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# Possibilities of developing novel potent antitumor agents from the leaves of *Cryptomaria japonica*

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## Abstract

During past few decades cancer has remained as the largest cause of mortality worldwide and number of patients suffering from cancer has been increasing at a fast rate. Hence medical research during the last few decades has been concentrating on identification and characterization of new synthetic pharmacological compounds to overcome this enormous problem. Leaf extracts of coniferous plant *Cryptomeria japonica* being known for their strong antibacterial and antifungal functions were selected to determine their antitumor/anticancer potentialities.

Methanolic extract of leaves were tested to determine its antitumor action in standard murine model of Ehrlich Ascites Carcinoma (EAC). Graded doses of the extract were given intraperitoneally to batches of mice, who received EAC challenge after 3hr. Treatment with same amounts of extract was continued for 9 consecutive days. Protective capacity of the leaf extract was evaluated in animals.

Statistically significant protection was observed with respect to different parameters including tumor volume , tumor cell count , viable tumor cell count, non- viable tumor cell count , mean survival time and increase in life span. Simultaneously hematological parameters were restored in treated mice vis-à-vis untreated control animals. Furthermore, the extract revealed distinct cytotoxic property, which may be the relevant reason of its anticancer/antitumor function.

This study shows efficacy of methanolic extract of leaves of *Cryptomeria japonica* as a probable antitumor/anticancer agent. Phytochemical analysis of the extract showed presence of flavonoids, which are known to possess significant anticancer activity. Thus there is a definite possibility of developing novel anticancer drugs from such plant products.

**Keywords :** Cancer, Anticancer agent, Antitumor agent, Plant extract, *Cryptomaria japonica*,

## Introduction

Cancer has posed to be the most dreadful infection of human beings in the last few decades, and the number of patients suffering from various types of cancer had been rising steadily throughout the whole world [1]. This is a disease which is equally prevalent among people of both developed and underdeveloped countries. According to the latest report by the World Health Organization [2]. cancers figure among the leading infections worldwide with about 14 million new cases and 8.2 million cancer related deaths in 2012. The number of new cases is expected to rise by about 70% over the next two decades [2]. A major portion of drug research is therefore directed to the discovery, designing and recognition of new molecules to target every single form of cancer through different strategies. Since plants have the enormous potentiality to synthesize structurally diverse bioactive molecules, researchers of many Asiatic countries have been pursuing anticancer drug designing with new molecular targets from plant sources against cancer [3,4]. Hartwell [5]. in his review of plants used against cancer listed 3000 plant products that were reported for their distinct activity against various human ailments including abscesses, corns, calluses, polyps, “hard swellings” and tumors. Subsequently Graham et al [6] confirmed the observations of Hartwell [5]. However, an extensive research has been developed to determine anticancer effect of curcumin which is obtained from the rhizome of *Curcuma longa.* Curcumin had been reported to inhibit the growth of transplantable tumors in different animal models and increases life span of tumor-harboring animals. Curcumin thus exhibits a great promise as a therapeutic agent and is currently in human clinical trials for various conditions including multiple myeloma, pancreatic cancer, colon cancer and myelodisplastic syndromes [7,8]. Apart from curcumin plant derived

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agents include vinblastine, vincristine, camptothecin derivatives, topotecan and irinotecan and etoposide derived from epipodophyllotoxin. Many such new agents are highly promising and are in the process of clinical development based on selective toxicity against cancer related molecular targets [9,10]. Traditionally plants of diverse origin have long been used in the treatment of cancer [11,12].

Cryptomaria is a monotypic genus of conifer in the cypress family *Cupressaceae* with a single species *C. japonica,* which is endemic to Japan where it is known as Sugi. Sugi is regarded as the national tree of Japan and planted around temples and shrines. The large evergreen tree also grows is China, and Himalayan regions of India and Nepal and is cultivated in Britain and North America as an ornamental tree [13]. Essential oil obtained from the leaves of the plant possesses antifungal activity [14] and also antimicrobial action [15]. Repellancy bioassay had proved that the essential oil from the leaves could repel the primitive wingless insects that live on paper, cotton and cereals at a very low concentration [16]. Epicuberol obtained from the black heartwood of *C. japonica,* is effective against the phenotypic and functional maturation of human monocyte-derived dendrite cells *in vitro* [17]. Based on the available literature the present study was designed to determine the antitumor and cytotoxic/ anticancer activity of different solvent extracts of the leaves of *C. japonica.*

## Materials and Methods

### Collection of plant material

The leaves of *C. japonica* were obtained from Gangtok, Sikkim, India. Authenticity of the plant leaves was confirmed by Botanical Survey of India, Howrah, India. The leaves were washed thoroughly with running tap water to remove dirt particles present on the surface and then dried in sunlight. These were then ground to coarse powder with the help of a mechanical grinder and stored at 4 C in airtight bottles.

### Preparation of extracts

Powdered (50g) sample was soaked separately in 100ml of methanol, petroleum ether and chloroform using Soxhlet apparatus and allowed to stand for 48hr at room temperature for successive steps in extraction. The supernatants were then collected separately, allowed to concentrate *in vacuo* and kept in a desiccator for complete removal of solvents. Aqueous suspension of each powder was prepared with 2% Tween 80 before being used for treatment.

### Animals

Swiss strain of albino male mice, each weighting 18-20 gm were taken and allowed to remain in polypropylene cages with sawdust bedding in the departmental animal house. Animals were fed with standard dry pellets and water was supplied ad libitum. The animals were allowed to acclimatize in the new environment for one week before initiation of experiment. Prior approval was obtained from the Animal Ethical Committee of the University before commencement of the *in vivo* experiments.

### Detection of toxic effect of leaf extracts of C. japonica

Five batches of mice each having 5 animals were starved overnight and given orally graded doses of methanolic extract of *C. japonica* (MC). The doses were 0 (control), 0.5, 0.1, 1.5 and 2.0 mg/gm body weight. The animals were observed carefully for first 2h to detect any behavioral, neurological or autonomic change. The observation was continued up to 100 h for occurrence of any other form of toxicity including death. One-fifth of the maximum safe dose was selected as the challenge dose in subsequent studies [18]. Petroleum ether extract (PC) and chloroform extract (CC) of *C. japonica* were tested for their toxicity in the same manner.

### Tumor producing cells

Ehrlich Ascites Carcinoma (EAC) cells were procured from Chittaranjan National Cancer Institute, Kolkata, India. Maintenance of EAC cells were carried out by repeated intraperitoneal injections in mice on every $9^{th}$ day [19]. The ascitic fluid was drawn with the help of a sterile syringe and number of tumor cells was determined in a Neubauer haemocytometer. Viability of such tumor cells was performed by trypan blue dye exclusion assay and the suspension containing >90% viable cells was used for transplantation.

### In vivo experiments for determinations of antitumor action of C. japonica extracts

Five groups of mice with 10 animals in each were taken and injected intraperitoneally with increasing amounts of MC (µg/gm): 0 (saline control), 100, 200, 400. After 3 h each mouse was given intraperitoneally $2x10^6$ EAC cells. This was recorded as Day 1; administration of MC was continued for 9 consecutive days more. After 24h of last injection, 5 mice from each group were sacrificed, their blood was collected individually to determine various biochemical and hematological parameters. The remaining 5 animals of each group were kept in their cages, served with normal food and water and observed till death to evaluate the efficacy of MC. Similar studies were performed with PC and CC.

### Action of C. japonica extracts on progress of EAC tumor in mice

The following parameters were recorded to determine host response to the extracts:
tumor volume, packed cell volume, total tumor cell count, viable tumor cell count, non-viable tumor cell count, median survival time and increases in life span.
The ascitic fluid from each mouse was collected individually from the peritoneal cavity and volume of fluid was measured in a graduated centrifuge tube and packed cell volume was determined by centrifugation of the entire fluid at 1000 rpm for 5 min.
To determine the tumor cell count, ascitic fluid was taken in a WBC pipette and diluted 100 times; a drop of such a cell suspension was fixed on a Neubaeur counting chamber (Haemocytometer), and number of cells was counted in 64 small squares.
Subsequently, cells were stained with trypan blue (0.4% in normal saline); viable cells failed to get stained while dead cells were uniformly stained. Numbers of viable and dead cells were counted.

## Haematological studies

Estimation of RBC, WBC and haemoglobin was performed with blood samples drawn from the heart following standard procedures [20,21].

## Haemoglobin estimation

Heparinized blood of about 0.1 mL amount was taken in Sakli's Hemoglobinometer and was diluted with 0.1N HCl until the colour was matched with the standard protocol. Reading was recorded and expressed in g/100ml of blood.
Simultaneously number of erythrocytes was counted by diluting the blood sample (1:200) with diluting fluid using Thoma pipette, and after vigorous mixing, a droplet of the mixture was discharged under a cover slip of Neubauer hemocytometer and the corpuscles were allowed to stand for 3 min.
The number of blood cells in 80 small squares was counted under light microscope and the number of cells in 1 cu mm of undiluted blood was calculated.
The total count of leucocytes were obtained by diluting the blood in 1:20 ratio with a diluent fluid. The Neubauer hemocytometer was filled with the mixture and number of cells in four corner blocks (each block subdivided in 16 sq) was determined and total leukocyte count/cu mm of blood was calculated.

## Detection of cytotoxic action of plant extract in EAC cell line

About 1 $10^6$ viable cells of EAC cell line were suspended in 0.1 ml of phosphate buffer saline (PBS; 0.2 M at pH 7.4) in a series of tubes. To the tubes were added increasing amounts of MC (µg/ ml): 0 (control), 100 200, 400, 800 in a final volume of 1 ml with addition of PBS where required. After incubation at 37 C for 30 min viability of cells was determined by employing the method of Boyse [22] with the help of trypan blue. Cytotoxicity of MC was calculated by determining inhibition % and $IC_{50}$ values.

## Statistical analysis

All the experimental values are being presented here as mean ± S.E.M. The data were statistically evaluated by one- way analysis of variants (ANOVA) which was followed by post hoc Dunnett's test using SPSS software; $p< 0.05$was considered as significant and $p<0.01$ was taken as highly significant.

# Results

## Effect of application of plant extracts in Swiss albino mice

Batches of mice taking increasing amounts of MC, PC and CC were all found to survive for 100 h, thus tolerating the extracts even at the highest test doses. Accordingly the amounts taken for determining the protective efficacy of each extract were (µg/gm): 100. 200 and 400 per mouse. Of the three extracts MC showed maximum protective capacity *in vivo* and hence detailed study of MC is being presented here.

## Inhibitory action of MC on progression of tumors produced by EAC

Treatment on growth pattern of EAC tumors by MC revealed statistically significant data. There were definite reduction in volumes of tumor size and packed cells in the treated groups vis- a –vis EAC control group (Table 1). Number of viable cells became lesser with gradually increasing doses of MC. Simultaneously non-viable cell count increased with higher doses of MC (Table 1).

**Table 1:** Effect of methanolic extract of *C. japonica* (MC) on growth of EAC tumors

| Group | Ascitic tumor volume (ml) | Packed cell volume (ml) | Tumor cell count ( $10^7$ / ml ) | | Total cell count ( $10^7$/ml) |
|---|---|---|---|---|---|
| | | | Viable (%cell Count) | Non viable (% cell count) | |
| EAC (control) | 4.3± 0.95 | 2.2± 0.17 | 14.15± 0.21 (98.74%) | 0.18± 0.07 (1.26%) | 14.33±0.28 |
| Treatment with MC (100 µg/gm) | 2.08± 0.03 | 0.75±0.12 | 6.25± 0.52* (85.38%) | 1.07±0.04* (14.62%) | 7.32±0.56* |
| Treatment with MC (200 µg/gm) | 1.55±0.08 | 0.59±0.06 | 7.0± 0.51* (80.83%) | 1.66±0.04* (19.17%) | 8.66±0.55* |
| Treatment with MC(400 µg/gm)) | 1.02±0.05 | 0.31±0.08 | 4.25±0.51* (70.71%) | 1.76±0.04* (29.28%) | 6.01±0.55* |
| Mitomycin C (20 µg/gm) | 0.79±0.18 | 0.45±0.02 | 6.65± 0.23* (96.23%) | 0.26± 0.08* (3.77%) | 6.91±0.31* |

Values are mean ± S.E.M; n= 5 in each growth. Treatment with MC was carried out for 9 days.* $p< 0.01$ for the treated groups versus EAC control; where the statistical significance was performed by one way ANOVA followed by post hoc Dunnett's test.

## Activity of increasing amounts of MC on blood cell counts of EAC infected mice

Actual counts of haemoglobin in normal mice was 14.5 ± 0.2, those of RBC and WBC were 5.8±0.21 and 7.8±0.25 respectively. Such counts in the EAC bearing animals were 9.8 ± 0.11 for haemoglobin; 3.9 ± 0.3 for RBC and 16.6 ± 0.5 for WBC. However, in the MC treated groups the counts were significantly restored and with 400µg/gm of MC all the counts came fairly close to those of normal mice (Table 2).

**Table 2:** Effect of MC (200 µg/gm and 400 µg/gm ) on haematological parameters of mice infected with EAC.

| Group | Hb ( gm %) | RBC (million/$mm^3$) | WBC ($10^3$ cells/$mm^3$) |
|---|---|---|---|
| Normal mice | 14.5± 0.2 | 5.8± 0.21 | 7.8±0.25 |
| EAC (Control) | 9.8±0.11 | 3.9±0.3 | 16.6±0.5 |
| Treatment with MC (200 µg/gm) | 12.1±0.18 | 4.2±0.1 | 12.3±0.2 |
| Treatment with MC (400 µg/gm) | 13.2±0.3 | 5.4±.021 | 8.6±0.23 |

Values given above are mean ± S.E.M; n= 5 in each group. Treatment with MC was made for 9 days. In EAC control group *vs* normal control group, $p< 0.01$; in treated groups *vs* EAC control group, $p< 0.01$; statistically significant values were obtained by performing one way ANOVA followed by Dunnett's test.

## Determination of in vitro cytotoxic activity of MC

There was a slow but steady increase in percentage of inhibition in the EAC cell line with serially increasing amounts of MC (Figure. 1). Such a progress in inhibition proved that the cytotoxic effect of MC on EAC cell line was dose dependent.

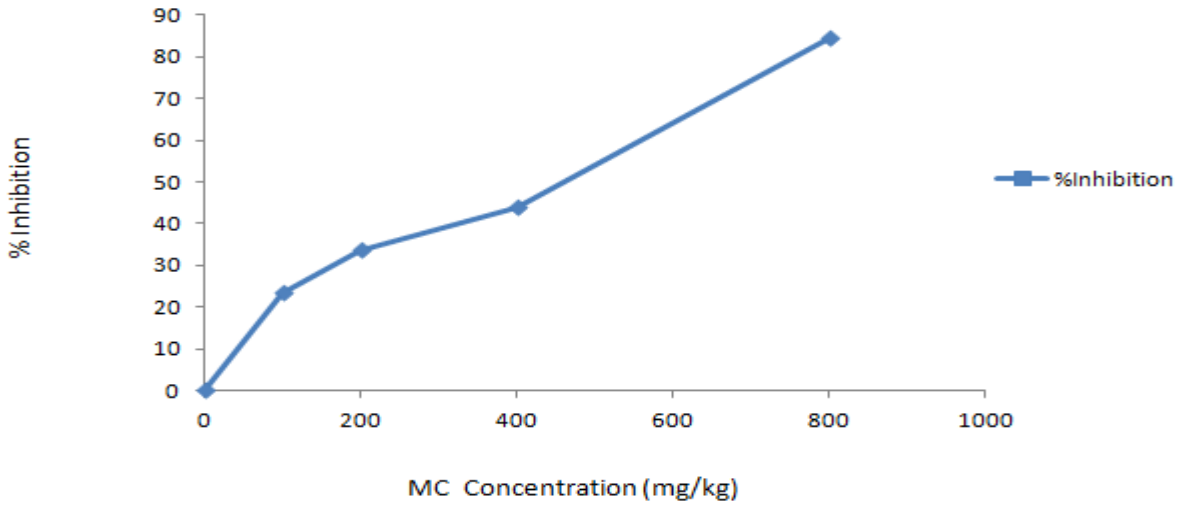


**Figure. 1** Effect of methanolic extract of C. japonica on *in vitro* EAC cell line

## Discussion

Cancer is a form of ailment that involves uncontrolled proliferation of tumor cells. A simple and straightforward animal model for the evaluation of anticancer activity of a substance is Ehrlich Ascitis Carcinoma (EAC). In this system implantation of cancer cells produce a local inflammatory reaction with progressive vascular permeability that results in an intense edema formation, cellular migration and accumulation of ascitic fluid[23]. Ascitic fluid being the direct source of nutrition of tumor cells its aggregation in the peritoneum is a necessity for the survival of tumor cells[24]. Therefore, in this model regression of the rapidly growing carcinoma and prolongation of life span of infected animals show a measure of the intensity of action of an anticancer agent[25]. Treatment of infected mice with MC showed significant reduction not only in total tumor cell count but also in the volumes of ascitic tumor cells in packed cells (Table 1). Furthermore, there was a definite decrease in viable cell count coupled with increase in non-viable cells with concomitant increase in the amounts of MC. Moreover, the gradual heightened response in the life span of infected mice receiving serially increasing doses of MC convincingly support the anticancer effect of this extract, since it is known that prolongation of life span is a positive criterion for evaluating the anticancer effect of an agent[26].

A very major problem in therapy of cancer is myelosuppression and anaemia[27]. Anemia recognized in tumor bearing mice is primarily due to iron deficiency or due to myelopathic and hemolytic states in cancerous animals[27]. In this study it was observed that there was a definite restoration in the number of WBC and RBC along with hemoglobin percent in MC treated mice, which was also dose dependent (Table 2). This confirms that MC possesses protective action on the hemopoetic system of experimental mice.

Many anticancer agents are known to produce prominent cytotoxic effects when applied on cancer patients as therapeutics[28]. However, in this study it was found that MC had a direct cytotoxic activity when compared to the EAC controlled group. Therefore, the mechanism involved in the anticancer property of this plant extract is probably due to its cytotoxic potentiality.

## Conclusion

This study elaborately represent the potentiality of methanolic extract of *C. japonica* leaf as probable antitumor/anticancer phytochemical. Although the petroleum ether and chloroform extracts of the same leaves were also effective in protecting mice infected with EAC, the methanolic extract certainly had distinctly better statistically significant activity in the entire protocol of the present investigation. Preliminary phytochemical analysis has revealed presence of flavonoids in this extract; the flavonoids are known to be often endowed with anticancer activity[29]. Thus, the present study opens up the possibilities of developing novel anticancer drugs from such plant products. Further, studies are in progress to determine the exact mechanism of action of MC at the molecular level.

## Authors' contributions

LB and AD carried out the cytotoxic and haematological studies. PS carried out *in vivo* studies and also participated in the cytotoxic studies. PK participated in the design of the study and performed the statistical analysis. SGD participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

## Conflict of Interest

The authors declare no conflict of interest.

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