Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(4): 704-707
Received: 17-05-2018
Accepted: 21-06-2018

Dhara R Bhatt
Department of Pharmacognosy, K.B. Institute of Pharmaceutical Education and Research, Gandhinagar, Gujarat, India

Khushboo D Jethva
Department of Pharmacognosy, K.B. Institute of Pharmaceutical Education and Research, Gandhinagar, Gujarat, India

Maitreyi N Zaveri
Department of Pharmacognosy, K.B. Institute of Pharmaceutical Education and Research, Gandhinagar, Gujarat, India

Correspondence
Dhara R Bhatt
Department of Pharmacognosy, K.B. Institute of Pharmaceutical Education and Research, Gandhinagar, Gujarat, India

Cytotoxic activity of alcoholic extract and its fractions of *Eulophia nuda* tubers on MCF7 cell line

Dhara R Bhatt, Khushboo D Jethva and Maitreyi N Zaveri

Abstract
The global burden of breast cancer is increasing with alarming rates among the women worldwide. The currently available therapies for breast cancer are associated with number of side effects. Hence there is a need to find the better alternative drugs for the treatment of breast cancer with fewer side effects. *Eulophia nuda* is one such terrestrial orchid which has been traditionally used for the treatment of tumours. In the present study, the alcoholic extract of *E. nuda* tuber and its different fractions were studied *in-vitro* for their cytotoxic activity using MTT assay on the MCF7 cell line. The results showed that the chloroform fraction at the concentration of 1000 µg/ml showed maximum cytotoxicity of 73.50% among all the fractions which was less than the cytotoxic activity of the alcoholic extract of *E. nuda*, 80.77%. The results suggested that the cytotoxic activity could be due to the synergistic action of the phytoconstituents present in the plant. 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,* cytotoxicity, MTT assay, MCF7 cell line

Introduction
Breast cancer is the highest and the most common of all the cancers, found among Indian females. The age adjusted rate of breast cancer is as high as 25.8 per 100,000 women and mortality rate is 12.7 per 100,000 women. As per the study, young age has been found as a major risk factor for breast cancer in Indian women. Breast cancer projection for India during time periods 2020 suggests the number to go as high as 1797900 [1]. The most effective and the recommended option for the treatment of breast cancer is chemotherapy. The major drawback of the currently available chemotherapeutic drugs are their narrow therapeutic indices and various side effects. To overcome this, current research is focused is on the complementary and alternative medicine. There is an increase in the literature supporting the use of complementary and alternative medicine (CAM) for the treatment of cancer. It is mainly used to control the treatment-related symptoms and to alleviate the side effects which helps in increasing the survival rate and quality of life in breast cancer patients [2].

The complementary and alternative medicine is inclusive of varied therapies for the treatment of cancer, out of which a large percentage is contributed towards the use of plant-based products. The currently available and most commonly used plant derived products in the market are vinca alkaloids, taxanes, epipodophyllotoxins and camptothecin derivatives. Plants have a huge potential to serve as a source of new drugs and various chemicals that may act as chemoprotective against cancer [3]. One such perennial terrestrial herb with underground tubers, *Eulophia nuda,* belonging to family *Orchidaceae*, has been traditionally used for the treatment of tumours. The herb is distributed in the central and Southeast Asian regions. In India, it is found throughout the Himalayan regions, from Nepal to Assam, and in Deccan from Konkan southwards. The tubers are reported to have number of medicinal uses. The tubers are used against tumours, bronchitis and scrofulous glands of the neck [4-7]. In Thailand, the tuber is traditionally used for the treatment of skin rash and rheumatoid arthritis [8]. *E. nuda* tuber is also reported to have demulcent and anthelmintic action [9]. The tubers also used as an aphrodisiac, for the treatment of acidity, piles and stomach ailments [10, 11]. The anti-proliferative activity of a phenanthrene derivative compound 9, 10-dihydro-2,5-dimethoxyphenanthrene-1, 7-diol isolated from *E. nuda* against human cancer cells has also been reported [12]. In the present study the petroleum ether, chloroform, ethyl acetate, *n*-butanol, ethanol and water fractions of the alcoholic extract of tubers of *E. nuda* were simultaneously evaluated for their cytotoxic activity along with the cytotoxicity of alcoholic extract of *E. nuda*, against MCF7 breast carcinoma cell line using MTT assay.

Materials and Methods

Plant material

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.

Preparation of *Eulophia nuda* extract and fractions

The dried and finely grounded powder of *E. nuda* tubers was extracted with alcohol using Soxhlet apparatus. The extract was evaporated to dryness. The dried extract was labelled and stored in an airtight container at 4˚C for further use. The alcoholic extract of the tubers of *E. nuda* was further fractionated successively with petroleum ether, chloroform, ethyl acetate, *n*-butanol, ethanol and water based on their polarity. The prepared fractions were labelled and stored in an air tight container at 4˚C for further use.

Chemicals and Reagents

All the chemicals and reagents used were procured from HiMedia and Sigma Aldrich. The labware and consumables were procured from Tarsons India Pvt. Ltd.

Cell culture maintenance

The MCF7 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), 1% antibiotics solution (penicillin and streptomycin) in a humidified atmosphere of 5% CO_2 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. The stock cultures were grown in 25 cm^2 tissue culture flasks and all experiments were carried out in 96 well flat bottom micro titre plates.

MTT Assay [13, 14]

Cells were seeded in a 96-well flat-bottomed plate and incubated for 24 h at 37°C and in 5% CO_2 atmosphere in an incubator. The MCF7 cells were exposed to the alcoholic extract and six different fractions of alcoholic extract of *E. nuda* tuber i.e., petroleum ether, chloroform, ethyl acetate, *n*-butanol, ethanol and water, at four different concentrations of 100, 250, 500 and 1000 µg/ml for 48 h. The solvent DMSO (1 %) treated cells served as a control and 5FU was used as a standard for MCF7 cell line. Cells were then treated with MTT reagent (0.5 mg/ml as final concentration in phosphate buffer saline) for 4 h at 37°C in the 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 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 MTT assay for the cytotoxicity assessment of the alcoholic extract of *E. nuda* along with its six different fractions based on their polarity i.e., petroleum ether, chloroform, ethyl acetate, *n*-butanol, ethanol and water, was carried out at four different concentrations of 100, 250, 500 and 1000 µg/ml on MCF7 cell line. The results of the MTT assay are as shown in the Figure 1. The results showed that the petroleum ether, chloroform, ethyl acetate and *n*-butanol fractions of the alcoholic extract of *E. nuda* showed significant cytotoxicity at the concentration of 1000 µg/ml and 500 µg/ml. From the different fractions of the alcoholic extract of *E. nuda*, maximum cytotoxicity of 73.50% was observed in the chloroform fraction at the concentration of 1000 µg/ml. The MTT assay was also performed using the alcoholic extract of the tubers of *E. nuda*. The cytotoxicity of the different fractions alcoholic extract of *E. nuda* was found less as compared to the cytotoxicity of the alcoholic extract, which was 80.77% at the concentration of 1000 µg/ml.

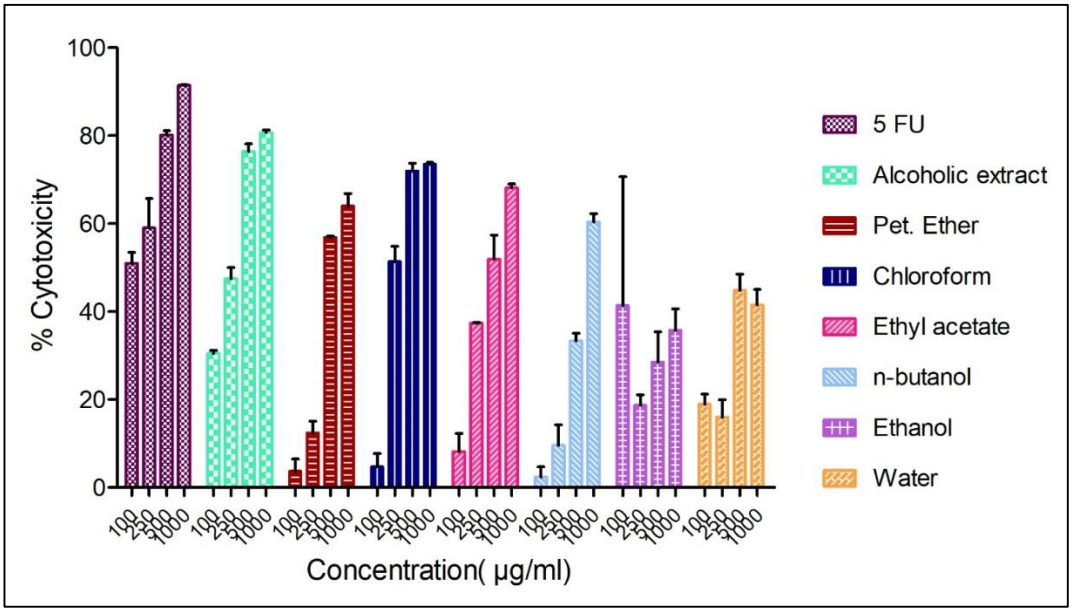

Fig 1: Cytotoxic activity of the alcoholic extract of *E. nuda* tubers and its fractions

Discussion

Breast cancer is the largest occurring cancers among the women worldwide in today's time [15]. The currently available chemotherapy and radio therapy are reported to have severe side effects on the overall health of the patients, which includes toxicity to the normal cells and rapid clearance of the drug from the tumour tissues. Hence, there is a need to find the safe and effective alternative treatment for the treatment of breast cancer. Nature has served as a reservoir for the medicines to the mankind since the ancient times. There are numerous drugs which are reported to have activity against various diseases [16]. In the literature it is mentioned that the herbs which are reported for their anti-cancer activity are also having other uses like immuno-modulators, antioxidants, etc., which helps in the treatment of cancer by improving the overall health of the patient. The phytoconstituents present in the plants are mainly responsible for the therapeutic activity of the plants. They generally suppress the growth of the cancerous cells by reducing the oxidative stress responsible for the cellular damage. This could be due to the antioxidants present in the plants. There are also reports of the naturally occurring antioxidant compounds which possess anti-cancer activity [17-19]. *Eulophia nuda*, belonging to the family *Orchidaceae*, is one such medicinally important orchid which possess many therapeutic activities. It contains various phytoconstituents like flavonoids, total phenols, carotenoids and Vitamin C. The plant is also reported to have antioxidant property, which supports its use in cancer [20]. In the present study an attempt was made to study the cytotoxicity of different fractions of the *E. nuda* tuber simultaneously with the cytotoxicity of the alcoholic extract of *E. nuda tuber*. The *in-vitro* cytotoxicity study was performed using MTT assay on the breast carcinoma cell line MCF7. The MTT assay was performed using six different fractions of alcoholic extract of *E. nuda* tuber based on their polarity i.e, petroleum ether, chloroform, ethyl acetate, *n*-butanol, ethanol and water along with the alcoholic extract of *E. nuda*, using four different concentrations of 100, 250, 500 and 1000 µg/ml. The yellow coloured MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) converts the viable cells with active metabolism into a purple coloured formazan product, which is dissolved in the solvents like iso propanol or dimethyl sulfoxide and quantified using a spectrophotometric method [21]. The results showed that the petroleum ether, chloroform, ethyl acetate and *n*-butanol fractions of the tubers of *E.nuda* showed significant cytotoxicity at the concentration of 1000 µg/ml and 500 µg/ml, but the maximum cytotoxicity of 73.50% was observed in the chloroform fraction at the concentration of 1000 µg/ml. Although the fractions of alcoholic extract of *E. nuda* tuber showed significant cytotoxic activity, the cytotoxic activity of the fractions was less as compared to the whole alcoholic extract of the tuber, which was found to be 80.77% at the concentration of 1000 µg/ml. This results supports the cytotoxicity of *E.nuda* reported by Shriram 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 [12]. The obtained results are suggestive of the fact that the cytotoxic activity could be a result of the synergism of various phyto constituents present in the *E. nuda* tuber. Many plants are studied for their anti-cancer activity, which includes studies involving single pure compounds, fractions as well as whole herb for their activity. However, there are several reports in the literature which are reported to show a significant activity when used as whole herb rather than using isolated active ingredients. This supports the synergistic action of the plants [22]. There are reports that the bioactive fractions are more potential, for example the synergism of the fractions of *Barleria prionitis* [22]. It is also reported that the when two or more agents are combined the toxicity or other side effects of a single pure compound can be overcome [23]. For example, the combination of the glycyrrhizin and saponin fractions of ginsenosides was found to be effective in reducing ulcerative colitis in male Wistar/ST rats caused by 2,4,6-trinitrobenzene sulfonic acid, whereas individually they were found to be ineffective [25]. Thus, the results of the study support the use of *E. nuda* in cancer and also its was found that the probable action of the tuber could be due to the synergistic activity.

Conclusion

Eulophia nuda has been traditionally used for the treatment of cancer. The present in-vitro cytotoxicity study supported the use of *E. nuda* tubers in the treatment of breast cancer. Moreover, the results also indicated that the cytotoxic activity could be due to the synergistic action of the phytoconstituents present in the plant. Further *in-vivo* and *in-vitro* studies could be performed on *E. nuda* using different models and bio-assays to establish its use and explore its potential for the treatment of cancer. *E. nuda* can further be studied for its anti-cancer activity in combination with other medicinal plants, which could be helpful in developing a formulation for the treatment and management of breast cancer.

Acknowledgement

The authors are thankful to DST- INSPIRE, New Delhi, India, for providing the financial assistance for the project.

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