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Research Article

Cytotoxicity Assessment of *Adenanthera Pavonina* Extracts in Brine Shrimp Larvae and Cancer Cell Lines

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ABSTRACT

Plants having many bioactive materials are present in nature. Only a small percentage of these bioactive compounds have been studied.. In the present study, chloroform, ethyl acetate, acetone, methanol and ethanol extracts of *Adenanthera pavonina* leaves are used for cytotoxicity study against *Artemia salina* and cancer cell lines, HCT116, NCIH460, U251 and MCF7. The chloroform extract showed high growth inhibition of MCF7 cancer cell lines while ethanol extract showed low growth inhibition against all the cell lines. The LD_{50} values of brine shrimp assay correlates well with the GI_{50} values of the extracts against cancer cell lines as shown in many previous studies. The toxicity of the extracts suggests the presence of bioactive compounds in the extracts and hence provides a scope for further fractional analysis to identify potential anticancer compounds within.

Key words: Cytotoxicity, plant extracts, brine shrimp, cancer cell lines, anti-cancer activity

INTRODUCTION

Plants are considered to be one of the natural living treasures. A huge reservoir of bioactive compounds exists in many species of plants, but only a small percentage have been examined and continue to be an important source of drugs. The screening of natural sources, such as plant extracts in search of new pharmacologically active principles led to the discovery of many clinically useful drugs that play a key role in the treatment of human diseases. Many new drugs are developed from plant sources. Morphine was isolated from opium produced from cut seed pods of the poppy plant (*Papaver somniferum*) approximately 200 years ago. Few drugs developed from natural sources have undoubtedly revolutionized medicine, like antibiotics (e.g. penicillin, tetracycline, erythromycin), antiparasitics (e.g. avermectin), antimalarials (e.g. quinine, artemisinin), lipid control agents (e.g. lovastatin and analogs), immuno-suppressants for organ transplants (e.g. cyclosporine, rapamycins), and anticancer drugs (e.g. paclitaxel, irinotecan) (Harvey, 2008).

Cancer, is projected to become the major cause of death in this century. There are at least 2,50,000 species of plants of which, more than 1,000 plants have documented anticancer properties (Mukherjee *et al*., 2001). There are many bench top bioassays which help in screening of natural products having cytotoxic activity. *In vivo* lethality is a basic bioassay in which a simple zoologic organism is used as a convenient monitor for screening in the discovery of bioactive compounds from nature. The use of brine shrimp (*Artemia salina*) for toxicology assay has many advantages. It is a rapid (24 h) assay and is inexpensive as it requires no aseptic techniques. It is useful in statistical validation since it uses large number of organisms. This experiment does not require animal serum and animal rights advocates have not yet objected to the use of artemia in experimental work. Self–reliance and rapid results are important advantages in this bench top assay (McLaughin and Rogers, 1998). A number of novel antitumor and pesticidal natural products have been isolated using this bioassay (Meyer *et al*., 1982; McLaughlin *et al.*, 1991; Sam, 1993). The plant extracts with toxicity against *A. salina* can be screened for anticancer activity using *in vitro* cell line assays. This will help us to screen crude extracts with bioactive compounds which has anticancer properties.

Adenanthera pavonina belongs to the family Mimosaceae, is an important medicinal plant from the Indian subcontinent. This species is endemic to Southern China and India. Various parts of this plant have also been used in traditional medicine for the treatment of asthma,

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boil, diarrhoea, gout, inflammations, rheumatism, tumour and ulcers, and as a tonic (Watt *et al*., 1962; Burkill, 1966; Kirtikar *et al.*, 1981). In this study the brine shrimp lethality and anticancer property of crude extracts of the leaves of *Adenanthera pavonina* against five cancer cell lines was studied. Additionally, the study was used to evaluate the suitability of the brine shrimp method as a preliminary assay to test natural products for pharmacological activity.

MATERIALS AND METHODS

Collection

Fresh leaves of *Adenanthera pavonina* were collected from the suburbs of Chennai, Tamil Nadu, India. The taxonomic authentication was done by Dr. P. Jayaraman, Plant Anatomy Research Centre, Tambaram, Chennai. The voucher specimen number is PARC/2014/2022. The collected plants were washed with running tap water, again washed with distilled water, air dried, homogenized to a fine powder and stored in air-tight bottles.

Preparation of crude extract

Dried plant material of *A. pavonina* was extracted with chloroform, ethyl acetate, acetone, ethanol and methanol separately. They were kept on a rotary shaker for 9 days, changing the solvents once in three days to elute maximum amount of extracts from the plants. The supernatant was collected by filtration using Whatman no.1 filter paper and the filtrate was evaporated at room temperature to retain thermo labile compounds in the extract. The extract was stored at 4^{o}C in airtight sterile vials for further studies.

Brine shrimp cytotoxicity assay

Brine shrimp cytotoxicity assay was performed using the method described by Meyer *et al*. (1982). A 24 h LC_{50} bioassay was performed in a multi-well test plate using nauplii of the brine shrimp *Artemia salina*. The test was conducted according to the standard operating procedure (35% salinity) with three replicates for each treatment and ten nauplii per replicate. Artificial sea water (ASW) was prepared by dissolving commercially available salt for sea water preparation (Red Sea, Israel) as per the instructions given. Brine shrimp eggs (*Artemia salina*), obtained from Ocean Nutrition International, USA, were incubated in ASW under a 60 W lamp, providing direct light and warmth. Since light has a triggering effect on the onset of the hatching (Sorgeloos, 1973), illumination was provided throughout the experiment. Air was bubbled through the suspension from the bottom of the hatching vessel to keep all the cysts in continuous motion (Sorgeloos and Persoone, 1975). After an incubation time of 24 h, the hatched nauplii were separated from the shells and remaining cysts were transferred to fresh ASW, using a Pasteur pipette. This was facilitated by attracting the shrimps with a light source. To the twelve-well plate containing 3 mL of ASW, 10 nauplii were added using Pasteur pipette. To the wells containing nauplii, aliquots from stock solution of extracts (dissolved in dimethyl sulfoxide) was added to make three different concentrations *viz*. 10, 100 and 1000 µg/mL. DMSO and potassium dichromate were used as negative and positive controls, respectively. All plates were incubated for 24 h at room temperature. Number of dead nauplii were counted after 24 h with the help of magnifying glass. When there was mortality in the control, the percentage of mortality (% M) was calculated as: % M = percentage of survival in the control - percentage of survival in the treatment. In the present study no mortality was observed in the control experiment. So, the percentage mortality (%M) was calculated by dividing the number of dead nauplii by the total number, and then multiplied by 100%. This is to ensure that the death (mortality) of the nauplii is attributed to the bioactive compounds present in the plant extracts. LC_{50} was calculated using Finney method (Finney, 1952).

Antiproliferative effect of extracts on cancer cell lines

The growth inhibitory activity of the five solvent extracts of *Adenanthera pavonina* were evaluated against cell line panel consisting of NCI-H460 (lung cancer), MCF7 (breast cancer), HCT116 (colon cancer), and U251 (brain cancer cell) using sulphorhodamine B (SRB) assay. The cell lines were obtained from the American Type Culture Collection (ATCC). The cell lines were routinely maintained as monolayer cell cultures in Roswell Park Memorial Institute medium containing heat inactivated fetal bovine serum (10%, Gibco), glutamine, penicillin and streptomycin solution (1%, L-glutamine, penicillin and streptomycin). Briefly, 100 µl of cell suspension were plated in each well of 96-well plates, and incubated for 24 h at 37°C in a humidified CO_2 (5%) incubator. The stock solutions of the five solvent extracts were prepared in dimethyl sulfoxide (DMSO) as a vehicle and various dilutions of the crude extracts (1, 0.5, 0.25, 0.125, 0.0625, 0.0312, and 0.0156 mg/mL) were added (100 µl) in each well. After 48 h of incubation, cold (4°C) trichloroacetic acid (50%, 100 µl) was added gently and left for 1 hour at 4^{o}C, followed by washing with distilled water and air drying at room temperature. To each well, SRB solution 100 µl was added and kept in dark for 30 min, the unbound stain was washed off with acetic acid (1%) and air-dried at room temperature. The protein bound stain was solubilised with 10 mM tris-base (pH 10.2) with shaking for 5 min followed by the measurement of the absorbance at 515 nm using a microplate reader. The absorbance for the blanks including blank test substance and control (without drug) were used to calculate the growth inhibitory effect of the test compounds. Camptothecin was used as positive control. GI_{50} which is the concentration of the extract or camptothecin causing 50% growth inhibition of cells was determined.

RESULTS

Brine shrimp lethality assay

The number of dead nauplii were counted after 24 h and the percentage toxicity was calculated for three different concentrations of the five different solvents used (Figure 1). The LD_{50} value of ethanol was 1387 µg which was the highest while the LD_{50} of chloroform was the lowest (256 µg). The LD_{50} values of ethyl acetate, acetone and methanol were 602, 681 and 910 µg, respectively.

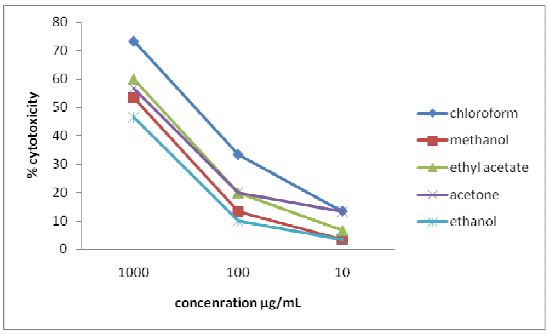

Fig. 1: Cytotoxicity of different solvent extracts of *A.pavonina* on *Artemia salina*

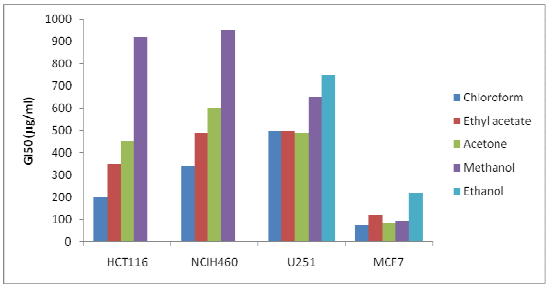

Fig. 2: GI_{50} values of different solvent extracts of *A. pavonina* on HCT 116, NCIH460, U251, and MCF7 cancer cell lines; Note: The GI_{50} values of ethanol extracts against HCT116 and NCIH460 >1000 µg.

SRB assay for anticancer activity

The GI_{50} values of the all the extracts are shown in Figure 2. All the extracts showed good growth inhibition of breast cancer cell line (MCF7). The GI_{50} value of chloroform showed the lowest of 75 µg against MCF7, while ethanol extract showed a high value of more than 1000 µg against HCT116 and NCIH460. The growth inhibition was less than 1000 µg for ethanol extract against U251 (750 µg) and MCF7 (220 µg). The growth inhibition shown by acetone and ethyl acetate extracts were almost similar against all the four cell lines. The GI_{50} values of camptothecin which was used a positive control were 20µg for HCT116, NCIH460, U251, and 49 µg for MCF7.

DISCUSSION

Brine shrimp lethality assay is considered to be the best assay when compared to other assays for rapid means of standardization of bioactivity in heterogeneous botanical products (MaLaughlin *et al*., 1998). In the present study, chloroform, ethyl acetate and acetone extracts of the *Adenanthera pavonina* showed good brine shrimp larvicidal activity. In a previous study, alcoholic extract of *Pistacia lentiscus* showed most prominent activity with LC_{50} 2.5 µg. The plant extracts *Aristolochia indica* (Aristolochiaceae), *Boswellia serrata* (Burseraceae), *Ginkgo biloba* (Ginkgoaceae), *Garcinia cambogia* (Clusiaceae), and *Semecarpus anacardium* (Anacardiaceae) exhibited significant brine shrimp lethality with LC_{50} values 13, 18, 21, 22, and 29.5 µg, respectively. According to Krishnarajua *et al.* (2005) the degree of lethality was found to be directly proportional to the concentration of the extract. Maximum mortalities took place at a concentration of 1000 µg/mL whereas least mortalities were at 10 µg/mL concentration. The present study also shows correlation between concentration and mortality.

Generally, hatched brine shrimp nauplii can survive for up to 48 h without food (Lewis, 1995) because they still feed on their yolk-sac (Pelka *et al*., 2000). Study was done during the early stages so that the need for feeding can be avoided, which could have possibly interfered with the experimental extract. It has been shown that *Artemia* is highly vulnerable to toxins at the early developmental stages (Sorgeloos *et al.*, 1978, Sleet and Brendel, 1985). This study was conducted for 24 h after hatching, which usually includes the early instar stages of nauplii. Usually at this stage in their life cycle the nauplii have reached their second and third instar and exhibit their greatest sensitivity to test compounds (Lewis, 1995). In the present study, the nauplii in their second and third instar were used for the experimental study. The LC_{50} values of brine shrimp assay showed cytotoxicity in all extracts. LC_{50} of ethanol extract was highest (1387 µg/mL). Other plant extracts have shown high values of LC_{50} in previous studies. *Trigonella stellata* and *Salvia dominica* are cytotoxic with LC_{50} values of 2.6 g/mL and 69 g/mL, respectively (Tawaha, 2006). Chloroform extract showed highest toxicity of LC_{50} value of 256 µg. In a previous study, the leaf extracts of *Lantana camara* and *Chromolaena odorata* and whole plant extract of *Euphorbia hirta* exhibited cytotoxic activity against the brine shrimp and considered as containing active or potent components. This is because their LC_{50} values are less than 1000 ppm or µg/mL (Olowa and Nuneza, 2013). In the current study, the GI_{50} value of chloroform extract of *Adenanthera pavonina* showed least values when compared with other extracts. In the present study, all the five extracts showed LC_{50} less than 1000 µg. Therefore, all the extracts were considered to have active components. As mentioned by Meyer *et al.* (1982), LC_{50} value of less than 1000 µg/mL is toxic while LC_{50} value greater than 1000 µg/mL is non-toxic.

The extracts having high LD_{50} values also show high GI_{50} values. The chloroform, acetone and ethyl acetate extracts showed low LD_{50} values and their GI_{50} values were less than 1000 µg/mL. From the pharmacological point of view, a good relationship has been found with the brine shrimp lethality test to detect anti-tumoral compounds in terrestrial plant extracts (Meyer *et al*., 1982; Solís *et al*., 1993; Mackeen *et al.*, 2000). Moreover, the brine shrimp lethality assay, which in most cases correlates reasonably well with cytotoxic and anti-tumor properties (Mclauglin *et al.*, 1993; Carballo *et al.*, 2002). Taxol ™, a new anti-tumor drug approved by FDA for treatment of ovarian, breast and non-small-cell lung carcinomas and originally isolated from the bark of *Taxus brevifolia* was discovered in this way (He *et al.*, 2001).

Conclusion

Significant dose dependant toxicity was observed in brine shrimp lethality assay as well as the *in vitro* assay conducted in cancer cell lines suggesting the presence of bioactive materials against cancer. The extracts of this

plant could be subjected to extensive chromatographic separation and purification processes to isolate individual compounds those were bioactive for the discovery of novel therapeutic agents. Moreover, the results of the present study seem consistent with the previously established correlation between cytotoxicity in cancer cell lines and brine shrimp lethality of plant extracts.

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