EVALUATION OF ANTICANCER ACTIVITY OF *CUCUMIS CALLOSUS* AGAINST EHRLICH'S ASCITES CARCINOMA BEARING MICE

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ABSTRACT

Objective: Our previous research isolated Cucurbitacin B (CuB) and ebenone leucopentaacetate (ELP) from methanolic fruit extract of *Cucumis callosus* (MFCC). The fruits of *C. callosus* (Rottl.) Cogn. (Family: Cucurbitaceae) plant have been traditionally used for antioxidant, anti-inflammatory, and antidiabetic actions. The objective of this research was to evaluate *in vitro* and *in vivo* anticancer effect of MFCC on Ehrlich Ascites Carcinoma (EAC) cell lines.

Methods: *In vitro* anticancer assay of MFCC and standard drug, 5-fluorouracil (5-FU) was evaluated using Trypan blue and 3-(4, 5-dimethylthiazol-yl)-2, 5-diphenyl tetrazolium bromide methods. *In vivo* anticancer activity of MFCC and 5-FU was also performed after 24h of EAC cells (2×10^6 cells/mouse) inoculation based on toxicity study for 9 consecutive days. The activity of the extract was assessed by the study of tumor volume, tumor weight, viable and non-viable cell count, hematological parameters, and biochemical estimations.

Results: The MFCC showed the direct antitumor effect on EAC cells in a dose-dependent manner with an IG_{50} value of 0.61 mg/ml. Furthermore, MFCC (350 mg/kg) exhibited significant (p<0.01) decrease in tumor volume, tumor weight, and viable cell count of EAC-treated mice. Hematological profile, biochemical estimation assay significantly (p<0.01) reverted to normal level in MFCC, and 5-FU treated mice.

Conclusion: The anticancer activity of fruits of C callosus is may be either due to the presence of CuB or/and ELP as phytoconstituent and the activity is comparable to standard drug 5-FU.

Keywords: Cucumis callosus, Anticancer, Ehrlich Ascites Carcinoma cell lines.

INTRODUCTION

Cancer is one of the largest causes of mortality in the world in the 20th century and Hussain *et al*. 2012 explored the pattern and trends of cancer in Odisha. A total of 74,861 cancer inpatients were registered at Acharya Harihar Regional Cancer Center, Cuttack, Odisha, for the years 2001–2011 [1,2].

Nature is always the great contributor toward this goal. Plants, vegetables, and herbs have been accepted as a source of cancer-preventing agent. Most of the pharmaceutical sectors throughout the globe carried out research to find a lead compound from the traditional systems of medicine which can block the development of cancer in a human [3,4].

Most of the cancer chemotherapeutic agents are associated with toxicity toward normal cells and tissues. Optimal dosing of cancer chemotherapeutic agents is often limited because of severe bone marrow depression as toxicity. It is a continuing challenge to design a therapy that is safer, effective, and selective [5,6]. Modern cancer biology focused on new anticancer drug development that may act in different mechanisms. In fact, those compounds having cytotoxic or cytostatic ability against cancer cells show a potential anticancer activity [7].

Evidence suggests that phytochemicals from fruits and vegetables may play an important role in reducing diabetes and cancer [8,9]. The proposed plant part in this study is fruits of *Cucumis callosus* (Rottl.) Cogn. (Cucurbitaceae). Fruits are oval or elliptical in shape and having bitter pericarp with numerous small seeds (Fig. 1) [10,11]. Tribal peoples of Balasore and Baripada district, Odisha, and East Midnapore district of West Bengal use fruits of *C. callosus* as a vegetable, during worship and for curing diabetes, epilepsy, inflammatory disorders, and diarrhea [12]. Ehrlich Ascites Carcinoma (EAC) cells are experimental tumor models used worldwide in cancer research. In 1907, Paul Ehrlich discovered this tumor in the mammary gland of a white mouse, and the tumor was named after him. It is a rapidly growing carcinoma with very aggressive behavior and is able to grow in almost all strains of mice. In ascites form, it has been used as a transplantable tumor model to investigate the antitumor effects of several substances [13,14].

This research evaluated the anticancer activity of *C. callosus* against Ehrlich's ascites carcinoma bearing mice. Our previous research isolated Cucurbitacin B (CuB) and ebenone leucopentaacetate (ELP) from methanolic pericarp extract of *C. callosus* [15]. CuB inhibits the growth of human malignant cells, both *in vitro* and *in vivo*, and has been shown to be effective against breast cancer, head, and neck squamous cell carcinoma, pancreatic cancer, hepatocellular carcinoma, osteosarcoma, and myeloid leukemia [16,17]. Consequently, natural and semisynthetic CuBs are proposed as a promising source for the development of new drugs for the prevention and treatment of various cancers. The objective of the research was to evaluate *in vitro* and *in vivo* anticancer effect of fruits of *C. callosus* on EAC cell lines.

METHODS

Drugs and chemicals

5-fluorouracil (5-FU), 3-(4, 5-dimethylthiazol-yl)-2, 5-diphenyl tetrazolium bromide (MTT), and Annexin V-FITC were purchased from HiMedia, Mumbai, India. All other reagents used were of analytical grade obtained from Merck Specialties Private Limited, Mumbai, India.

Ethics statement

All the experiments were conducted according to norms and guidelines of CPCSEA and Institutional Animal Ethical Committee (367001/C/CPCSEA) of University.

Plant material and extract preparation

The *C. callosus* fruits (15 kg) were collected and authenticated by M. S. Mondal, Botanical Survey of India, Kolkata, India, and the voucher specimen number was CNH/1-1(196)/2007/Tech-II/160. The methanol extract of the pericarp powder of dried fruits was prepared, and its yield was found to be 17.6% w/w.

Acute toxicity study

The LD_{50} dose of the extract was determined by administering the extract orally to male Swiss albino mice [18].

Assay for *in vitro* anticancer assay

Cell culture

EAC cells were obtained from Chittaranjan National Cancer Institute (CNCI, Kolkata, India) for *in vitro* study. 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 at the Pharmacology Research Laboratory of KL Deemed to be University.

Trypan blue exclusion assay

At first, different concentrations of MFCC (0.35, 0.45, 0.55, 0.65, and 0.75) mg/mL and 5-FU (0.02, 0.12, 0.22, 0.33, and 0.44) mg/mL were prepared. 1×10^6 EAC cells were suspended in 0.1mL of phosphate buffered saline (PBS, 0.2 M, and pH 7.4) and mixed with 100 mL of various aforementioned concentrations of the drug. The final concentration of drug solution was adjusted by PBS and incubated at 37°C for 3 h. After 3 h, equal quality of the drug-treated cells is mixed with Trypan blue (0.4%) and left for 1 min. It is then loaded in a hemocytometer, and the viable and non-viable count is recorded within 2 min. Viable cells do not take up color, whereas dead cells take up the color. However, if kept longer, live cells also generate and take up the color [19].

The percentage of growth inhibition is calculated using the following formula:

$$Growth\ inhibition(\%) = \frac{Total\ cells - Dead\ cells}{Total\ cells} \times 100$$

MTT assay

The MTT assay was used for quantitative determination of viable cells. The assay was based on the conversion of the yellow tetrazolium salt-MTT, to purple-formazan crystals by metabolically active cells. 96 well plates at a cell density of 2×10^6/ml per well in 100 µl of RPMI 1640 were allowed to grow in a CO_2 incubator for 24 h (37°C, 5% CO_2). The medium is then removed and replaced by fresh medium containing different concentrations of MFCC and 5-FU for 48 h. The cells are incubated for 24–48 h (37°C, 5% CO_2). Then, 20 µl MTT stock solutions (5 mg/ml in PBS) are added to each well and incubated for 5 h. The medium is removed and 200 µl dimethyl sulfoxide is added to each well to dissolve the MTT metabolic product. Then, the plate was shaken at 150 rpm for 5 min, and the optical density was measured at 560 nm. Untreated cells (basal) are used as a control of viability (100%), and the results are expressed as percentage viability (log) relative to the control [20].

$$\%\ viability = \frac{OD\ of\ test\ material}{OD\ of\ control} \times 100$$

% Inhibition=100−(% viability)

Assay for *in vivo* anticancer assay

Transplantation of tumor

Ascitic fluid was drawn out from EAC tumor-bearing mouse. The viable EAC cells were counted (Trypan blue indicator) under the microscope and were adjusted at 2×10^7 cells/ml. All mice were injected with EAC cells suspension (0.1 ml) (ip).

Treatment schedule

30 numbers of Swiss albino mice (20–25 g) were divided into five groups. Group I kept as normal saline control (5 ml/kg b.w, oral) and Group II kept as EAC control (2×10^6 cells/mouse, ip). After 24 h of EAC transplantation animals in Groups III and IV administered MFCC (350) and (450) mg/kg b.w, oral respectively; Group V administered 5-FU (30 mg/kg b.w, oral) once daily for 9 consecutive days. After administration of the last dose, 6 mice from each group were kept fasting for 18 h and blood was collected by cardiac puncture for the estimation of hematological and biochemical parameters. Antitumor activity of MFCC was assessed by observation of changes with respect to the parameters as per Haldar *et al.* 2010 [13].

Evaluation of apoptosis using fluorescence-activated cell sorting (FACS)

To understand the nature of cell death, we utilized double labeling techniques using annexin-V-FITC/PI to distinguish between apoptotic and necrotic cells (Fig. 2).

RESULTS

Acute toxicity study

The LD_{50} dose of MFCC was found to be 3500 mg/kg, b.w., p.o. in mice.

***In vitro* anticancer assay**

The *in vitro* anticancer assay of the MFCC showed a direct cytotoxic effect in a dose-dependent manner. As the concentration of the drug increased, the cytotoxicity also increased. Average 50% of the cytotoxicity (IG_{50}) was observed at the concentration of 0.61 mg/mL for MFCC and 0.346 mg/mL for 5-FU (Fig. 3).

***In vivo* anticancer assay**

Antitumor activity of MFCC and 5-FU against EAC tumor-bearing mice was assessed by tumor volume, tumor weight, cell count (viable and non-viable), mean survival time, and percentage increase in lifespan. The tumor volume, tumor weight, and viable cell count were found to be significantly increased, and non-viable cell count was significantly decreased in EAC control animals when compared with normal control animals (Table 1). Administration of MFCC at the doses of 350 and 450 mg/kg significantly decreased the tumor volume and viable cell count. Non-viable cell count was significantly higher in MFCC treated animals when compared with EAC control animals.

There was an increased level of white blood cell (WBC) and decreased the level of hemoglobin (Hb) and red blood cell (RBC) in EAC control group as compared to normal control group (Table 2). After treatment with MFCC at the doses of 350 and 450 mg/kg in EAC bearing mice significantly increased the RBC count, Hb content, and significantly reduced the WBC count as compared with the EAC control group.

Fig. 1: *Cucumis callosus* herb, whole fruits, and their cross-sectional parts
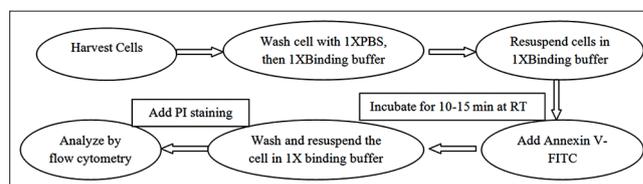

Fig. 2: Schematic representation of the Annexin-V FITC assay

Table 1: Effect of MFCC on tumor volume, tumor weight, total cell count, viable and non-viable cell count, MST and %ILS in EAC bearing mice

Parameters	EAC control	EAC+MFCC (350 mg/kg)	EAC+MFCC (450 mg/kg)	EAC+5-FU (30 mg/kg)
Tumor volume (ml)	2.75±0.23	1.72±0.16[#]	0.88±0.14*	0.65±0.15*
Tumor weight (g)	3.16±0.14	1.18±0.06[#]	0.92±0.07*	0.61±0.03*
Total cell (×10^7 cell/ml)	9.20±0.43	5.45±0.21*	3.70±0.15*	3.91±0.08*
Viable cell (×10^7 cell/ml)	8.92±0.42	3.78±0.31[#]	1.04±0.08*	0.60±0.08*
Non-viable cell (×10^7 cell/ml)	0.27±0.03	1.36±0.08[#]	2.65±0.07*	3.18±0.06*
Viable cell (%)	94.92	75.00	28.68	14.65
Nonviable cell (%)	4.07	26.00	74.43	85.25
MST (days)	18.00	27.50	33.00	38.00

Values are mean±SE from 6 observations in each group. One-way ANOVA between EAC control group and treated groups followed by Dunnett's test. p: [#]<0.05; *<0.01. MST: Median survival time, %ILS: Percentage increase life-span, MFCC: Methanolic fruit extract of *Cucumiμs callosus*, EAC: Ehrlich Ascites Carcinoma, SE: Standard error, ANOVA: Analysis of variance

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

Parameters	Normal control	EAC control	EAC+MFCC (350 mg/kg)	EAC+MFCC (450 mg/kg)	EAC+5-FU (30 mg/kg)
RBC (cell×10^6/μl)	5.18±0.20	2.83±0.13[a],*	4.04±0.24[b],[#]	5.17±0.10[b],*	6.08±18[b],[#]
WBC (cell×10^3/μl)	4.86±0.32	7.87±0.65[a],*	6.12±0.23[b],[#]	5.08±0.23[b],*	6.12±0.66[b],[#]
Hemoglobin (g/dl)	12.46±0.38	4.60±0.28[a],*	8.19±0.43[b],[#]	10.15±0.36[b],*	10.64±0.38[b],[#]

Values are mean±SE from 6 observations in each group. One-way ANOVA between EAC control group and the treated groups followed by Dunnett's test. [a]EAC control group versus normal group; treated groups versus EAC control group. p: [#]<0.05; *<0.01. MFCC: Methanolic fruit extract of *Cucumis callosus*, EAC: Ehrlich Ascites Carcinoma, SE: Standard error, RBC: Red blood cell, WBC: White blood cell, ANOVA: Analysis of variance

FACS

Our flow cytometry data revealed that, in comparison with control untreated EAC cells (Fig. 4), MFCC at the doses of 350 and 450 mg/kg increases the apoptosis level by 12.7 and 14.8%, respectively.

Statistical analysis

The data expressed as the mean±standard error were statistically analyzed using one-way ANOVA followed by Dunnett's *post hoc* test by GraphPad Prism software, version 5. p<0.05 was considered significant and p<0.01 as highly significant.

DISCUSSION

Our previous research evaluated hypoglycemic, antioxidant, and antihyperlipidemic potential of *C. callosus* fruit and also, proved that isolated CuB was the promising source for such activity [15,21]. It is well known that plants with antioxidant property can be effective for the treatment of cancer, liver damage, inflammation, etc. Hence, the effect of MFCC on EAC bearing mice satisfy the notion. It may be that the active phytoconstituent CuB reaches the target site. The plants with the genus *Cucumis* are found to be effective as anticancer agent only when they contain CuB. Thus presence of CuB or ELP may also be the reason for the MFCC to exhibit anticancer effect against EAC bearing mice.

In EAC tumor-bearing mice, a regular rapid increase in ascitic tumor volume was observed. Ascitic fluid is the direct nutritional source for tumor cells, and a rapid increase in an ascitic fluid with tumor growth would be a means to meet the nutritional requirement of tumor cells [22]. 5-FU is a key anticancer drug, with a broadspectrum of activity against various forms of tumors (of the brain, breast, gastrointestinal tract, ovary, liver, pancreas, etc.), alone or in combination with other chemotherapeutic agents [23,24]. Treatment with MFCC and 5-FU decreased the tumor volume, tumor weight, and viable tumor cell count of the tumor-bearing mice. Usually, in cancer chemotherapy, the major problems that are being encountered are a reduction in RBC and WBC count in blood [25]. Treatment with MFCC and 5-FU brought back the RBC and WBC count toward the normal levels. Hence, that was clearly indicated the protective action of MFCC on the hemopoietic system.

Cucurbitacins are immensely bitter taste compound and are found in many plants belonging to Cucurbitaceae family. CuB, D, E, I, and their derivatives have been studied for their anticancer activities [26]. Miliato *et al.* 2012 demonstrated *in vitro* and *in vivo* anticancer properties of Cucurbitacin isolated from *Cayaponia racemosa* [27]. The CuB exhibited strong cytotoxic effects against breast cancer cells in a dose-dependent manner and was shown to prominently alter the cytoskeletal network of breast cancer cells, inducing rapid morphological changes and improper polymerization of the microtubule network [28]. Furthermore, scientists discovered that CuB inhibited the tyrosine phosphorylation of STAT3, STAT5, and JAK2 in pancreatic cancer cell lines (Panc-1 and MiaPaCa-2) *in vitro* and in Panc-1 xenografts *in vivo*. Inhibition of the JAK-STAT pathway affected various downstream targets involved in progrowth signaling (e.g. c-myc, cyclins, and survivin) and apoptosis (e.g. p53, Bcl-xL, and Bcl- 2) [29,30]. The cytotoxic effect of MFCC against EAC cells is probably due to downregulation of JAK and STAT proteins in EAC cells. The apoptotic mechanism was observed by cell cycle analysis using FACS.

In the early stages of apoptosis, the cell membrane is still intact and impermeable to DNA binding dye PI. However, Annexin-V binds specifically with phosphatidylserine is translocated to the extracellular leaflet of the membrane. In contrast, during necrosis, because the cell membrane is ruptured, these cells take up both the fluorochromes. The MFCC induced cell death observed in this study can occur by two distinct modes - apoptosis and necrosis, which can be distinguished by morphological and biochemical features. Annexin-V-FITC/PI staining of MFCC-treated EAT cells resulted in an increase in Annexin$^+$/PI$^-$ and Annexin$^+$/PI$^+$ cells compared to the control (Annexin$^-$/PI$^-$), indicating apoptosis as a possible mode of cell death [31,32].

CONCLUSION

The present study demonstrates that the methanolic pericarp extract for *C. callosus* fruits has remarkable antitumor activity against Ehrlich's ascites carcinoma cells treated mice.

AUTHOR'S CONTRIBUTION

Conceived and designed the experiments: Siva Prasad Panda and Uttam Prasad Panigrahy. Performed the experiment: Siva Prasad Panda and A.R Reddy. Analyzed data: A.R Reddy and Uttam Prasad Panigrahy. All authors read and approved the final manuscript.
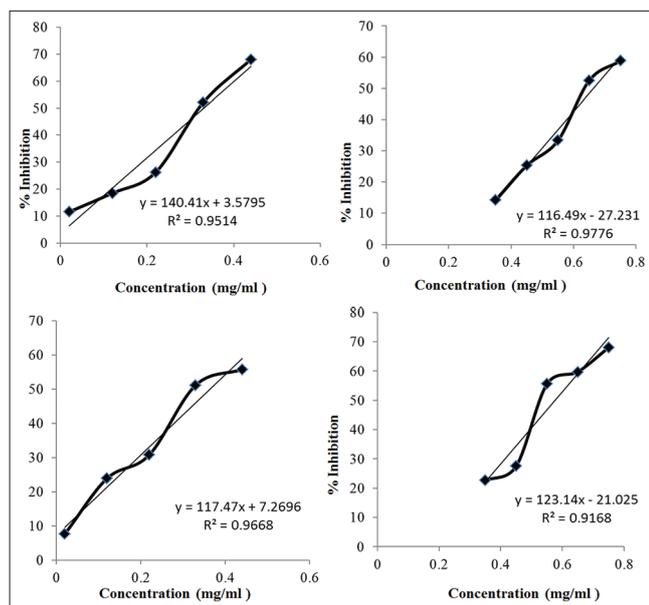
Fig. 3: Anticancer effect of methanolic fruit extract of *Cucumis callosus* and 5-FU on Ehrlich Ascites Carcinoma cell line by Trypan blue and 3-(4, 5-dimethylthiazol-yl)-2, 5-diphenyl tetrazolium bromide assay
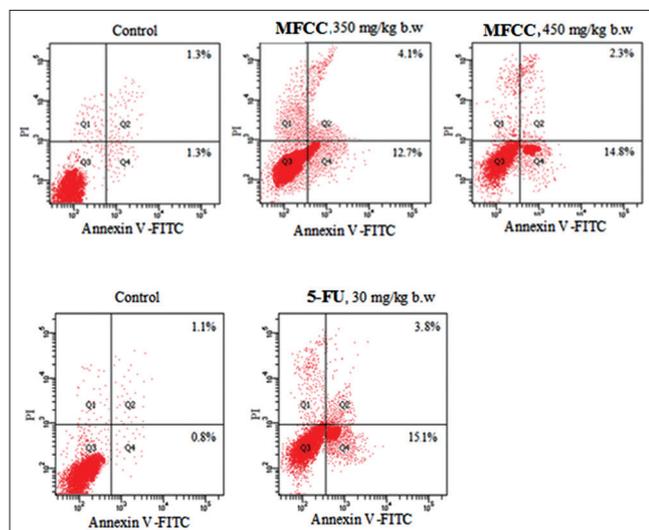
Fig. 4: Evaluation of percentage of apoptosis for methanolic fruit extract of Cucumis callosus and 5-FU

CONFLICTS OF INTEREST

The author declared that they have no conflicts of interest.

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