

EVALUATION OF ANTI CANCER POTENTIAL OF METHANOL EXTRACT OF *CURCUMA ZEDOARIA*.

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ABSTRACT

Objective: Evaluation of anti cancer activity of methanol extracts of *Curcuma zedoaria* against Ehrlich's ascities carcinoma (EAC) cell line in Swiss albino mice.

Method: *In vitro* cytotoxicity assay has been evaluated by using the trypan blue and MTT assay method. *In vivo* anti cancer activity was performed by using EAC cells induced mice groups (n=12), at the doses of 100 and 200 mg/kg b.w. respectively, half of the mice were sacrificed and the rest were kept alive for life span parameter. The anti cancer potential of MECZ was assessed by evaluating tumor volume, viable and nonviable tumor cell count, tumor weight, hematological parameters and biochemical estimations. Furthermore, antioxidant parameters were assayed by estimating liver tissue enzymes.

Result: Dose dependent cytotoxicity was observed in (* $p < 0.05$) Trypan blue and MTT assay method. *In vivo* anti cancer parameters like tumor volume, tumor weight, and viable cell count were decreased compared to the EAC control group. MECZ treated EAC cell-bearing mice had an increased life span to that of EAC control group. Hematological and serum biochemical profiles were restored to normal levels in MECZ-treated mice compared to the EAC control group. Among the tissue parameters lipid peroxidation, reduced glutathione, superoxide dismutase, and catalase toward normal levels compared to the EAC control group. In short,

Conclusion: MECZ exhibited remarkable antitumor activity in Swiss albino mice, which is attributed to its augmentation of endogenous antioxidant activities and cytotoxic nature.

Keywords: *Curcuma zedoaria*, Zingiberaceae, EAC cell line, antitumor activity, 5-Fluorouracil

INTRODUCTION:

Cancer is a disease in which a group of cells display uncontrolled growth, invasion (intrusion on and destruction of adjacent tissues), and metastasis (spread to other locations in the body via lymph or blood). The Ehrlich tumor was initially described as a spontaneous murine mammary adenocarcinoma. It is a rapidly growing carcinoma with very aggressive behaviour and can grow in almost all strains of mice. In the ascitic form, it has been used as a transplantable tumor model to investigate the antitumor effects of several substances [1]. Available cancer chemotherapeutic agents have shown deleterious effects on the host cells, especially bone marrow, epithelial tissues, gonads, and the reticular-endothelial system. So ongoing research is aiming at new therapeutic approaches which besides being effective in cancer therapy will also have reduced untoward effects on normal cells. Thus the current focus is on medicinal plants and their derivatives which are traditionally used all over the world. They are gradually becoming popular in modern society as natural alternatives to synthetic chemicals. *Curcuma* is a well-known genus of Zingiberaceae family. *Curcuma zedoaria* has components like essential oils, oil-resin, and other constituents with a wide spectrum of biological properties. Presence of extract and the essential oil has cineol, camphor, alpha-pinene, camphor and other compounds. *Curcuma zedoaria* has been used to treat coronary heart disease, liver cancer, anaemia, chronic, pelvic inflammation and helps to prevent leukopenia caused by cancer therapies. It is used to as an antioxidant [2]. Antioxidant compounds in food play an important role as a health protecting factor. Scientific evidences suggest that antioxidants reduce the risk of coronary diseases including cancer and heart diseases. Free

radicals are responsible for aging and causing various human diseases. Antioxidant substances which scavenge free radicals play an important role in the prevention of free radical-induced diseases. By donating hydrogen radical, the primary radicals are reduced to non-radical chemical compounds and are converted to oxidized antioxidant radicals. This action helps in protecting the body from degenerative diseases [3]. Medicinal plants are widely used as alternative medicines for cancer related diseases because it is believed to have active natural occurring compounds in killing cancer. However there are only limited studies on the efficacy and use of medicinal plants. Therefore it is important to identify the components which are responsible for the chemotherapeutic effects by destroying cancer cells [4]. Hence an attempt has been made to extract the phytochemicals present in the rhizome of the plant *Curcuma zedoaria* and carry out tests to determine its antioxidant and cytotoxic activities.

MATERIALS AND METHODS

DRUGS AND CHEMICALS

5-fluorouracil (5-FU) from Sigma (St.Louis, MO), Trichloroacetic acid (TCA) from Merck (Mumbai, India), thiobarbituric acid (TBA) and nitroblue tetrazolium chloride (NBT) from Loba Chemie (Mumbai, India), 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), phenazonium methosulfate (PMS), reduced nicotinamide adenine dinucleotide (NADH), and reduced glutathione (GSH) from SISCO (Mumbai, India) were obtained. All other reagents used were of analytical reagent grade and were obtained commercially.

Experimental Animals

Adult male Swiss albino mice approximately 8 wk of age with an average body weight of 20–25 g were used for the experiment. The mice were grouped and housed in polyacrylic cages (38 cm × 23 cm × 10 cm) with no more than six animals per cage. The animals were maintained under standard laboratory conditions (temperature 25°C–30°C and 55%–60% relative humidity with a 14-h/10-h dark/light cycle). They were allowed free access to standard dry pellet diet (Hindustan Lever, Kolkata, India) and water *ad libitum*. The mice were acclimatized to laboratory conditions for 7 d before commencement of the experiment.

Experimental Protocol

Preparation of Extract

The powdered material (650 g) was extracted with methanol by a Soxhlet extraction apparatus and the extract was evaporated to dryness *in vacuo* (at 35°C and 0.8 MPa) in a Buchi R-114 evaporator (yield 7.99%). The dry extract (MECZ) was kept in a vacuum desiccators until the use.

Acute Toxicity Study

The acute oral toxicity of MECZ in Swiss albino mice was performed as per Organisation for Economic Co-operation and Development guideline 425[5]. The LD₅₀ value of MECZ was determined at 1 g/kg body weight p.o. for mice.

In vitro Cytotoxicity Assay

Trypan Blue Inclusion Assay

The viability and non viability of cells were checked by trypan blue assay method as per standard protocol [6].

MTT Assay

MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide], assay was done as per Haldar *et al* 2015[7].

$$\text{Lysis (\%)} = 100 - (T - PC)/(C - PC) \times 100$$

Where T is the test, PC is the positive control, and C is the control. The IC₅₀ value was evaluated by linear regression analysis using GraphPad Prism software.

Transplantation of Tumor Cells

EAC cells were obtained from Chittaranjan National Cancer Institute (Kolkata, India). The EAC cells were maintained *in vivo* in Swiss albino mice by intraperitoneal transplantation of 2×10^6 cells per mouse after every 10 days. Ascitic fluid was drawn out from EAC tumor-bearing mice at the log phase of the tumor cells. Each animal received 0.1 ml of tumor cell suspension containing 2×10^6 tumor cells intraperitoneally [8].

In Vivo Anti cancer Activity

The Swiss albino mice (20 - 25)g body weight were divided in five groups out of which two groups were normal saline control and EAC control groups respectively. Except the normal saline control group all other groups were transplanted with EAC cell line (2×10^6 cells/animal) i.p. After 24 hours of EAC transplantation, groups other than normal saline control and EAC control were administered MECZ (50 mg/kg body weight and 100 mg/kg body weight) and reference drug 5-FU (20 mg/kg body weight) intraperitoneally for 9 consecutive days. 24 hours after the last dose followed by 18 hours of fasting, fifty percent of animals from each group were sacrificed and antitumor, hematological, serum biochemical and tissue antioxidant parameters were observed. Rest from each group were maintained with food and water *ad libitum* to determine the percentage increase in life span (% ILS) of the tumor host.

Tumor Volume, Packed Cell Volume and Tumor Weight

After dissection the ascitic fluid volume were collected from the peritoneal cavity of mice and measured in graduated centrifuge tube.

Packed cell volumes were determined by centrifuging at 1000 rpm for 5 mins. Weight of mice before and after the collection of ascitic fluid volume was measured and this gives the tumour weight [9].

Viable and Nonviable Tumor Cell Count

The ascitic fluid was taken in a white blood cell (WBC) pipette and diluted 100 times. Then a drop of the diluted cell suspension was placed on the Neubauer's counting chamber and stained with trypan blue (0.4 % in normal saline) dye.

Cells not stained were viable and the stained ones were non viable. Cell viability parameter was calculated by : Cell count = (number of cells × dilution factor) / (area × thickness of liquid film).

Percentage Increased Life Span

The effect of MECZ on tumor growth was monitored by recording the mortality of the experimental mice. The % ILS was calculated by the following formula:

$$\% \text{ ILS} = [(\text{mean survival time of treated group} / \text{mean survival time of control group}) - 1] \times 100$$

Where mean survival time = (day of first death + day of last death) / 2.

Hematological Parameters

Hematological parameters such as hemoglobin (Hb), red blood cell count (RBC), and WBC were measured by using an automated cell counter (Medonica CA 620). WBC count was significantly reduced in a dose dependent manner with respect to EAC control group. RBC count and hemoglobin content were found to be significantly restored to the normal level [10].

Biochemical Parameters

Serum biochemical parameters such as total proteins, serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), serum alkaline phosphatase (SALP), and serum bilirubin were analyzed by using commercially available kits (Span Diagnostics Ltd., Surat, India). SGPT, SGOT, SALP, and Bilirubin indicates the elevated level of liver functional enzymes in serum in EAC treated group with respect to normal animals. The total protein was found to be significantly decreased in the EAC control group as compared with the normal saline group. Administration of MECZ at the dose of 100 mg/kg and 200 mg/kg in EAC bearing mice significantly increased the total protein content as compared with the EAC control [11].

Tissue Antioxidant Parameters

GSH, Superoxide dismutase (SOD) and catalase (CAT) and Lipid peroxide activity in liver tissue were assayed as per the standard protocol [12].

Statistical analysis

The experimental results were expressed as mean ± standard error of mean (SEM). Statistical significance was analyzed by one-way ANOVA followed by Dunnett's post hoc test of significance. P values of <0.05 was considered as statistically significant.

RESULTS

Acute Toxicity Study

The oral LD₅₀ value of the methanol extract of *Curcuma zedoaria* in mice was 1000 mg/kg body weight.

Assay for *in vitro* cytotoxicity

In the assay for *in vitro* cytotoxicity study, Trypan blue inclusion assay and MTT assay on MECZ showed direct cytotoxic effect on the EAC cell line in a dose dependent manner with IC₅₀ value of 20 µg/ml and 54.17 µg/ml respectively. Fig.1 and Fig.2.

Tumor growth and survival parameters

Anticancer activity of MECZ against EAC tumor bearing mice was assessed by tumor volume, tumor weight, viable and non-viable cell

count, mean survival time and % increase in life span. The tumor volume, tumor weight and viable cell count were found significantly increased and non- viable cell count was significantly reduced (p <0.05) in EAC control animals when compared with normal control animals (Table 1). After administration of MECZ at the dose 100 mg/kg and 200 mg/kg, tumor volume, tumor weight and viable cell count were significantly decreased, where as non-viable cell count was significantly higher in MECZ content animal when compared with EAC control animals. Furthermore, the median survival time of EAC control animal was 21 days, whereas in the MECZ treated groups these were 28 days and 33 days, respectively. The reference drug 5-fluorouracil (20 mg/ kg) showed 43 days (Table. 1).

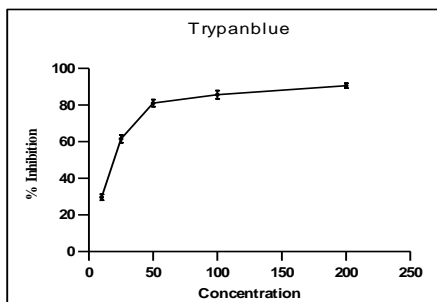


Fig.1: Cytotoxic effect of MECZ on *in vitro* EAC cell line by Trypan blue. Values are Mean ± SEM; where n = 6.

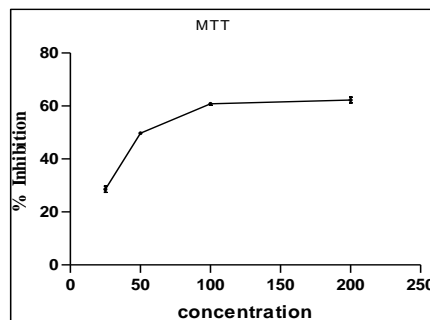


Fig.2: Cytotoxic effect of MECZ on *in vitro* EAC cell line by MTT assay. Values are Mean ± SEM; where n = 6.

Tissue antioxidant assay parameters

Lipid peroxidation results in the formation of ROS species and subsequently elevates the level of malondialdehyde (MDA) which induces cancer in liver tissues. In the present study the MDA level was significantly (P<0.05) increased in EAC control animals when compared with normal control animals. Interestingly, treatment with MECZ significantly reduced the MDA levels as compared with EAC control group (Fig. 3). The levels of Catalase, SOD and reduced GSH were significantly (P<0.05) decreased in EAC control group when compared with normal control group. Administration of MECZ in a dose dependent manner significantly raised the SOD (Fig. 4), reduced glutathione (Fig. 5), Catalase (Fig. 6) levels as compared with EAC control animals.

Table. 1: Effect of MECZ on tumor volume, tumor weight, total cell count, viable and nonviable cell count, mean survival time (MST) and percentage increase life-span (% ILS) in EAC bearing mice.

Parameters	EAC Control (2 × 10 ⁶ cells/mouse)	EAC + MECZ (100 mg/kg)	EAC + MECZ (200 mg/kg)	EAC + 5-FU (20 mg/kg)
Tumor volume (ml)	2.85±0.13	2.1±0.06 ^{a*}	1.29±0.09 ^{b*}	0.55±0.05 ^{b*}
Packed cell	2.04±0.07	1.35±0.8 ^{a*}	0.93±0.3 ^{b*}	0.29±0.01
Viable cell	8.92×10 ⁷ ±0.42	1.71×10 ⁷ ^{a*}	3.51×10 ⁷ ^{b*}	0.60×10 ⁷ ±0.08 ^{b*}
Non-viable cell	0.38×10 ⁷ ±0.04	2.15×10 ⁷ ^{a*}	1.96×10 ⁷ ^{b*}	3.21×10 ⁷ ±0.09 ^{b*}
MST(Days)	21	28 ^{a*}	33 ^{b*}	43 ^{b*}
%ILS	00	62	79	104.76

Statistical significance (p) calculated by one way ANOVA between EAC control group and the treated groups followed by Dunnett's test (* p < 0.05). Each point represents the mean ± S.E.M. (n = 6 mice per groups).

Haematological parameters

Haematological parameters of tumor bearing mice were found to be significantly altered to the normal control group. There was significantly (p<0.05) elevated level of WBC and significantly

reduced level of RBC and haemoglobin (Hb) in EAC control group as compared to normal control group (Table 2). But, treatment with MECZ at the doses of 100 mg/kg and 200 mg/kg in EAC bearing mice increased both the RBC and Hb content significantly while WBC count was reduced significantly when compared with the EAC control group. Table. 2.

Table. 2: Effect of MECZ on hematological parameters in EAC bearing mice

Parameters	Normal saline (5 ml/kg)	EAC control (2×10 ⁶ cells/mouse)	EAC + MECZ (100 mg/kg)	EAC+ MECZ (200 mg/kg)	EAC + 5-FU (20 mg/kg)
RBC (cell × 10 ⁶ /mm ³)	5.00 ± 0.20	1.03 ±0.09 ^{a*}	3.24±0.15 ^{b*}	4.12±0.17 ^{b*}	5.00 ±0.20 ^{b*}
WBC (cell × 10 ³ /mm ³)	4.12 ± 0.32	6.84 ±1.13 ^{a*}	5.59±0.52 ^{b*}	5.00±0.50 ^{b*}	4.89 ±0.33 ^{b*}
Hb. (g/dL)	10.48 ±0.38	4.00 ±0.14 ^{a*}	6.00±0.24 ^{b*}	8.50±0.30 ^{b*}	10.00 ±0.46 ^{b*}

Statistical significance (p) calculated by one way ANOVA between EAC control group and the treated groups followed by Dunnett's test (* p < 0.05). Each point represents the mean ± S.E.M. (n = 6 mice per groups).

Serum biochemical parameters

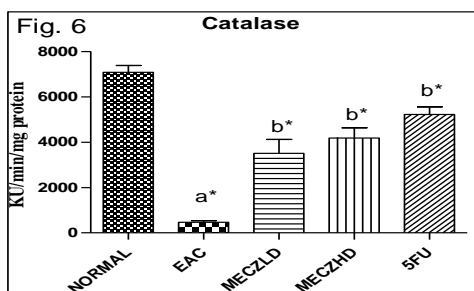
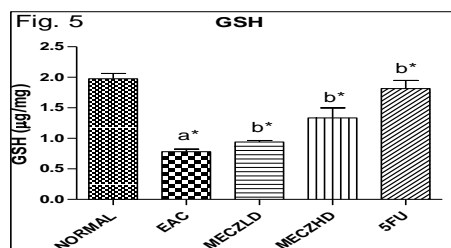
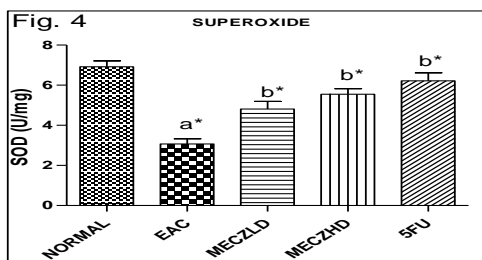
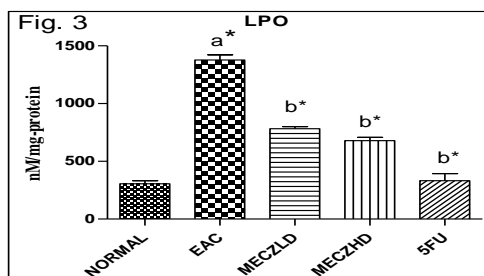
The serum biochemical parameters like amount of SGOT, SGPT and SALP in the EAC control group were significantly (P<0.05) increased as compared to the normal control group. The total protein content

was found to be significantly declined in the EAC control group when compared with the normal control group. Treatment with MECZ at the dose of 100 mg/kg and 200 mg/kg body weight significantly decreased the SGOT , SGPT and mg/kg) and SALP levels where as significantly increased the total protein content in a dose dependant manner as compared to EAC control groups (Table. 3).

Table.3: Effect of MEHS on serum biochemical parameters in EAC bearing mice

Parameters	Normal saline (5 ml/kg)	EAC control (2 × 10 ⁶ cells/mouse)	EAC + MECZ (100 mg/kg)	EAC + MECZ (200 mg/kg)	EAC + 5-FU (20 mg/kg)
SGOT (IU/L)	9.34±0.28	17.79±1.43 ^{a*}	9.41±3.10 ^{b*}	8.2±2.10 ^{b*}	9.45±0.12 ^{b*}
SGPT (IU/L)	14.86±1.02	37.44±3.11 ^{a*}	43.27±14.27 ^{b*}	43.27±10.04 ^{b*}	20.44±0.34 ^{b*}
SALP (IU/L)	75.33±9.34	194.87±7.68 ^{a*}	12.73±5.69 ^{b*}	19.58±2.87 ^{b*}	100.60±10.02 ^{b*}
TOTAL PROTEIN	8.95±0.92	5.69±0.93 ^{a*}	6.01±0.45 ^{b*}	6.53±0.18 ^{b*}	8.57±0.23 ^{b*}

Statistical significance (p) calculated by one way ANOVA between EAC control group and the treated groups followed by Dunnett's test (* p < 0.05). Each point represents the mean ± S.E.M. (n = 6 mice per groups).



Values are represented as mean ± SEM, where n=6. ^a normal control group vs EAC control group, *p < 0.05; ^b EAC control group vs All treated groups, *p < 0.05.

DISCUSSION

Chemotherapy is still a major challenge to the cancer patients because such highly potent drug can be toxic and less than 1% of injected drug molecules can reach their target cells, where as the rest may damage healthy cells and tissue[13]. Some of the common side effects include myelosuppression, alopecia, nausea, vomiting, anaemia due to their toxic effects on local tissues. Contrast to that, natural products offer protective and therapeutic actions to all cells with lower toxicity and are beneficial in facilitating nutrient repletion [14]. The present study was carried out to evaluate the antitumor activity of MECZ in EAC tumor bearing mice. EAC cells are rapidly growing adenocarcinoma with very aggressive behaviour and cells can proliferate in almost all strains of mice. The MECZ treated animals at the doses 100 and 200 mg/kg were observed to witness its anti tumour property. This was found to significantly reduce the tumor volume, packed cell volume, viable tumor cell count and brought back the hematological parameters towards normal levels as compared to EAC control group. This could be because of a direct toxic effect or indirect effect on tumour cell mediated by macrophage activation and vascular permeability inhibition [7]. Prolongation of the life span is one of the vital criteria for judging the value of any anticancer agent[14]. Therefore by looking at the increased life span of the treated animals it can be inferred that MECZ increases the life span of EAC tumor-bearing mice, which may be due to the prevention of tumor cell proliferation and the decrease in the ascitic fluid volume which serves as the nutritional source to tumour cells[7]. Reduction in RBC count, haemoglobin content and increased number of WBC cells are often exhibited in cancer. Restoration of these parameters to their normal levels proves the protective action of the extract on the haemopoietic system. Certain tissue cells contain characteristic enzymes, which enter into the blood only when the cells to which they are confined are damaged or destroyed. A significant quantity of these specific enzymes in the blood indicates the probable site of tissue damage. For this reason, serum enzymes have been studied as both early possible indicators of cancer and as an aid in following the progression and regression of the disease. In certain circumstances, they can be carcinogenic and may stimulate hepatotoxicity[11]. Cancer related hepatocellular damage leads to elevated levels of hepatic enzymes like SGPT, SGOT and lowered level of Total Protein. Extract brought back the enzymes to their normal level indicating hepatoprotective activity. Lipid peroxidation is a process of oxidative lipid degradation, in which cell damage occurs due to the formation of free radical electrons from the lipids in cell membranes. Malondialdehyde (MDA) is formed during oxidative degeneration as a product of free oxygen radicals which is accepted as an indicator of lipid peroxidation [15]. MDA, the end product of lipid peroxidation, was reported to be higher in cancer tissues than in nondiseased organ [16]. In EAC-bearing mice, the levels of lipid peroxide in liver were significantly elevated; however, this was reduced to near normal levels in the MECZ-treated groups. This may be a sign of the decline in free radical production and subsequent reduction in oxidative stress, which is the main risk factor of the ailment[7]. The cells are also equipped with enzymatic antioxidant mechanisms that play a vital role in the obliteration of free radicals. It has been reported that a decline in SOD activity in EAC-bearing

mice may be due to the loss of Mn²⁺-associated SOD activity in EAC cells and the loss of mitochondria, which ultimately leads to a decrease in total SOD activity in liver [17]. The inhibition of both SOD and CAT activity as a result of tumor growth was also reported [18]. Similar findings were observed in the present investigation with EAC-bearing mice. The administration of MECZ not only elevated SOD and CAT levels in a dose-dependent manner but also returned lipid peroxide and GSH content to their normal levels, which confirms the antioxidant and free radical scavenging property of MECZ [7].

CONCLUSION

The methanol extract of *Curcuma zedoaria* possess significant anti cancer activity both *in vitro* and *in vivo* and increased the life span of tumor bearing mice. Further investigations are in progress in our laboratory to identify the active principles involved in this anti cancer activity.

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CONFLICT OF INTEREST

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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