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CYTOTOXICITY OF *MUSSAENDA PHILIPPICA* AGAINST *ARTEMIA SALINA* AND CANCER CELL LINES

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ABSTRACT

Many herbs are evaluated for their different bioactivity, but there had been limited screening of the bioactive compounds from the ornamental plants. The ornamental plant, *Mussaenda philippica* of the family Rubiaceae, is known for its colourful sepals. The toxicity of the leaf extracts of *M. philippica* was evaluated by a preliminary assay using *Artemia salina* and anticancer activity was assessed using in vitro studies against two cancer cell lines, NCI-H460 and HCT116. The LD50 value of the ethanol extracts showed a high value of 856.91µg/mL against brine shrimp and the chloroform extract showed a low value of 520.40µg /mL. The chloroform, ethyl acetate and ethanol extracts showed a significant growth inhibition of both the cancer cell lines. Both the cytotoxicity studies suggest the presence of bioactive compounds in the extracts.

KEY WORDS: **cytotoxicity, brine shrimp, cancer cell lines, ornamental plant.**

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INTRODUCTION

Since long time, evidence has accumulated to demonstrate promising potential of medicinal plants used in various traditional, complementary and alternative systems especially for cancer treatment[1]. Many herbs have been evaluated in clinical studies and are currently being investigated to understand their anti-tumour actions against various cancers. More than 60% of currently used anticancer agents are derived in one way or another from natural sources [2,3]. Some of the plant sources from which anti-cancer agents have been isolated include *Taxus brevifolia*, *Curcuma longa*, *Betula alba*, *Catharanthus roseus*, *Cephalotaxus* species, *Podophyllum* species, *Camptotheca acuminate*, *Erythroxylum pervillei*, *Ipomoeca batatas*, and many others. Still research is going on in different parts of the world attempting to explore the bioavailability of anti-cancerous compounds in unexplored plant species. Moreover, many cancer patients who are burdened by drug-induced toxic side effects, have now turned to seek help from the complementary and alternative medicine hoping for a better cure. According to an estimate, 50% of breast cancer and 37% of prostate cancer patients use herbal products [4]. Among the different types of plants, ornamental plants offer aesthetic value and ecological balance in ecosystems. Most ornamental plants however are utilized more for their beauty as they impart different colours to the surroundings. However, there had been a limited screening of the bioactive compounds from the ornamental plants. It is therefore worthwhile to set a screening of the anticancer activity of these plants, validate their folkloric use and establish their potential as nutraceuticals and pharmaceuticals. *Mussaenda philippica* is an ornamental plant of the family Rubiaceae, commonly known as Flag Bush is found throughout South East Asia. Due to the colourful nature of the plant it is usually used for landscaping. It is used as a medicinal plant in India and other South Asian countries. It is reported to have analgesic[5] and anticonvulsant activity[6]. The antibacterial activity of the leaves of *M. roxburghii* has been studied[7]. Considering the pharmaceutical values of the *M. philippica*, in the present study the cytotoxicity of the leaf extracts were studied. As a preliminary assay, the toxicity was assessed using brine shrimp lethality assay and the anticancer potential of the extracts was evaluated by *in vitro* assay against two cancer cell lines, NCI-H460 (lung cancer cell line) and HCT116 (colon cancer cell line).

MATERIALS AND METHODS

(i) Collection of samples.

Fresh leaves of *Mussaenda philippica* 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/2015/3131. 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 **.**

(ii) Preparation of crude extracts.

Dried plant material of *M. philippica* were extracted with chloroform, ethyl acetate and ethanol separately. They were kept on a rotary shaker for 9 days, changing the solvents once in three days to elute the 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.

(iii) Brine shrimp lethality assay

The brine shrimp cytotoxicity assay was performed using the method described by Meyer *et al*[8]. A 24-h LD50 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)* 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[9], 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[10]. After an incubation time of 24 h, the hatched nauplii were separated from the shells and remaining cysts using a Pasteur pipette and were transferred to fresh ASW. 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 a 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 was 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 is 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. The method of Finney[11] based on the regression of probit mortality as a function of the logarithms of the doses of extracts allows the determination of the LD50. Statistical analysis was performed via BioStat Pro 5.9.8 software.

(iv) In vitro cytotoxicity assay

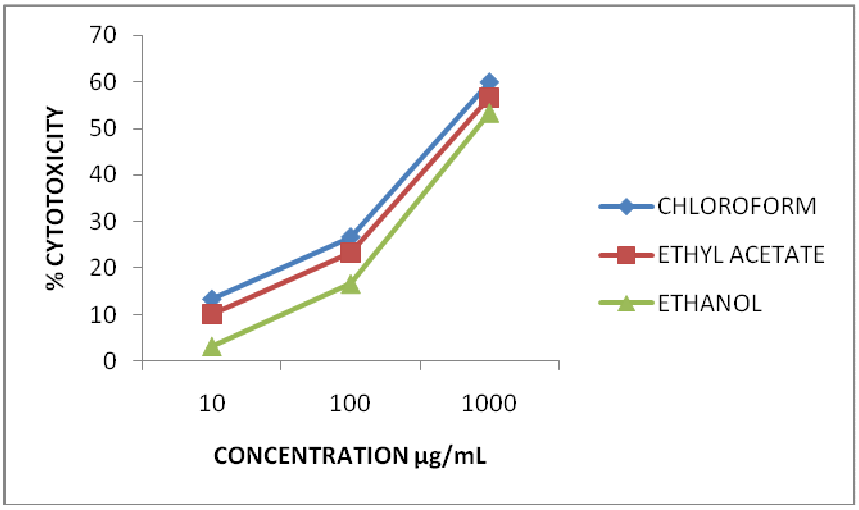
The growth inhibitory activity of the three solvent extracts of *M. philippica* were evaluated against cell line panel consisting of NCI-H460 (lung cancer), and HCT116 (colon cancer), using sulphorhodamine B (SRB) assay. The cell lines were obtained from the American Type Cuture Collection (ATCC). The cell lines

were routinely maintained as monolayer cell cultures in Roswell Park Memorial Institute (RPMI) medium containing fetal bovine serum (10%), and 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°C, followed by washing with distilled water and air dried at room temperature. To each well, SRB solution 100 µL was added and kept in the dark for 30 minutes, the unbound stain was washed off with acetic acid (1%) and air-dried at room temperature. The protein bound stain was solubilized with 10mM 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 of 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. GI50 which is the concentration of the extract or camptothecin causing 50% growth inhibition of cells was determined.

RESULTS

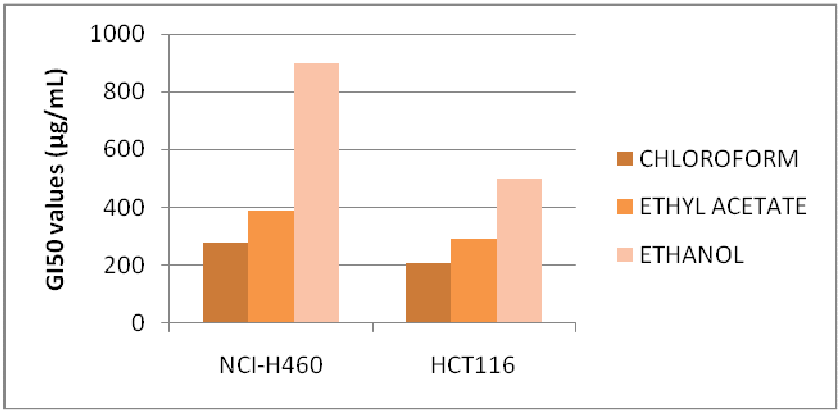
The number of dead nauplii were counted after 24 hours of treatment and the percentage toxicity was calculated for three different concentrations. (Figure 1). The LD50 value of ethanol was 856.91µg which was the highest while the LD50 of chloroform was the lowest (520.40µg). The LD50 values of ethyl acetate against brine shrimp was 683.57µg. The LD50 values of all the extracts were significantly ($p<0.05$) higher than the LD50 value of potassium dichromate (170.25µg) which was used as positive control.

Figure 1
Cytotoxicity of different solvent extracts against brine shrimp



The GI50 values of the all the extracts are shown in Figure 2. The GI50 value of chloroform showed the lowest of 210 µg/mL against HCT116 and this reflected its high anti-proliferative activity of the extract. Ethanol extract showed a high value of 900µg/mL against NCIH460. The growth inhibition was 500µg/mL for ethanol extract against HCT116. The values were significantly ($P<0.05$) higher when compared to the positive control. The GI50 value of camptothecin which was used a positive control was 20µg/mL for HCT116 and 18µg/mL for NCI-H460.

Figure 2
GI50 values of the chloroform, ethyl acetate, and ethanol extracts against NCI-H460 and HCT116.



DISCUSSION

Many studies have reported different biological activities of the extracts from different parts of *Mussaenda* species. Traditionally, the roots are useful for cough, jaundice and when chewed acts as an appetizer[12]. The pharmacological activities reported from *Mussaenda* species were diuretic, antiphlogistic, antipyretic and effective in laryngopharyngitis, acute gastroenteritis and dysentery[13] and also anti-fertility activity[14]. Many new compounds are isolated from Ethyl acetate extract of the stems of *Mussaenda erythrophylla* ((Rubiaceae). β-sitosterol, 5 hydroxy-7, 4'-dimethoxy flavones, 3- iso cumaryloxy – cyclopropane-1-oic acid and 4 -hydroxy-3-methoxy cinnamic acid were isolated and their structures were elucidated by IR and NMR spectroscopic method[15]. In the present study, all the different leaf extracts showed significant lethality of brine shrimp in 24 hours. Chloroform extract showed a mortality rate of 60% at 1mg/mL while ethanol showed a mortality of 53.3% at 1mg/mL concentration. The LD50 value of ethanol extract showed the highest of 856.92µg/mL. In an earlier study with M. *roxburghii*, both the petroleum-ether and carbon tetrachloride soluble fraction of crude methanol extract of leaf demonstrated strong cytotoxic activity with LC50 value of 0.52 and 0.62 µg/ml, respectively[7]. In another study, 50% ethanolic extract, obtained from dried powdered plant material of *Croton bonplandianum* was very effective against brine shrimp and showed LC50 at the concentration of 46.7mg/lit[16] .In a previous study, ethanol and polyphenol extracts of *Portulaca quadrifida* exhibited significant effect against HT-29 cell lines and were found less effective against normal L-6 cell lines indicating the cancer specific activity which was further supported by the DNA fragmentation assay[17]. Lakshmi et al.,[18] have studied the antioxidant and antitumor activity of *Mussaenda philippica* sepal extract against *in vivo* colon cancer and breast cancer in mice. The ethyl acetate extract of *Mussaenda philippica* sepal extract exhibited antitumor and antioxidant effect by modulating lipid peroxidation and augmenting antioxidant defense system in Caco2 and MCF7 bearing mice. In an earlier study, the methanolic extract of *Mussaenda philippica* against isoniazid and rifampicin induced hepatotoxicity in experimental rats showed hepatoprotective effect[19]. In the present study, the chloroform leaf extract has shown good growth inhibition of both NCI-H460 and HCT116 cell lines. Ethyl acetate extract showed a moderate activity towards both the cell lines. In an earlier study, the cytotoxicity profile of *Combretum rupicola* when compared with doxorubicin (used as positive control) showed significant power of action. Ethyl acetate extract of C. *rupicola* exhibited antiproliferative activity with total growth inhibition varying from 65 to 194 µg mL-1 against MCF-7(breast), PC-3(prostate), 786-0 (renal) and U251 (glioma). The most significant activity was observed against MCF-7 (TGI 65.9 µg mL-1 , breast cancer)[20]. In both the assays performed in this study, the chloroform extract has shown a better cytotoxicity compared with ethyl acetate and methanol extracts of M. *philippica*.

CONCLUSION

In the toxicity assay against brine shrimp larvae, all the extracts exhibited significant toxicity suggesting the presence of compounds having cytotoxic properties. The LD50 and GI50 values reflected the promising efficacy of anticancer potential of the extracts. Thus, this plant extracts can be subjected to extensive chromatographic separation and purification processes to isolate bioactive compounds for the discovery of novel anticancer agents. Moreover, further *in vivo* studies can also be done to prove the synergistic effect of the compounds found naturally in the extracts. This study also supports the correlation between the toxicity shown towards brine shrimp and the cancer cell lines as previously studied.

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