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CYTOTOXICITY OF TAMILNADIA ULIGINOSA RETZ.TIRVENG & SASTRE (RUBIACEAE)

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ABSTRACT

Cytotoxicity assays are widely used by the pharmaceutical industry to screen for cytotoxicity in compound librariesFor the cytotoxic effect of the fraction against the brine shimpnaupili was tested and relative toxicity was analysed. The significant lethality indicated the presence of potent cytotoxic components in the fraction. This may be mainly due to the presence of the active phyto constituents in the plant, particularly in the fruits observed during the present study.

Keywords: Cytotoxicity, Brine Shrimp Lethality Assay Tamilnadiauliginosa.

INTRODUCTION

Pharmacology is the scientific study of the preparation, qualities, and uses of drugs. It is also dealing with the effects of drugs on living organisms. Traditional and folk medicines play an important role in health services around the globe. Plant as illustrated throughout the history of civilization has served as the major source of medication for the treatment of human ailments. Plants have potent biochemicals and components of phytomedicines. Since time immemorial, man is able to obtain a marvelous assortment of industrial chemicals from them. A large number of medicinal plants and their purified constituents have shown beneficial therapeutic potentials including antioxidant capacity, the ability to scavenge free radicals (Khalafet al., 2007). The role of free radicals has been recognized not only in carcinogenesis, but also in chronic inflammatory responses such as rheumatoid arthritis, aging, vascular disorders like the endothelial and myocardial injury during the ischemia and reperfusion (Bolli, 1991; Ferrari and Ceconi, 1991).

Phytochemicals produced by plants have antimicrobial activity and used for the development of new antimicrobial drugs (Nascimento*et al*., 2000). In order to promote the use of medicinal plants as potential sources of antimicrobial compounds, it is important to thoroughly investigate their composition and activity and thus validate their use (Nair, 2006). It has been shown that *in vitro* screening methods could provide the needed preliminary observations to select crude plant extracts with potentially useful properties for various pharmacological investigations.

Cytotoxicity is the quality of being toxic to cells. Cytotoxicity assays are widely used by the pharmaceutical industry to screen for cytotoxicity in compound libraries. Researchers can either look for cytotoxic compounds, if they are interested in developing therapeutics those target rapidly dividing cancer cells; or they can screen "hits" from initial high-throughput drug screens for unwanted cytotoxic effects before investing their development as a pharmaceutical. Assessing cell membrane integrity is one of the most common ways to measure cell viability and cytotoxic effects. Compounds that have cytotoxic effects often compromise cell membrane integrity. Vital dyes, such as trypan blue or propidium iodide are normally excluded from the inside of healthy cells.

The brine shrimp bioassay is a simplest, less expensive and easily achievable method replacing cell lines bioassay in order to determine the toxicity of plant extracts by the estimation of their medianlethality concentration LC_{50} (Meyer *et al*.,1982; Piccardi*et al*.,2000). This method is normally conducted to draw inferences on the safety of the plant extracts and further depict trends of their biological activities and considered as a useful tool for the preliminary assessment of toxicity (Solis *et al*., 1993). Furthermore, from the pharmacological perspective, a good correlation has been found with brine shrimp lethality test to detect anticancer compounds in plant extracts (Mackeen*et al*., 2000).

Several plant extracts and plant products have been shown to possess significant anti–tumour activities (Bhattacharya *et al*., 1997). There is a growing interest in the pharmacological evaluation of various plants used in Indian traditional system of medicine.

MATERIALS AND METHODS

For pharmacological analysis methanolic fractions of fruit was used. Methanolic fractions were prepared as per the column chromatography method given below.

Cytotoxicity assay

a. Lethality bioassay

Five different concentrations of the plant extracts (1, 2.5, 5, and 10 µg/ml) in 5% DMSO were prepared. Each extract concentration to be tested was dispensed into10 ml volume glass vials. Each concentration was tested in

triplicate. After labeling the glass vials properly, ten living shrimps were added to each vial with the help of a Pasteur pipette. About 10 ml of DMSO in sea water and different concentrations of potassium permanganate (as in the sample vials) were taken as negative and positive controls respectively. The vials were incubated for 24 h. Larvae were considered dead if they did not exhibit any internal or external movement during the observation. The larvae were not provided with any food. To ensure that the mortality observed in the bioassay could be attributed to bioactive compounds and not due to starvation, the dead larvae in each treatment were compared with the dead larvae in the negative control. After 24 h, the vials were examined using a magnifying glass and the number of surviving larvae was counted. The percentage of mortality was calculated for each concentration. The concentration-mortality data were analyzed statistically. The concentration-mortality relationship of the plant product indicates its effectiveness and is usually expressed as a median lethal concentration (LC_{50}). The LC_{50} value was determined according to (Rahman *et al*., 2009).

b. Cytotoxicity on DLA cell line

Cytotoxicity activity of *T.uliginosa*fruit extract was assessed by determining the percentage of viability of Daltons Lymphoma Ascites (DLA) cells using trypan blue dye exclusion technique. DLA cells were grown in the peritoneal cavity of healthy mice weighing 25-30g by injecting the suspension of cells ($1x10^6$cells/Ml). For this, tumour cells were aspired from the peritoneal cavity of the mice and washed with PBS (0.2ml, pH7.4) and centrifuged for 15min at1, 500 rpm. The pellet was suspended with PBS and the process was repeated three times. Finally, the cells were suspended in a known quantity of PBS and the cell count was adjusted to $1x10^7$ cells/ml. This cell suspension (0.1ml) was dispensed in 0.8ml of phosphate buffer and incubated with different concentrations of column fractionated fruit extract (10-200mg/ml) at 37°C for 3h. After 3h, the trypan blue dye exclusion test was performed. The viability percentage was determined and the IC_{50} value was calculated (Tapsell*et al*., 2006).

Antitumour activity

Male and female Swiss albino mice (average weight 20-25g) were purchased from Small Animal Breeding Station, Kerala Veterinary and Animal Sciences University, Mannuthy, Thrissur, Kerala, India. They were housed in well ventilated sterile polypropylene cages in the animal house of Amala Cancer Research Center. Mice were maintained at controlled temperature and relative humidity (60+10%) and provided 12 h light/dark cycles. They were fed with normal pellet rat chow (SaiDurga Feeds and Foods, Bangalore, India). Experiments were started after acclimatization of the animals for one week as per the instructions prescribed by the Committee of Ministry of Environment and Forest, Government of India, and implemented through the Institutional Animal Ethical Committee for the purpose of Control and Supervision of Experiments on Animals.

a. Effect of *T.uliginosa* fruit extract on solid tumor bearing animals

Swiss albino mice (6-8 weeks) weighing 25-30g was divided in to four groups of six animals each: Group I: Control (DLA cells alone), Group II: Cyclophosmamide 10 mg/kg body weight (reference drug), Group III: *T.uliginosa* fruit extract 100 mg/kg body weight+ DLA cells, Group IV: *T.uliginosa* fruit extract 500 mg/kg body weight+ DLA cells. DLA cells ($1x10^6$ cells/animals) were injected subcutaneously on the right hind limb of mice to produce solid tumour.

After 24 h of tumour inoculation, different doses of the drugs were given consecutively for 10 days. Cyclophosphamide (10 mg/kg body weight) was used as standard drug. The diameter of the hind limb was measured using Vernier calipers from the 7th day onwards on every 3rd day up to 30days.

b. Effect of *T.uliginosa* fruit extract on ascites tumor bearing animals

Swiss albino mice (20-25 g) were divided in to four groups and each group contained six animals: Group I: Control (DLA cells alone), Group II: Cyclophosmamide 10 mg /kg body weight (reference drug), Group III: *T.uliginosa* fruit extract 100 mg/kg body weight + DLA cells, Group IV: *T.uliginosa* fruit extract 500mg /kg body weight+ DLA cells.

Viable DLA cells ($1x10^6$cells) in 0.1 ml of PBS were injected into the peritoneal cavity of mice. Different concentrations of the crude drug were administered orally for 10 days after tumor injection. Cyclophosphamide (10 mg/kg body weight) was used as standard. The death pattern of animals due to tumor burden was noted and the percentage increase in life span was calculated.

Percentage increase in life span= (T-C/C) X 100

Where T and C are mean survival of treated and control mice respectively.

RESULTS

Cytotoxicity study

a. Brine Shrimp Lethality Assay (BSLA)

Cytotoxicity study of *T.uliginosa* fruit extract showed dose dependent effects. Cytotoxicity and mortality of brine shrimp increased with increase in concentration of the extract (Table :1).

Table:1 Details of Brine shrimp Cytotoxicity study of *T. uliginosa* fruit extract.

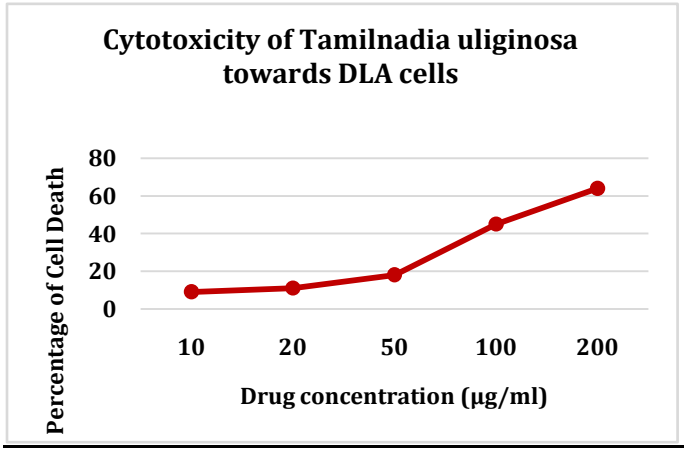
Concentration	Total no. of brine shrimps	No. of shrimps alive	No. of shrimps dead	% of mortality
Extract 1.00	10	9	1	10%

2.50	10	7	3	30%
5.00	10	4	6	60%
10	10	3	7	70%
Control				
1.00	10	0	0	0
2.50	10	0	0	0
5.00	10	9	1	90%
10	10	0	10	100%

b. In vitro cytotoxicity using DLA cells

In vitro cytotoxicity study using DLA cells also showed dose dependent-toxicity (Fig.61, 62 & 63). Drug concentration at 10mg/ml showed 9% cell death, it reached a maximum of 64% at 200mg/ml fruit extract. The concentration required for 50% death of DLA cell lines (IC50) was found to be 159µg/ml.

Cytotoxicity of *T.uliginosa* towards DLA cells



DISCUSSION

Cytotoxicity study

a. Brine Shrimp Lethality Assay (BSLA)

Plant derived natural products such as phenols and flavonoids have received considerable attention in recent years due to their diverse pharmacological properties including cytotoxic and cancer chemo preventive effects. The cytotoxic activity of the methanol extracts of *T.uliginosa*was tested using brine shrimp lethality assay (BSLA). This assay has been routinely used in the primary screening of the extracts as well as isolated compounds. BSLA assesses the toxicity towards brine shrimp, which could also provide an indication of possible cytotoxic properties of the test materials (Laughlin *et al*., 1991). As noted previously, brine shrimp lethality bioassay indicates cytotoxicity as well as a wide range of pharmacological activities such as antimicrobial, pesticidal and antitumour. Brine shrimp nauplii have been utilized in the analysis of pesticide residues, mycotoxins, stream pollutants, anesthetics, dino flagellate toxins, morphine like compounds, carcinogenicity of esters and toxicants in marine environment. In the present investigation, varying degrees of lethality were observed on exposure to different dose levels of the test samples. The degree of lethality was found to be directly proportional to the concentration of the extracts tested. In other words, mortality increased gradually with an increase in concentration of the test samples. Most of these compounds are known free radical scavengers, reactive species quenchers, hydrogen donors, antioxidant enzyme activators, detoxification inducers, normal cell differentiation promoters, tumor production and proliferation cell inhibitors and apoptosis inducers. The fractionated methanol extract of *T.uliginosa*with an LC_{50} value of 5µg/ml appears to be highly effective either equal to or even better than the standard, potassium permanganate with an LC_{50} value of 8.21µg/ml. An extract having LC_{50} below 20µg/ml is generally considered as a potent bioactive extract. BSLA is known to have a good correlation with the results obtained for human solid tumor cell lines. The inhibitory effect of the extract might be due to the toxic compounds present in the active fractions that possesses ovicidal and larvicidal properties. The metabolites either affect the embryonic development of the eggs (Manilal*et al*., 2009). Thus, the results of the present study could be utilized to determine a possible relationship between brine shrimp lethality and other cytotoxicity assays. The brine shrimp lethality assay is used as a preliminary screening assay and therefore the results of the present study may be used to focus research in future on to the particular plant part, plant extract/fraction to prioritize for further fractionation and isolation of bioactive compounds. In order to understand the mechanism of cytotoxicity better, further *in vitro* cytotoxicity assays involving specific cancer cell lines should be conducted using the active fractions/ compound.

b. Cytotoxicity against DLA Tumour Cell Lines

Anticancer drugs are used to treat malignancies or cancerous growths. Examination of traditionally used natural products has culminated in the development of a variety of drugs that are medicinally proven for their therapeutic effectiveness against a wide range of disease (Craig, 1999). One of the requisites of cancer chemo preventive agent is elimination of damaged or malignant cell through cell cycle inhibition or induction of apoptosis with less or no toxicity to normal cells (Srivastava, 2006). The greatest impact of plant derived drugs is observed in the area of antitumor research, where compounds such as taxol, vinblastine, vincristine and camptothecin have dramatically improved the effectiveness of chemotherapy against some of the cancers (Rates, 2001). The use of herbal medicines or dietary agents is being increasingly utilized as an effective way for the management of many cancers (Miyoshi *et al*., 2003).

In the present study, we have evaluated the potent antitumor activity of the plant *T.uliginosa*which showed cytotoxicity against DLA tumor cell lines. The cytotoxic activity of *T.uliginosa*fruit extract against DLA cell lines partially explains its significant antitumor activity against

solid and ascites tumour. Administration of the extract is found to increase the life span of the ascites tumour bearing animals. The results of the present study proved that the *T.uliginosa* fruit extract can reduce ascites as well as solid tumor development demonstrating its potential anticancer activity.

Antioxidants scavenge free radicals and are associated with reduced risk of cancer. So this medicinal plant can be considered as promising sources of anticancer agents for medicinal and commercial uses (Kumar *et al*., 2011). The exact nature of the compounds or definite mechanism of action of these compounds in producing the above effects needs further experimentation. This work offers scope for further research in phytochemical analysis and development of novel anticancer drugs.

CONCLUSION

For the cytotoxic effect of the fraction against the brine shimpnaupili was tested and relative toxicity was analysed. The significant lethality indicated the presence of potent cytotoxic components in the fraction. This may be mainly due to the presence of the active phyto constituents in the plant, particularly in the fruits observed during the present study.

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