Acutely oral toxicity of Methanolic Extract of *Pandanus odoratissimus* leaves in Albino Mice as per OECD 425 TG

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ABSTRACT

Anticancer activity of Methanolic Extract of *Pandanus odoratissimus* (MEPO) was studied against Ehrlich Ascites Carcinoma (EAC) cells in Swiss albino mice. Anticancer activity of MEPO was examined by determining the body weight, average tumor weight, tumor cell volume, cell count, viable, mean survival time, life span and hematological parameter. The crude extract increased the life span of EAC treated mice and restored the hematological parameters. Thus, the present study reveals anticancer potential of MEPO against EAC in experimental models.

Key words: *Pandanus odoratissimus*, crude extract, anticancer activity, EAC cell, Vinblastine

INTRODUCTION

Natural products such as plant extract, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug discoveries because of the unmatched chemical diversity they can provide (Cos et al., 2006). According to the World Health Organization (WHO), more than 80\% of the world’s population relies on traditional medicine for their primary healthcare needs. This has captured the interest of many researchers to explore local medicinal plants for valuable medicinal traits. Several studies indicate that medicinal plants contain compounds like peptides, unsaturated long chain fatty acids, aldehydes, alkaloids, essential oils, phenols and water or ethanol soluble compounds. These compounds are significant in therapeutic application against human and animal pathogens, including bacteria, fungi and viruses (Pavrez et al., 2005; Khan et al., 2003). In recent years, numerous drug resistances in human pathogenic microorganisms have developed due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases. On the other hand, free radicals are known to be the major cause of various chronic and degenerative diseases. Oxidation is a natural process in organisms for the production of energy to fuel biological cycles. Conversely, the uninhibited production of oxygen-derived free radicals is involved in the onset of many diseases such as arthritis, atherosclerosis, rheumatoid and cancer as well as in many degenerative diseases related with aging (Halliwell and Gutteridge, 1984). Traditional Indian medicinal plants have been in use from time immemorial and their utility has been increasing day by day in the present scenario. Naturally obtained compounds are considered safer and easily biodegradable than synthetic compounds and the problem of drug resistance observed in synthetic drugs is also reduced (Chanchal and Balasubramaniam, 2011). Plants and plant products represent a source of leads for many pharmaceutical products, phytochemical compounds and secondary metabolites in plants have been used in treating a number of human ailments. Drugs obtained from medicinal plants comprise 25\% of total drugs in developed countries and about 80\% in developing countries (Joy et al., 1998). Cancer is the leading cause of mortality across the world and the failure of conventional chemotherapy to effect major reduction in the mortality indicates that new approaches are essentially needed (Kapadia et al., 2002). An extremely promising strategy for cancer prevention by only method is chemotherapy, which is defined as the use of synthetic or natural agents to block the development of cancer in humans (Gupta et al., 2004). A variety of bioactive compounds and their derivatives have been shown to
inhibit carcinogenesis in a number of experimental system involving initiation, promotion and progression (Ho et al., 1994). Plants, vegetables and herbs are used as folk drugs and traditional medicines that have been accepted currently as one of the main sources of cancer chemoprevention drug (Abdulaev, 2001). Pandanusodoratissimus L. is said to be a restorative, deodorant, indolent and phylactic, promoting a feeling of wellbeing and acting as a counter to tropical lassitude. It may be chewed as a breath sweetener or used as a preservative in foods. It is also said to possess healthful properties, including antiviral, anti-allergy, antiplatelet, anti-inflammatory, antioxidant and antitumor activity (Kirtikar and Basu, 2000).

MATERIALS AND METHODS

Chemicals and reagents

Methanol(Chemid), Sodium nitroprusside (Chemco), Trypan blue (Vetek), N-(1-Naphthyl ethylene diamineDihydrochloride (Himedia), 5-Flourouracil (Chemco).

Collection of plant material

Leaves of Pandanusodoratissimus L. were collected from Gurmitkal, near Gulbarga, north Karnataka, India. The botanical identification was made by Dr. Shiddamallya N, Scientist, National Ayurveda Dietetics Research Institute (NADRI), Bangalore. A voucher specimen was deposited in department (RRCBI-12749).

Preparation of plant extracts

The extract was obtained by infusion and maceration from 200g of plant material. The material was weighed chopped and extracted with solvent. The infusion was prepared with 50 gm of dried leaves in 2 × 200 ml of methanol respective to its temperature and solid matter was removed by filtration. After this preliminary step, the same plant material was extracted in boiling distilled water at the same condition and the maceration was obtained following the aforementioned process at room temperature 28°C overnight. The solvent was removed by rotary evaporation. The yield (w/w) of the infusion and maceration of methanol were found to be 3.78 and 1.78 respectively in terms of collected plant materials.

Animal studies

Albino mice of Swiss strains of either sex were used to the present study. The animals were procured from the listed suppliers of animal breeding center Mahaveera Enterprises A.F plot no. 9 and 18, S.No: 127 and 150Peerzadiguda, GhatkesarMandal. Mice weighing 25-30 g were used for the study. The temperature in the experimental animal room was 22°C(±3°C) and the relative humidity was between 50-60%. These animals were fed pellet diet (CFTRI, Mysore) and drinking water and Ad libitum. They were kept in 12 h light/dark cycle and maintained for at least 5 days prior dose to acclimatizing to laboratory conditions. The animals’ experimental protocol has been approved by our Institutional Animal Ethics Committee (IAEC) of LuqmanParmacy College, ring road, Gulbarga, Karnataka, approval registration no: 346/CPCSEA.

Acute toxicity study

The acute oral toxicity study was carried out in accordance to OECD Test Guidelines 425 (Up and Down Procedure), albino mice, weighing 28 ± 4 g having age 8–10 weeks were randomly selected. Animals were kept under standard conditions for five days. Limit test was performed using MEPO(2000 mg/kg p.o.) as single dose and mice were kept without food for 3-4 h prior to dose but had access to water ad libitum. The dose was administered to individual mice according to body weight. The animals were closely observed for first 30 min, then for 4 h. Food was provided after 1-2 h of dose. After observing survival of treated animals, 4 additional mice were administered with the same dose under same conditions. The same procedure was followed for vehicle treated control group of 5 mice to whom 1% CarboxymethylCellulose (CMC) gel was administered in same manner as that of treated group. The animals were observed closely for any toxic effect within first 6 h and then at regular intervals for a total period of 14 days (OECD, 2008).

In-vitro anti cancer activity

MTT assay

MTT [3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide] is a pale yellow substrate that is cleaved by living cells to yield a dark blue formazan product. This process requires active mitochondria and even fresh dead cells do not cleave significant amount of MTT. Thus the amount of MTT cleaved is directly proportional to the number of viable cells present, which is qualified by colorimetric methods. Briefly, the extract was dissolved in DMSO and serially diluted with complete medium to get the concentration in a range of test concentration. DMSO concentration was kept <0.1% in all the samples. Cellline maintained in appropriate conditions were seeded in 96 well plates and treated with different concentration of the test samples and incubated at 37°C, 5% CO₂ for 96 h. MTT reagent was added to the wells and incubated for four 4 h; the dark blue formazan product
formed by the cells was dissolved in DMSO under a safety cabinet and read at 550 nm. Percentage inhibition was calculated and plotted against the concentrations. Graph (4.42 versions) software was used to calculate the IC$_{50}$ values.

**Trypan blue dye exclusion assay**

Trypan blue is a vital stain that leaves non-viable cells with a distinctive blue color when observed under microscopy. Viable cells have intact cell membrane and hence do not take dye from their surroundings. From this, one can easily distinguish between viable and non-viable cells, so the former are unstained, small, and round, while the latter are stained and swollen. Cell suspensions were prepared by incubating for 24 h in 50% CO$_2$ wherein 300µl of extract was added. After incubating for 48 h, 100µl of cell suspension and 100µl of 0.4% trypan blue solution were taken in an eppendorf tube, mixed thoroughly and allowed to stand for 15 min. Coverslip was placed and Pasteur pipette was used to transfer a small amount of trypan blue- cell suspension. The edge of the coverslip was carefully touched with the pipette tip and each chamber was allowed to fill by capillary action. Starting with a chamber of the haemocytometer all the cells in the 1 mm center square and four 1mm corner squares were counted and percentage inhibition was calculated and plotted with concentrations and Graph Pad Prism (5.0 version) software was used to calculate IC$_{50}$ values (Aditya et al., 2013; Ravikumar et al., 1987).

**In-vivo anticancer activity**

**Ehrlich Ascites Carcinoma (EAC) tumour cells induced anti-cancer study**

Thirty mice were divided into 5 groups and were treated as mentioned below:

- **Group I**: Normal control (oral dose of 10 ml/kg.b.wt. sodium CMC suspension 1%).
- **Group II**: EAC control (oral dose of 10 ml/kg.b.wt. sodium CMC suspension 1%).
- **Group III**: EAC induced + Reference drug (oral dose of 20 mg/kg.b.wt. 5-Flourouracil).
- **Group IV**: EAC induced + methanolic extract (100 mg/kg.b.wt).
- **Group V**: EAC induced + methanolic extract (200 mg/kg.b.wt).

All the treatments were given orally at 24h after tumor inoculation and continued daily once for 14 days.

**Hematological analysis**

On the 15$^{th}$ day half of the animals from each group were anesthetized and blood samples were collected in EDTA containing tubes by retro-orbital puncture method for the evaluation of hematological parameters like Hemoglobin (Hb), RBC and WBC content, Neutrophils (N), Lymphocytes (L), Monocytes (M), and Eosinophils (E). The remaining half animals in each of the groups were observed for the Mean Survival Time (MST) and percent increase in their life span.

**Histopathological analysis**

The vital organs isolated from sacrificed mice were fixed in 10% formalin and embedded in paraffin wax. Paraffin sections were made at 5mm and stained with hematoxylin and eosin. The slides were studied under a light microscope and images of tissues were captured and magnified for further study.

**RESULT**

**Acute toxicity study**

The body weights of test animals of both control and MEPO treated groups were found to be increased progressively throughout the study (Table 1).

**In-vitro anticancer activity**

The MEPO was subjected to MTT assay and Trypan blue
dye exclusion as say against EA Ccelllines. MEPO showed an IC$_{50}$ concentration 140.95µg/ml against EAC cell line (Figure 1). In Trypan blue dye exclusion assay MEPO showed an IC$_{50}$ concentration 150.01µg/ml respectively (Figure 2).

In-vivo anticancer activity

Ehrlich Ascites Carcinoma (EAC) cells induced anticancer study

Effect on tumor growth

The average life span of EAC tumor control animals was observed. When compared to EAC control groups and other groups, MEPO showed a significant change in average life span of animals than in EAC control groups. The average life span of 5-FU treated animals was found to be 33.33%, which indicate its potential anticancer nature (Figure 3). The anticancer nature of MEPO was further confirmed by the significant reduction in the body weight of test animals than EA Control mice. The MEPO treated groups showed a significant decrease in the tumor volume, tumor weight, viable cell count and a significant increase in the non-viable cell count as compared to EAC bearing mice (Figure 4).

Hematological analysis

EAC tumor bearing mice showed significant increase in
WBC (Figure 5) and RBC (Figure 6) as compared to the normal groups. Treatment with MEPO group significantly reversed EAC tumor induced changes in the haematological studies (Figure 7). The standard 5-FU also showed similar observations. The architecture of liver, spleen, lung and kidney cells was observed in histopathological studies (Figure 8).

**DISCUSSION**

The current study was conducted to assess the acute toxicity of aqueous methanolic extract of *Pandanus odorattissmus* leaves in animal model by following OECD guidelines 425 (Gregus and Klaassen, 2001) as the acute oral toxicity study is necessary to determine the safer dose range to manage the clinical signs and symptoms of the drugs (Auletta, 1995). In this study, mice rather than rats were used because it is scientifically documented that lethal dose data collected from mice might be more appropriate to anticipate the toxic effects in human beings (SUNECE, 2017). The toxic outcomes of drugs on vital body organs are exposed by clinical signs and symptoms which are principal observations among other toxicity indicators (Friedman et al., 2017). The extract was found to be safe up to 2000mg/kg. No animal was found dead while some changes in behavioral pattern...
Figure 5: WBC

Figure 6: RBC

Figure 7: Effect of Hematological parameters in in-vivo anticancer study.
like increased respiration, increased somatomotor activity, convulsion, tremor and itching were observed in treatment group in first 24 h. During 14 days of acute toxicity evaluation period, it was observed that food and water intake were normal with non-significant body weight variations. It suggests the normal processing of lipids, carbohydrates and protein metabolism inside animals body because these nutrients play a major role in different physiological functions of the body (Ramaiah, 2011; Ozer et al., 2008).

Hematological parameters are sensitive markers of the physiological changes in response to any environmental pollutant or toxic stress in animals (Chunlaratthanaphorn et al., 2007). Blood platelets have a vital role in the process of blood coagulation. This study showed remarkable elevated levels of platelet count indicating hemostatic activity of tested extract sample (Sillanaukee, 1996). Statistically, significant elevated WBC count and lymphocytes suggest its defending potential against the microorganisms and also its contribution to enhance

Figure 8: Histopathology of control and methanolic extract treated groups at limit dose (2000 mg/kg).
cellular inflammatory process. These results are supported by the study of different researchers (Adedapo et al., 2004; Adeoye et al., 2015). Liver, kidney, heart, lungs and spleen are the vital organs of our body which are the major targeted area of any toxic substance metabolically (Ogunlana et al., 2013). When animals were sacrificed at the end of study, there were no lesions found on macroscopic examination of heart, kidney and liver in comparison with vehicle control group. Hepato cellular damage may result in increased cell membrane permeability and cause release of amino-transferases into blood stream (Travlos et al., 1996, Alimba et al., 2012).

CONCLUSION

Pandanusodorattissmus leaves showed good cytotoxic effect on EAC cell line in both MTT assay and Trypan blue dye exclusion assay. The extract showed significant reduction in the percent increase in body weight, tumor volume, tumor weight, viable cell count and increased non-viable cell count. These results suggest that Pandanusodorattissmus leaves exhibit significant anticancer activity. Further studies are needed to determine the active component responsible for its activity.

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