.3	0	0	0	0
TCE VI	20	5.3	19.7	9.9	0	0	0	0
CPA VI	18.5	—	18.2	1.6	0	0	0	0

$*p<0.0001$; $**p<0.001$; $***p<0.002$; $\dagger p<0.05$ (when compared with SPS). SPS: sterile physiological saline; CPA: cyclophosphamide. TCE I & CPA I: treatment of 50 mg/kg TCE and 25 mg/kg CPA at stage I; TCE II & CPA II: treatment of 50 mg/kg TCE and 25 mg/kg CPA at stage II; TCE III & CPA III: treatment of 50 mg/kg TCE and 25 mg/kg CPA at stage III; TCE IV & CPA IV: treatment of 50 mg/kg TCE and 25 mg/kg CPA at stage IV; TCE V & CPA V: treatment of 50 mg/kg TCE and 25 mg/kg CPA at stage V; TCE VI & CPA VI: treatment of 50 mg/kg TCE and 25 mg/kg CPA at stage VI. To evaluate the antineoplastic action of TCE during various stages of tumor development, tumors were inoculated and allowed to grow for 1, 3, 6, 9, 12 and 15 d and for reasons of clarity these days have been arbitrarily designated as stage I, II, III, IV, V and VI, respectively.

ticancer activity only when administered in the early stages of tumor development, which is validated by the body weight changes (Table 2). None of the animals treated with CPA at various stages of tumor development survived beyond 36 d post-tumor cell inoculation (Table 2).

The survival of EAC mice declined in a stage specific manner. The MST for the tumor bearing animal that received CPA at stages I, II, III, IV, V and VI was approximately 29, 26, 24, 21, 19 and 18 d, respectively (Table 2), whereas AST was approximately 29, 26, 24, 21, 19 and 18 for stage I, II, III, IV, V and VI respectively. An IMLS, of 58, 39, 26 and 13% was observed for stages I, II, III and IV respectively. The administration of CPA at the late stages (*i.e.* V and VI) proved ineffective in increasing the IMLS. The IALS decreased in a stage specific manner in the CPA group which was approximately 56, 47, 28, 17, 5 and 1%, for stage I, II, III, IV, V and VI, respectively (Table 2).

Treatment of mice with 50 mg/kg TCE at stages I, II, III, IV, V or VI resulted in an increase in the MST approximately up to 55, 48, 43, 35, 23 and 20 d, whereas AST also elevated to approximately 56, 43, 39, 31, 24 and 20 d, respectively, when compared with CPA treatment (Table 2). Despite an increase in IMLS and IALS after TCE treatment, a stage-specific decline was evident in IMLS and IALS and the lowest values were observed for stage VI. The IMLS of approximately 192, 153, 129, 84, 24 and 5% while the IALS of approximately 213, 137, 117, 70, 33 and 10% was recorded for stage I, II, III, IV, V and VI, respectively after 50 mg/kg TCE treatment (Table 2).

TCE was effective in arresting the weight gain in the animals due to remission of tumor, especially during the early stages of tumor growth. TCE was found to be efficient even in the mid stages of tumor progression in comparison with CPA. The use of TCE even during the late stages of tumor development proved effective in reducing the tumor burden and weight gain than that of CPA treatment (Fig. 1). The administration of TCE was better than CPA as 33 and 17% survivors ($p<0.05$) were observed at the end of 120 d for stage I and III respectively, while no survivors were reported for CPA group. There was an increase of 26, 29, 31, 34, 36 and 37 d approximately in MST, while the AST elevated approximately by 28, 30, 33, 35, 37 and 38 d for stage I, II, III, IV, V and VI, respectively, when compared with the CPA group (Table 2).

Biochemical Analyses. Glutathione (GSH) The GSH concentration of EAC cells, remained unaltered with time in

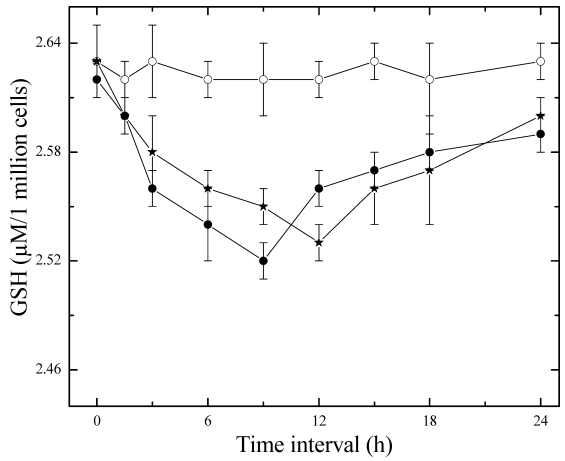

Fig. 2. Effect of the Treatment of 50 mg/kg of Dichloromethane Extract of *Tinospora cordifolia* (TCE) in Comparison with Cyclophosphamide (CPA) on the Alteration in the Glutathione Content in Mice Transplanted with Ehrlich Ascites Carcinoma

SPS (open circles); CPA (closed circles) and TCE (stars). SPS: sterile physiological saline.

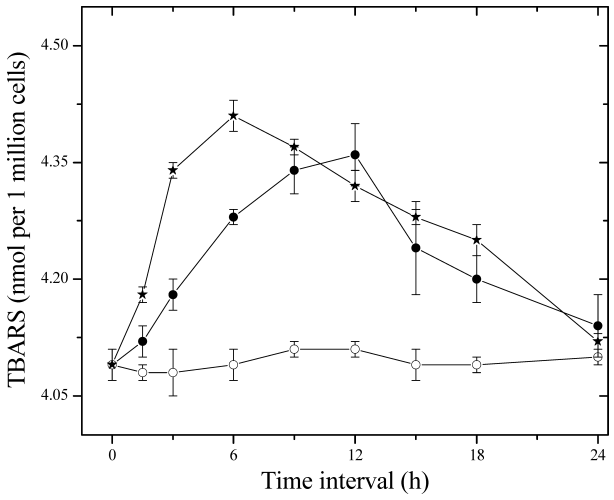

Fig. 3. Effect of the Treatment of 50 mg/kg of Dichloromethane Extract of *Tinospora cordifolia* (TCE) in Comparison with Cyclophosphamide (CPA) on the Alteration in the Lipid Peroxidation in Mice Bearing Ehrlich Ascites Carcinoma

SPS (open circles); CPA (closed circles) and TCE (stars). SPS: sterile physiological saline.

EAC mice treated with SPS. Treatment of EAC mice with CPA treatment caused a time dependent decline in the GSH concentration in EAC cells till a nadir was reached at 9 h post-treatment. A further increase in the assay time caused a significant elevation at 12 h post-treatment, thereafter a steady state was reached (Fig. 2). Similarly, TCE treatment showed a steady decline in GSH concentration in EAC cells and a nadir was reached at 12 h post-treatment. An elevation in the GSH concentration showed a gradual elevation thereafter up to 24 h. However, the concentration of GSH was 2.6 folds lower than the SPS group (Fig. 2).

Lipid Peroxidation (LPx) The baseline levels of lipid peroxidation in EAC cells remained unaltered with assay time in mice treated with SPS. Treatment of EAC mice with CPA caused a time dependent increase in the LPx of tumor cells till a peak was reached at 12 h post-treatment. A further increase in the assay time caused a significant and gradual decline in the lipid peroxidation up to 24 h post-treatment (Fig. 3). The pattern of elevation of LPx after TCE treatment was similar to that of CPA treatment except that the peak elevation was observed at 6 h post-treatment and LPx was higher in TCE group than that of CPA group (Fig. 3).

DISCUSSION

Humans have always relied on nature for survival since ancient times, which has been their main source of food, protection, clothing, transportation and remedies.[26,27] Natural products have been regarded as important sources that could produce potential chemotherapeutic agents.[28] Plant-derived compounds, in particular have a special place in anticancer therapy and some of the new chemotherapeutic agents currently available for use in a clinical setting include paclitaxel, vincristine, podophyllotoxin and camptothecin, a natural product precursor for water-soluble derivatives.[1,29—31] Obviously natural products are extremely important as sources of medicinal agents. Although there are some new approaches to drug discovery, such as combinatorial chemistry and computer-based molecular modeling design, none of them can replace the importance of natural products in drug discovery and development. Guduchi has been reported to be non-toxic in acute toxicity studies *in vivo*, with almost no side effects. The studies on the antineoplastic action of TCE *in vivo* are lacking. Therefore, it was desired to screen the antineoplastic activity of TCE in Swiss albino mice transplanted with Ehrlich ascites carcinoma.

The administration of various doses of TCE caused a dose dependent retardation in the tumor development, as is evident by the inhibition in body weight gain and increase in the life span (MST and AST). This indicates to the effectiveness of TCE in restricting tumor cell multiplication and growth. The reports regarding the antineoplastic action of dichloromethane extract of *T. cordifolia in vivo* are lacking. However, it has been reported to reduce clonogenic survival in cultured HeLa cells in a dose dependent manner earlier.[11] A similar effect has been reported earlier in mice treated with the extract of *Alstonia scholaris*.[21,32—34] Other Indian medicinal plants like *Ervatamia heyncana*, *Rubia cordifolia*, *Tylophora indica*, *Hygrophila spinosa* and *P. hexandrum* have been reported to possess antineoplastic activity in different tumor models *in vivo* and *in vitro*.[35—40] Of all the TCE doses tested, the highest anticancer activity was observed for 50 mg/kg, where 100% healthy survivors were observed at the end of 110 d, while 33% survivors were observed at the end of 120 d. In contrast, no survivors were reported for the SPS or CPA groups at the end of 120 d. Similar observations have also been reported earlier for *Tylophora indica*, *Alstonia scholaris* and *Aegle marmelos* that had an optimal activity only at a particular drug dose beyond which, it was either ineffective or toxic.[21,33,40]

Human beings suffering from various neoplastic disorders come for clinical evaluation and treatment in the different stages of tumor development. Therefore, an effective anticancer agent should be able to kill tumor cells efficiently during any stage of the tumor. An effort has been made to screen the antineoplastic activity of 50 mg/kg TCE at various stages of tumor development with respect to the efficacy of

25 mg/kg of CPA. The results from the stage specific evaluation show that both TCE and CPA retarded the increase in the body weight gain in animals due to tumor development and increased the survival during the early stages effectively. This may be due to the effective killing of EAC cells during the early stages of tumor development by TCE, where the tumor burden is less when compared to late stages. TCE was effective even during mid and late stages of tumor development, while CPA treatment proved ineffective. This was evident by increased MST and AST during various stages of tumor development, when compared with CPA treatment. The effectiveness of TCE was better than CPA, where 33.3 and 16.7% survivors were observed at the end of 120 d for stage I and III respectively, while no survivors were reported for CPA treatment. The studies of the anticancer activity of plants at different stages of tumor development are scanty. However, the information on the treatment in advanced stages of tumor development is lacking. The extract of *Alstonia scholaris*, has been reported to inhibit EAC growth in various stages of tumor development.[21)]

T. cordifolia has been found to contain alkaloids, diterpenoid lactones, glycosides, steroids, sesquiterpenoid, phenolics, aliphatic compounds and polysaccharides. Alkaloids like berberine, palmatine, tembetarine and magnoflorine have been isolated from the stem of *T. cordifolia*. The roots of *T. cordifolia* are also reported to contain other alkaloids like berberine, choline, tinosporin, isocolumbin, palmatine, tetrahydropalmatine and magnoflorine.[13—18)] *T. cordifolia* has also been found to contain an immunomodulator arabinoglycan.[41)] The anticancer activity exhibited by TCE can be attributed to the presence of berberine as it has been isolated from TCE (data not shown). Berberine has been shown to have an anticancer effect on mice bearing Ehrlich ascites carcinoma at the dose of 10 mg/kg b. wt.[32)] Berberine has been reported to reduce the *in vitro* growth of brain tumor cells, teratocarcinoma cells and HepG2 cells as also inhibition of the secretion of alpha-fetoprotein by HepG2 cells.[42)] Even though berberine is one of the major alkaloids present in the stems of *T. cordifolia*, it can be inferred that there may be other important constituents in the dichloromethane extract, which might have contributed to the enhanced antineoplastic action of TCE than the positive control CPA.

The exact mechanism of action of TCE is not known. The antineoplastic activity cannot be attributed to a single mechanism but several mechanisms may be operational simultaneously for effective tumor cell kill. TCE may have caused DNA damage in the EAC cells leading to the cytotoxic effect. This contention is supported by our earlier study where TCE has been found to increase micronuclei in a concentration dependent manner in HeLa cells.[11)] The reduced GSH contents and increased lipid peroxidation by TCE may also have contributed to the killing of EAC cells. Increased concentration of GSH in the tumor cells have been reported to make the tumor refractory to treatment, while depletion of glutathione has been reported to enhance the cell death and apoptosis of the tumor cells along with the loss of essential sulfhydryl groups that result in an alteration of the calcium homeostasis and eventually loss of cell viability.[43,44)] The lipid peroxidation is another important event related to cell death and has been reported to cause severe impairment of membrane function through increased membrane permeability and membrane protein oxidation and eventually cell death by damaging the cellular DNA.[45,46)] The changes in the membrane fluidity coupled with the damage to DNA by TCE may have been responsible for the killing of tumor cells and prolongation of life span in EAC mice. The cytotoxic effect of TCE may be due to inhibition of topoisomerase II. TCE contains berberine that has been found to be a topoisomerase II inhibitor.[47)] Other topoisomerase II inhibitors like etoposide and teniposide have been found to exert antineoplastic activity.[48)]

CONCLUSIONS

From our study it is clear that the TCE is a composite mixture of various alkaloids including berberine. The presence of other alkaloids may have a combinatorial effect on the antineoplastic action of TCE. Encouraging results of pre-clinical and clinical trials of this extract and its other constituents could become a part of standard cancer treatment protocols and a powerful weapon for the treatment of cancer.

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