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Research Article

Evaluation of anticancer activity of Indian Earthworm *Lampito mauritii*

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ABSTRACT

The dry powdered earthworm *Lampito mauritii* was tested for its toxicity at different concentrations (10, 20, 40, 80,160 and 320 µg/ml) for MTT assay in HT 29 colorectal cancer cells. The IC_{50} for HT 29 was 80 µg/ml. There was a tremendous percentage of inhibition for HT 29 cells recorded upto 82.25 % at 320 µg/ml in 48 hours. Treated HT29 cells at 80 µg/ml concentration showd 47.67 % cell cycle arrested at G2/M phase. This result concluded that the sample arrested cell cycle at G2/M phase and at the same time these treated HT29 cells undergoes/ induced apoptosis. Under fluorescent staining with AO/EB, the apoptotic morphological changes could be detected more significantly in the earthworm powder treated than in the untreated cells. The results showed signs of necrosis at 320 µg.ml-1 but morphological changes of typical apoptosis was observed at 80 µg/ml concentration. The anticancer activity of the earthworm powder produced by the earthworm *L. mauritii* seems to be a promising new direction in drug delivery research.

Key words : *Lampito mauritii*, HT 29 cells, Flow cytometric assay, Fluorescent dye staining,

INTRODUCTION

Prevention and early detection are key factors in controlling and curing colorectal cancer. Indeed, colorectal cancer (CRC) is the second most preventable cancer after lung cancer. When the cancer is identified early, initial treatment often lead to an excellent outcome. Colorectal cancer is the cancer that occurs in the large intestine and rectum. It is the third most common cancer in men and the second in women. The burden of CRC has rapidly risen in some economically developed Asian countries like Japan, South Korea and Singapore [1&2]. Fortunately, the age adjusted incidence rates of CRC in all the Indian cancer registries are very close to the lowest rates in the world .Although many people think of colorectal cancer as a disease that primarily affects men, it is also common among women. Today, the average person has about a 1 in 20 chance of developing colorectal cancer during his or her life. The choice of treatment for colorectal cancer largely depends on the stage of the disease (ie) how large the tumor has grown, how deep it has invaded the layers of the colon or rectum. Treatment options include surgery, radiation therapy, chemotherapy, and combinations of these approaches. Although these various therapeutic procedures have decreased the mortality rate of this disease, more effective therapies are still warranted, especially for cases of metastatic disease. The chemotherapeutic regimens in use are not effective enough to destroy the cancer cells or alternatively result in significant side effects, such as bone marrow suppression, gastrointestinal mucosal damage and neuropathy. Thus, it is necessary to find new promising therapeutic agents. Earthworms have been widely used in traditional Chinese medicine for thousands of years. However, it is only during the past few decades, with the development of biochemical technologies, that research on the pharmaceutical effects of earthworms has been initiated. Recently earthworm extract was found to have an anti-tumor effect [3-7].Previously crude protein preparation extracted from whole earthworm tissue of *Eisenia foetida*, named No.2 of earthworm extraction (EE2), could suppress tumor growth in mice inoculated with sarcoma 180 and Ehrlich ascites tumor [8].Subsequent studies demonstrated that EFE showed significant anti-tumor activity in hepatoma cells both *in vitro* and *in vivo*, which may be because EFE could induce apoptosis of

hepatoma cells and inhibit the expression of matrix metalloproteinase-2(MMP-2)[9-12].The present study was aimed to test the anticancer activity of the earthworm powder obtained from *Lampito mauritii* in HT-29 cells.

MATERIALS AND METHODS

Earthworms

Matured earthworms of *Lampito mauritii* (Megascolecidae, Oligochaeta) were obtained from Gopal farm house,Panikkampatti ,Karur district, Tamil Nadu, India. These earthworms were maintained in large pits that contained mixture of soil and cow-dung. The temperature was maintained at 25 ± 2ºC. The worms were acclimatized for at least 15 days before pounded into powder.

Preparation of Earthworm Powder

Earthworms in the size ranging between 15 and 18 cm were alone chosen for this investigation. 500 sexually matured, clitellated (900 mg/worm) earthworms *Lampito mauritii* (Kinberg) were obtained from the container. They were washed with running tap water and then fed with wet blotting paper for 18-20 hours for gut clearance. The gut cleared worms were again washed with distilled water. The worms were kept in a plastic trough tightly covered with polythene cover and exposed to sunlight for three days to kill the earthworms. Mucus and coelomic fluid oozed out that digested the dead worms forming a brown colored paste and this was freeze dried into powder.

***In vitro* cytotoxicity assay of *L.mauritii* powder**

In vitro cytotoxicity assay of *L.mauritii* powder was determined by using the procedure described by Mosmann[13]. The HT29 cells were grown in minimal essential medium, supplemented with 4.5 g/L glucose, 2 mmol/L L-glutamine and 5% fetal bovine serum (growth medium) at 37 °C in 5% CO_2 incubator. The MTT assay used to determine the inhibitory effects of test compounds on cell growth *in vitro*. In brief, the trypsinized cells from T-25 flask were seeded in each well of 96-well flat-bottomed tissue culture plate at a density of 5×10^3 cells/well in growth medium and cultured for 48 hours at 37 °C in 5% CO_2 to adhere. Test Compounds were incubated in RPMI media without FBS & antibiotics for 24 hours. After 48 hours incubation, the supernatant was discarded and the cells were pretreated with growth medium and were subsequently mixed with different concentrations of test compounds (10, 20, 40, 80,160,320 µg/ml) in triplicates to achieve a final volume of 100 µL and then cultured for 48 hours.After incubation with compounds, the media is removed from the wells and added 100µl/well (50µg /well) of the MTT working solution and incubated for 3to 4 hours. After incubation with MTT reagent, the media is removed from the wells and then 100 µl of DMSO was added to rapidly solubilize the colored formazan product. After 30 min incubation, the absorbance of the culture plate was read at a wavelength of 572 nm. The inhibition rate of the EWP on HT29 cells were calculated by % of Inhibition = 100 - (Sample/ Control) x100

Flow cytometric assay

Culture 1.8 $x10^6$ cells (HT29) in a 6-well plate containing 2 ml of complete media. After 24 hrs of incubation, remove the media and wash one time with 1X PBS. Add 0 and 80 µg/ml of control and test sample respectively in 1 ml / well and incubated in 5% CO_2 at 37 ^oC. After 24 hrs of treatment, the adhesive cells and suspended cells were harvested, pooled and pelleted. After washing with PBS, cells were treated with 70% ethanol .After 1 hour centrifuged at 1500 rpm for 5 min at room temperature and then discarded the supernatant . Again wash with PBS, cells were incubated for 1 hour at room temperature in 500 µl of propidium iodide (PI) solution containing 0.05 mg/ml PI, 0.1 mm EDTA, and 0.05 mg/ml RNase A in PBS. The percentage of cells in various stages of cell cycle in the sample treated and un-treated populations were determined using FACS Canto II (BD Biosciences, San Jose, CA) and analyzed by Flow Jo 7.5.5 (Tree Star Ashland OR). The tests were repeated three times.

Fluorescent dye staining

HT29 cells were plated in 24-well plates (10^5 cells / well) and incubated overnight in a humidified 5% CO_2 incubator at 37°C for 24 hours. The adhesive cells and suspended cells were harvested, pooled, pelleted and resuspended in 200 µl medium. Then 15 µl of a mixture of fluorescent dyes containing 100 µg/ml acridine orange and 100 µg/ml ethidium bromide (AO/EB) was added to the cells and mixed gently. The live, apoptotic and necrotic cells were observed under the fluorescent microscope at a magnification of 40**X**.This experiments were repeated twice.

RESULTS AND DISCUSSION

***In vitro* cytotoxicity assay of *L.mauritii* powder**

The cytotoxicity of the EWP was studied on HT 29 colon cancer cells by exposing them at different dosages at 10-320µg/ml (2 fold variations). The graph was plotted with % of inhibition (absorbance at y-axis) against the concentration of the drug (x-axis). At low dilution rates (10µg/ml) the viability was not affected by the EWP, however at greater concentration (320 µg/ml) 82% growth inhibition or cytotoxicity was observed (Table 1& Figure 1). The IC50 concentration was determined (drug concentration that is required to reduce half of the cells from the total population) to be 80µg/ml.

Table No. 1: "Inhibition rate of EWP on HT 29 cells"

Conc. µg/ml	OD 590 nm	% Inhibition
0 (control)	1.02	0.00
10	0.87	14.29
20	0.65	28.34
40	0.45	42.40
80	0.32	51.20
160	0.18	68.50
320	0.06	82.25

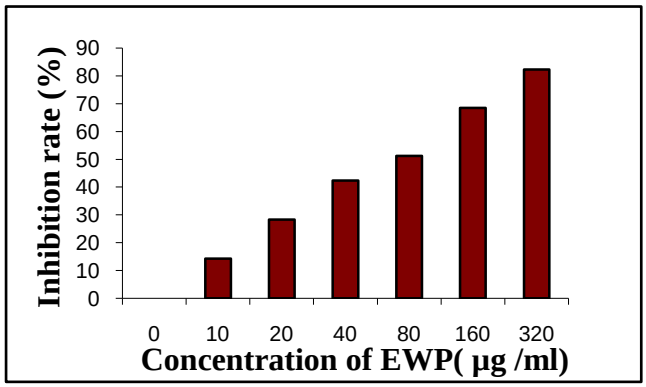

Figure. 1. Inhibition rate of HT 29 cells under the treatment of EWP (*Lampito mauritii)* by MTT assay at the end of 24 hours.

Flow cytometric assay

In contrast to untreated, treated HT29 cells at 80 □ g/ml concentrations showed (Fig.2) 47.67 % cell cycle arrested at G2/M phase. This result concluded that the sample arrested cell cycle at G2/M phase and at the same time these treated HT29 cells undergoes/ induced apoptosis.

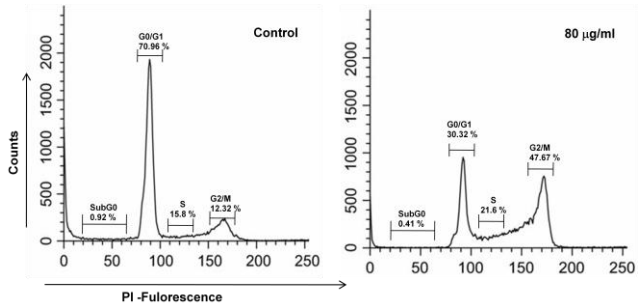

Figure 2: Cell cycle analysis shows 47.67 % cell cycle arrest at G2/M phase.

Fluorescent dye staining

Acridine orange is a cell-permeable dye that intercalates into DNA and results in a green color change. Ethidium bromide enters cells with disrupted membrane integrity and intercalates into RNA and double-stranded DNA to appear orange. Thus differential uptake and binding of these dyes allows us to identify cells in the early and late stages of apoptosis and necrosis. When seen under fluorescent microscope, early apoptotic cells show the presence of green patches of fragmented and condensed chromatin, and late apoptotic cells showed the presence of orange patches of fragmented and condensed chromatin. By comparison, viable cells were uniformly green and necrotic cells were uniformly orange. Acridine orange (AO) – ethidium bromide (EB) staining were done for the HT 29 cells treated with sub-lethal concentration 80 µl.ml^{-1} of earthworm powder for 24 hrs. The type of cell death induced by the sample can be studied by using fluorescent staining for assessment of morphological changes. The Figure exhibits the morphological changes of apoptosis including cell shrinkage and chromatin condensation as compared to control cells. The live, apoptotic and necrotic cells were monitored under the fluorescent microscope. From the results of Figure 3. we found that in HT 29 cells, live cells were seen in the control group (green color), both early and late apoptotic cells were seen in the presence of 80 □ g/ml sample, nearly all cells were late apoptotic cells. These cytological changes indicated that the cells were committed to a specific mode of cell death,probably apoptosis.

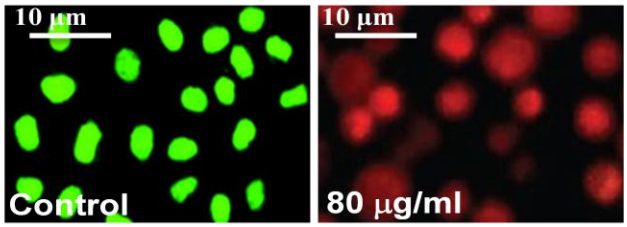

Figure 3: HT29 cells were stained by AO/EB and observed under fluorescence microscope: a control group; b in the presence of 80 µg/ml sample.

Animals have been used as medicinal resources for the treatment and relief of a myriad of illnesses and diseases practically in every human culture. From the time of Charles Darwin till date, earthworms have been fascinating organisms by virtue of their behavior, as source of food with richness of proteins, minerals and fatty acids, and wide use in traditional medicines of different countries for their several lead compounds bringing relief to human ailments. Very few people are aware of the earthworm's long association with medicine despite the availability of relevant literature right from 14th century. Doctors who practice folk/traditional medicine in countries like India, Burma and China claim that earthworms are sources of bioactive compounds, which are found new uses in production of 'life saving medicines' for cardiovascular diseases, cancer, and inflammation. Apart from the anticoagulatory and fibrinolytic activities of earthworm medicines, studies also indicated that the coelomic fluid of earthworms exhibits other biological functions, including bacteriostatic, proteolytic, cytolytic (hemolytic) and mitogenic activities [14-15] .Later, attention has been focused on the earthworm's antitumor activity. There have been numerous articles demonstrating certain components extracted from earthworms possess anti-tumor effects. However, due to the different extracting methods used by various researchers, the ingredients derived from earthworm vary significantly in different studies. The research on the pharmaceutical effects of earthworms has been initiated along with the development of biochemical technologies. Many bioactive molecules which can be consider as potential drug have been detected in the earthworm paste. These molecules exhibited different activities, such as fibrinolytic, anticoagulative, anticancer, antimicrobial and thus may be exploited for the treatment of variety diseases [16]. The whole animal extract of *Pheretima posthuma* has active principles involved in strong fibrinolytic activity and considerable cytotoxic and

anti-tumor activity [17]. Using the MTT assay, it was found that Earthworm Fibrinolytic Enzyme (EFE)could inhibit the proliferation of several cancer cell lines *in vitro*, including gastric cancer cell line SCG7901, esophagus cancer cell line Eca-109, cervical cancer cell line HeLa, leukemia cell line K562, among others. The results suggested that the anti-tumor spectrum of EFE was relatively wide [8- 9]. Inducement of apoptosis in SiHa cells *in vitro* by the CF was a function of dose and time period [18]. Current study has been confirmed that the earthworm powder of *L.mauritii* has potent anticancer activity as revealed by the MTT assay,flow cytometric and fluorescent studies carried out in HT 29 cells. The anticancer activity of the earthworm powder produced by the earthworm *L. mauritii* seems to be a promising new direction in drug delivery research. Our report on anticancer activity of the *L. mauritii* is novel as it is being mostly available in the field in most part of Asia, especially the Indian subcontinent.

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