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In-vitro cytotoxicity studies of the therapeutic orchid: *Eulophia nuda*

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Abstract

The global burden of breast cancer is increasing with alarming rates. The current research trend in the cancer is focused towards finding the safe and effective therapy from the traditionally used medicinal plants. *Eulophia nuda* is one such terrestrial orchid which has been traditionally used for the treatment of tumours. In the present study, the alcoholic, hydro alcoholic and aqueous extracts of *E. nuda* tubers were studied *in-vitro* for their cytotoxic activity using two different models for cytotoxicity, brine shrimp lethality assay and MTT assay. The MTT assay was performed on the breast carcinoma cell line MCF7 and on the non-cancerous Vero cell line. The results suggested that the alcoholic extract of *E. nuda* showed the most significant cytotoxicity, 80.77% on the MCF7 cell line at the concentration of 1000 µg/ml. The extract did not show any cytotoxicity on the Vero cells, and hence it could be considered safe to the normal cells. Further *in-vitro* and *in-vivo* studies are necessary to establish the use of *E. nuda* for the treatment and management of the breast cancer.

Keywords: *Eulophia nuda*, breast cancer, cytotoxicity, brine shrimp lethality assay, MTT assay

Introduction

Breast cancer is one of the most common cancers occurring among the females around the world. The incidences of breast cancer are increasing in the developing countries and by the year 2030, the global burden of breast cancer is expected to cross 2 million. The data from the global and Indian studies shows a significant increase in the incidence and cancer -associated morbidity and mortality of breast cancer. In India, the incidence of breast cancer is increasing rapidly due to the changes in the reproductive risk factors, dietary habits and increasing life expectancy [1-5]. The drawback of the currently available therapies for the breast cancer is that they are having severe side effects and are very costly. Therefore, there is need for safe and cost-effective therapy for the treatment of breast cancer [6].

The recent trend in cancer research is moving towards the traditionally used herbal remedies in efforts to discover new therapeutic agents which are devoid of the side effects associated with current cancer therapies [7]. *Eulophia nuda*, a perennial terrestrial herb with underground tubers, has been traditionally used for the treatment of tumours. The herb is found in central and Southeast Asian regions. In India, it is found in the Himalayan region, from Nepal to Assam, and in Deccan from Konkan southwards. The tubers are reported to be used against tumours, scrofulous glands of the neck and bronchitis [8-11]. In Thailand, this orchid is used in the traditional medicines for the treatment of skin rash and the raw tubers are consumed in order to cure rheumatoid arthritis [12]. *E. nuda* tuber is also reported to have demulcent and anthelmintic action [13]. The tubers also called as 'Salep', which are used as an aphrodisiac [14]. In addition, *E. nuda* tubers are also used for the treatment of acidity, piles and stomach ailments [15]. The present work was undertaken to evaluate the *in-vitro* cytotoxic activity of *E. nuda* extracts using brine shrimp lethality assay and MTT assay on MCF7 and Vero cell lines.

Materials and Methods**Preparation of *Eulophia nuda* extracts**

Fresh tubers of *Eulophia nuda* were obtained from the forest regions of Dang district, Gujarat, India. The tubers were washed with water, dried in shade, finely powdered and stored in air tight containers for further use. The voucher specimen was deposited at Department of Pharmacognosy, K. B. Institute of Pharmaceutical Education and Research, Gandhinagar, Gujarat, India. Three different extracts, alcoholic, hydroalcoholic (30:70) and aqueous extracts were prepared. Ten gram g of the dried, finely grounded powder of *E.nuda* tubers was repeatedly extracted by reflux, using 150 ml of solvents, alcohol, hydroalcohol (30:70) and water.

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The extract was filtered and evaporated to dryness. The dried extract was labelled and stored in an airtight container at 4°C for further use.

Cell culture maintenance

The MCF7 and Vero cell line, was procured from National Centre for Cell Sciences (NCCS), Pune, Maharashtra, India. The cells were cultured in Minimum essential medium (MEM) (Eagle) with non-essential amino acids, supplemented with 10% Foetal Bovine Serum (FBS), antibiotics 1 % (penicillin and streptomycin) in a humidified atmosphere of 5% CO₂ at 37 °C until confluent. The cells were dissociated with Trypsin – EDTA solution. The cells were sub-cultured when they reached 70-80% confluency and the media was changed every two to three days. All the above reagents and chemicals were procured from HiMedia. The stock cultures were grown in 25 cm² tissue culture flasks and all experiments were carried out in 96 well flat bottom micro titre plates (Tarsons India Pvt. Ltd.).

Brine shrimp lethality assay ^[16-17]

Brine shrimps (*Artemia salina*) were hatched using brine shrimp eggs in a perforated plastic bottle kept in a beaker, filled with sterile artificial seawater, under constant aeration for 48 h and continuous light exposure. The artificial seawater was prepared using sea salt 38 g/L and the pH was adjusted to 8.5 using 1 N NaOH. Ten nauplii were drawn through a micro-pipette and were transferred in each well of 24-well plate containing 2.0 ml of artificial seawater. In each well, 0.5 ml of the *E. nuda* extracts containing 1% DMSO were added and maintained at room temperature for 24 h under the light. After 24 h, live nauplii were counted and percentage lethality and LD₅₀ values were calculated using Graph Pad Prism. Experiments were conducted in triplicates along with control i.e., 1% dimethyl sulfoxide (DMSO) in artificial seawater, prepared alcoholic, hydroalcoholic and aqueous extracts of *E. nuda* at four different concentrations of 100, 250, 500 and 1000 µg/ml and 5 Fluorouracil (5FU) as a standard.

MTT Assay ^[18-19]

Cells were seeded in a 96-well flat-bottomed plate and incubated for 24 h at 37°C and in 5% CO₂. Both the cell lines, i.e. MCF7 and Vero were exposed to the alcoholic, hydroalcoholic and aqueous extracts of *E. nuda* at four different concentrations of 100, 250, 500 and 1000 µg/ml for 48 h. The solvent DMSO treated cells served as a control and 5FU was used as a standard for MCF7 cell line and Cisplatin was used as standard for Vero cell line. Cells were then treated with MTT reagent (0.5 mg/ml as final concentration)

for 4 h at 37°C in dark. Then all the media and MTT reagent was removed from the wells and 200µl DMSO solvent was added to each well to dissolve the formazan crystals. The optical density (OD) was recorded at 570 nm using a Microplate (ELISA) reader. The percentage cytotoxicity for MCF7 cells and the percentage cell viability for Vero cells was calculated.

Statistical analysis: The data is expressed as mean ± standard error of the mean (SEM). Statistical calculations were performed by applying one-way analysis of variance (ANOVA) followed by Tukey Test, using Graph Pad Prism software. The results were considered statistically significant if the P < 0.05.

Results

The brine shrimp lethality assay for the cytotoxicity assessment of the alcoholic, hydroalcoholic and aqueous extracts of *E. nuda* was carried out at four different concentrations of 100, 250, 500 and 1000 µg/ml. The results of the brine shrimp lethality assay of the three different extracts of *E. nuda* are as shown in Figure 1. The present data showed that the alcoholic extract of *E. nuda* showed toxicity with 50% mortality at the concentration of 1000 µg/ml on the brine shrimp larvae. The hydroalcoholic and aqueous extract of the *E. nuda* extract did not show any cytotoxicity on the brine shrimp larvae. The LD₅₀ values of the alcoholic extract of *E. nuda* was found to be 964.1 µg/ml whereas, the LD₅₀ values of the hydroalcoholic and aqueous extracts were found to be greater than 1000 µg/ml.

The MTT assay for the cytotoxicity assessment of the alcoholic, hydroalcoholic and aqueous extracts of *E. nuda* was carried out at four different concentrations of 100, 250, 500 and 1000 µg/ml, on two different cell lines, MCF7 and Vero. The results of the cytotoxicity of *E. nuda* extracts on the MCF7 cell line are as shown in Figure 2. The data suggests that the alcoholic extract of the *E. nuda* extract showed more cytotoxicity as compared to that of hydroalcoholic extract, while the aqueous extract of *E. nuda* did not show cytotoxicity on the MCF7 cells. The alcoholic extract of the *E. nuda* at the concentration of 1000 µg/ml showed the most significant cytotoxicity of 80.77 % on the MCF7 cells, having the IC₅₀ value of 285.1 µg/ml. Further the alcoholic, hydroalcoholic and aqueous extracts of *E. nuda* were also tested on the non – cancerous Vero cell line using MTT assay at four different concentrations of 100, 250, 500 and 1000 µg/ml. The results of the MTT assay on the Vero cell line are as shown in the Figure 3. None of the above three extracts of *E. nuda* showed cytotoxicity on the Vero cell line.

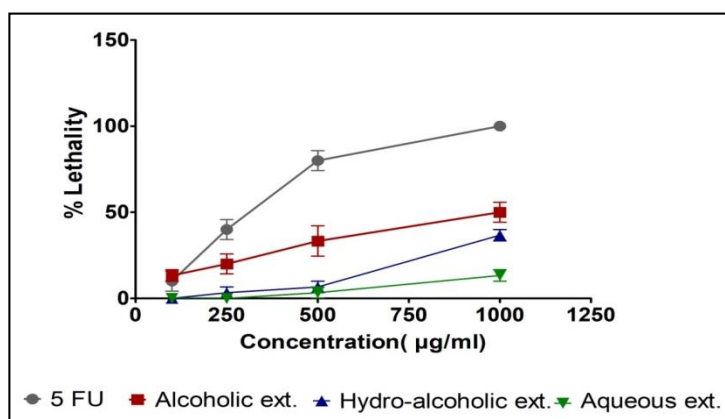


Fig 1: % Lethality of *E. nuda* extracts using brine shrimp lethality assay

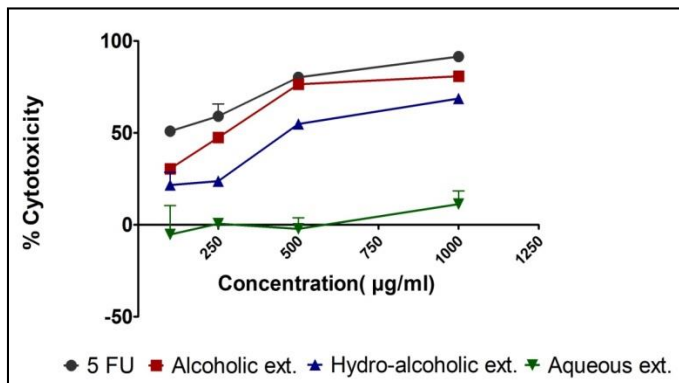


Fig 2: % Cytotoxicity of *E. nuda* extracts using MTT assay on MCF7 cells

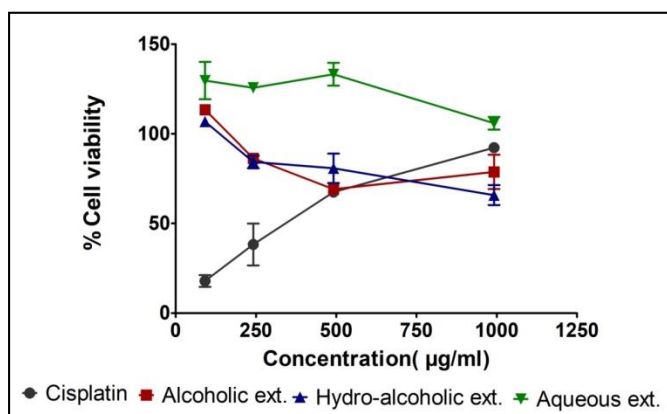


Fig 3: % Cytotoxicity of *E. nuda* extracts using MTT assay on Vero cells

Discussion

In the current time, breast cancer is the most commonly occurring cancers amongst the women around the world [20]. The currently available therapies for the breast cancer are the chemotherapy and radiotherapy. But they are reported to have severe side effects on the patients. The major side effect of the chemotherapy is that, along with the cancerous cells it also shows toxicity to the normal cells. Another side effect is the rapid clearance of the drug from the tumour tissues, which creates the need to find the safe and effective alternative treatment for breast cancer. Since the ancient times, nature has served as a source of medicines to the mankind. Many modern drugs have been isolated from the nature [21]. In the present study one such therapeutic orchid, *Eulophia nuda*, was studied for its cytotoxic activity *in-vitro* by using brine shrimp lethality assay and MTT assay on the human breast carcinoma cell line, MCF7 and the non-cancerous Vero cell line. Three different extracts, alcoholic, hydroalcoholic and aqueous extracts were studied at four different concentrations of 100, 250, 500 and 1000 µg/ml for its cytotoxic activity. The results of the brine shrimp assay indicated the presence of the cytotoxic components, which supports the use of *E. nuda* for its anti-tumour activity. According to Meyer *et al.*, crude plant extract is toxic (active) if it has an LD₅₀ value of less than 1000µg/ml while non-toxic (inactive) if it is greater than 1000 µg/ml [16]. The LD₅₀ values of the alcoholic extract of *E. nuda* was found to be 964.1 µg/ml, which was less than 1000 µg/ml and hence supporting its cytotoxic activity. Further, the prepared extracts of *E. nuda* were studied for their *in-vitro* cytotoxic activity using MCF7 breast cancer cell line and Vero cell line via MTT assay. In the MTT assay, the viable

cells with active metabolism convert the yellow coloured MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into a purple coloured formazan product which can be quantified using a spectrophotometric method [22]. The alcoholic extract and the hydro-alcoholic extract of *E. nuda* showed the significant cytotoxic activity of 80.77% and 68.67% respectively at the concentration of 1000 µg/ml. This results supports cytotoxicity of *E. nuda* reported by Shiram V. *et al.*, 2010. It also supports the claims laid by the folklore in Western Ghats region in India for using this orchid against tumours and cancer [23]. Moreover, the extracts of *E. nuda* did not show any cytotoxicity to the normal cells of Vero cell line *in-vitro*, and hence it can be considered safe to be used for the treatment of cancer.

The literature suggests that the herbs which are reported for their anti-cancer use, are also reported for their other uses, like immuno-modulators, antioxidants, etc., which not only helps in preventing cancer but also acts as chemo-protective. This helps in the overall wellbeing of the patient by improving the quality of life. The antioxidant potentials of these plants, mainly contributed by their bioactive compounds, have been closely linked to their abilities to suppress growth of cancer cells, likely through reduced oxidative stress, which may play a role in the development and progression of cellular damages underlying cancerous growth. As such, it has been suggested that antioxidant supplementation may reduce breast cancer recurrence and mortalities and through bioassay systems and animal studies, there have been indications that numerous naturally-occurring antioxidant compounds possess anti-cancer properties [24-26]. *E. nuda* contains considerable amounts of total phenols, flavonoids, vitamin C and carotenoids. The plant also indicates broad spectrum of antioxidant properties mediated by effective scavenging of various free radicals and subsequently inhibiting the lipid peroxidation, which supports its use for the treatment of cancer [27]. The above reported phytoconstituents of *E. nuda* supports its use in the treatment of cancer.

Conclusion

The results of the present study showed that the tubers of *Eulophia nuda* exhibited moderate to potent cytotoxicity on the human breast carcinoma cell line, MCF7 and was also found non-cytotoxic to the normal cells of Vero cell line. These results suggest that *E. nuda* can be further subjected to the various *in-vitro* and *in-vivo* studies to explore its potential use in the treatment of cancer. Thus, by understanding the complex synergistic interaction of various constituents of anticancer herbs, the herbal formulations can be designed to attack the cancerous cells without harming normal cells of the body.

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