

## Brine shrimp lethality and cytotoxicity assay of *Araucaria bidwillii* Hook in human carcinoma cell lines

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### SUMMARY

The leaf extracts of *Araucaria bidwillii* Hook. (Araucariaceae) were evaluated for their cytotoxic effect in various human cancer cell lines. Preliminary investigation by brine shrimp lethality assay indicated that LC<sub>50</sub> value of various successive extracts were found to be less than 1000 µg/ml, where the ethyl acetate extract showed maximum activity of less than 100 µg/ml. Further cytotoxic evaluation of various leaf extracts of *Araucaria bidwillii* Hook was carried out in four different human cancer cell lines - acute myeloblastic leukemia (HL-60), chronic myelogenous leukemia (K-562), breast adenocarcinoma (MCF-7) and cervical epithelial carcinoma (HeLa). Cytotoxicity was assessed by trypan blue dye exclusion method and 3-(4,5-dimethyl thiazole-2yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay. From the present investigation it was found that the ethyl acetate and methanol extract of *Araucaria bidwillii* Hook was found to be more effective in leukemic cell lines and was less effective in MCF-7 and HeLa. The IC<sub>50</sub> value of the ethyl acetate extract in leukemic cell lines was found to be 28.18 and 34.64 µg/ml and methanol extract was found to be 33.11 & 39.81 µg/ml. It can be concluded that various extract from the leaves of *Araucaria bidwillii* Hook. posses cytotoxic activity tested in brine shrimps and various human carcinoma cell lines.

**Key words:** *Araucaria bidwillii* Hook; Brine shrimp lethality bioassay; Human cancer cell lines; Cytotoxic activity

### INTRODUCTION

Higher plant-derived natural products have long been and will continue to be extremely important as source of medicinal agents for treating degenerative diseases. In addition to the biologically active plant-derived secondary metabolites, which have found direct medicinal application as drug entities, many other bioactive plant compounds have

proven useful as “leads” or model compounds for drug syntheses or semisyntheses (Fransworth, 1984; Tyler, 1988). Bioassay methods for the screening and testing of plant extracts have varied over the years but eventually they evolved into pre clinical trials (Geran *et al.*, 1972; Suffness and Dourous, 1979; Mukherjee, 2003). The advent of bioassay for the monitoring of plant extracts, fractions and isolated natural compounds obtained from botanicals is frequently included in natural product research and screening of medicinal plants through these bioassays has simplified research in botanicals (Mclaughlin, 1991). A rapid, robust and inexpensive *in-vivo* Brine Shrimp Lethality bioassay has been

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used for screening during bioactivity-directed isolation of biologically active natural compounds (Meyer *et al.*, 1982; McLaughlin, 1991; Sam, 1993; De Rosa *et al.*, 1994; Mukherjee, 2002). This assay provides a guide before stepping into isolation of phytoconstituents particularly to screen the anti-tumour activity. The plant *Araucaria bidwillii* Hook (Araucariaceae) popularly known as 'monkey puzzle' or 'Bunya - Bunya' is an important group of living gymnosperms dating back of Jurassic period of 195 million years ago having considerable economic and nutritive significance (Guha *et al.*, 1971; Hora, 1981; Dhanasekaran *et al.*, 1993). In India this evergreen genus of *Araucaria bidwillii* Hook is commonly distributed in southern part of hills area of Nilgiris and northern part of Himalayan regions. The seeds are of high edible value for Australian aborigenes and are rich in fat content (Guha *et al.*, 1971; Hora, 1981). The Lahu tribal groups of Northern Thailand use this plant to cure insomnia in children (Anderson, 1972). Psychopharmacological, antipyretic and analgesic activity of this plant has been reported by various workers (Dhanasekaran *et al.*, 1994; Dahanukar *et al.*, 2000). Several claims in the folklore practices with the tribal communities of Nilgiris hills of the southern part of India based on the use of the leaves as well as the exudates of this plant for the treatment of anxiety, mental disorder, inflammation, malnutrition has been reported through ethnobiological survey by the author and his group (Mukherjee, 2001; Rajan *et al.*, 2002). Therefore the present investigation was carried out to screen the various successive extracts of the *Araucaria bidwillii* Hook in brine shrimp lethality bioassay method as a preliminary screening, further the extracts were screened for their cytotoxic effect in various human cancer cell lines.

## MATERIALS AND METHODS

### Plant material

The fresh leaves of *A. bidwillii* Hook were collected from Government Botanical Garden, Udahgamandalam,

India, and authenticated by Dr S. Rajan, Field Botanist, survey of medicinal plants and collection unit, Government of India, Udahgamandalam. A voucher specimen (JUNPSL 2002-001) of this plant material has been retained in the School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata 700 032.

### Preparation of extracts

The shed dried hand grinded coarse leaves of *A. bidwillii* Hook were first defatted with petroleum ether (40-60°C) in the soxhlet extraction apparatus. The marc obtained was extracted subsequently with chloroform, ethyl acetate and followed by methanol as described by the method (Ilyas *et al.*, 1978). The solvent evaporated by rotary vacuum evaporator. The extracts were then freeze-dried which was further used for screening purpose. The yields of the extracts were 2.98% w/w (petroleum ether), 3.16% w/w (chloroform), 2.85% w/w (ethyl acetate) and 5.86% w/w (methanol).

### Phytochemical investigation of the plant

The successive extract obtained with different solvent was subjected to qualitative analysis where the presence of major phytoconstituents like steroids and terpenes (petroleum ether extract), steroids and terpenes (chloroform extract), flavonoids (ethyl acetate extract) and saponins, carbohydrate and tannins (methanol extract) were found. The presence of phytoconstituents was reconfirmed by thin layer chromatography (TLC) studies.

### Chemicals

RPMI 1640 medium, fetal bovine serum, gentamycin, penicillin, streptomycin, trypan blue and agarose were purchased from GIBCO. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], doxorubicin (adriamycin), NP-40, and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO). Other reagents were of analytical grade and were procured locally. Brine shrimp *Artemia salina* cysts were obtained from

laboratory of aquaculture and artemia reference centre, Belgium.

### Brine shrimp lethality assay

#### Hatching of brine shrimp eggs

This assay uses brine shrimp, *Artemia salina* Leach, which is used to determine the toxicity of plant extract. Brine shrimp eggs of *Artemia salina* Leach were hatched in artificial sea water (ASW) (aqueous solution of NaCl 3.8% w/v) and incubated at 25°C. The starting pH of the ASW was 8–8.5. After 48 hours of hatching the larvae (nauplii) were collected and used of BSL bioassay McLaughlin (1991).

#### Assay procedure

The brine shrimp lethality assay of the successive leaf extract of *Araucaria bidwillii* was carried out by the method described by Mayer *et al.* (1982). All the extracts were tested at concentration levels of 1 g/ml to 10 µg/ml. Each test was done in six replicates. A suspension of 10 nauplii (100 µl) was added into each well of a 24 well microplate and covered microplate was incubated for 24 h at room temperature. After this period the number of dead nauplii in each well was counted using binocular microscope. Potassium permanganate (100 µg/ml) was used as a standard (positive control) and a control reaction was carried out with out the sample (negative control). The results were calculated statistically against their respective controls. The statistical method of probit analysis was used to calculate the concentration of the extract or fraction that would kill 50% of brine shrimps with in the 24 hrs exposure, i.e. the LC<sub>50</sub> with the 95% confidence intervals (Finney, 1971). The extracts were considered bioactive when LC<sub>50</sub> was 1000 µg/ml or less.

#### Cell cultures

Four human cancer cell lines were used for the present investigation. Acute myeloblastic leukemia (HL-60) and chronic myelogenous leukemia (K562) cells were maintained in RPMI1640 supplemented with 15% heat inactivated fetal bovine serum and

gentamycin (40 µg/ml), penicillin (100 units/ml) and streptomycin (10 µg/ml). Breast adenocarcinoma (MCF7) and cervical epithelial carcinoma (HeLa) cells were maintained in MEM supplemented with similar concentrations of serum and antibiotics as stated above. Cells were grown at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air.

#### Cell viability and cytotoxicity

Viability of cells was determined by trypan blue dye exclusion method and cytotoxicity was assessed by the MTT assay. Exponentially growing cells ( $1 \times 10^4$ ) were plated in 96-well plates and after 48 h of growth the cells were treated with a series of concentrations of the various successive extracts of *Araucaria bidwillii* Hook (20, 40, 80, 120, 160 µg/ml dissolved in DMSO (final concentration 0.1%). Control cells were treated with DMSO alone and positive controls with various amounts of doxorubicin. Incubation was carried out at 37°C for 48 h. Cells were then exposed to 0.2% trypan blue and were counted in a hemocytometer. MTT solution was added to each well (1.2 mg/ml) and incubated for 4 h. The reaction results in the reduction of MTT by mitochondrial dehydrogenases of viable cells to form a purple coloured formazan product. The formazan product was dissolved in DMSO and the amount was estimated by measuring absorbance at 570 nm in an ELISA plate reader. (Francis and Rita, 1986)

## RESULTS

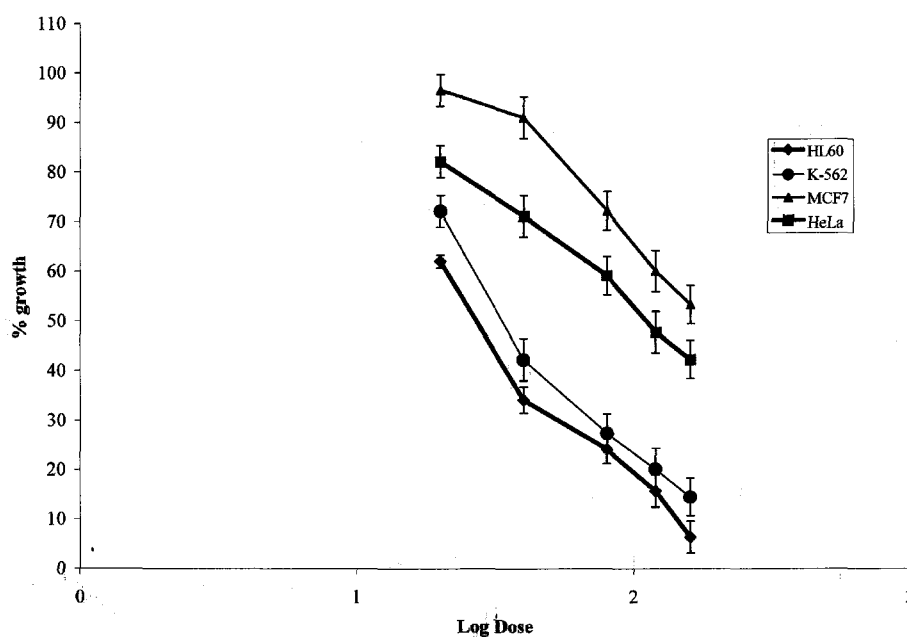
### Brine shrimp lethality assay

The result of the brine shrimp lethality assay of successive leaf extracts of *A. bidwillii* has been represented in the Table 1. Among all the four extracts were found to posse's brine shrimp lethality of the various concentrations from 10 µg/ml-1000 µg/ml compared with that of the standard potassium permanganate (LC<sub>50</sub> 40.8 µg/ml). Interestingly LC<sub>50</sub> value of the successive extracts of the leaves of *Araucaria bidwillii* was <1000 µg/ml. The order of

**Table 1.** Lethal dose (LC<sub>50</sub>) effect of different extracts of *Araucaria bidwillii* Hook in Brine shrimp lethality bioassay

Treatment	LC <sub>50</sub> (µg/ml)		
	10 (µg/ml)	100 (µg/ml)	1000 (µg/ml)
Control			
Potassium permanganate	<i>P</i> < 0.01	<i>P</i> < 0.001	<i>P</i> < 0.001
Petroleum ether extract	ns	ns	ns
Chloroform extract	ns	<i>P</i> < 0.05	<i>P</i> < 0.05
Ethyl acetate extract	ns	<i>P</i> < 0.001	<i>P</i> < 0.001
Methanol extract	ns	<i>P</i> < 0.05	<i>P</i> < 0.001

Each dose was done in triplicate. ¶ - no mortality of the brine shrimp. n.s - not significant. *P* < 0.05, *P* < 0.01, *P* < 0.001 statistically significant against respective sample controls.



**Fig. 1.** Effect of Ethyl acetate extract of *Araucaria bidwillii* Hook in various cancer cell lines by MTT assay.

LC<sub>50</sub> value of successive extracts was found to be ethyl acetate extract < methanol extract < chloroform extract.

#### Cell viability and cytotoxicity

The results of cell viability and cytotoxicity of ethyl acetate and methanol extracts of the *A. bidwillii* in various cancer cell lines were shown in the Fig. 1-4. Potent cytotoxic activity was observed in the ethyl acetate and methanol extract of the *A. Bidwillii* Hook in acute myeloblastic leukemia (HL-60) and chronic myelogenous leukemia (K562) cell lines. The MTT assay, IC<sub>50</sub> value of the ethyl acetate extract

was found to be 28.18 and 34.64 µg/ml and methanol fraction was found to be 33.11 & 39.81 µg/ml. The cell viability assay the IC<sub>50</sub> of the ethyl acetate extract was found to be 24.54 & 30.90 and methanol extract found to be 36.30 & 41.68 in acute myeloblastic leukemia (HL-60) and chronic myelogenous leukemia (K562) cell lines.

#### DISCUSSION

Cancer continues to represent the largest cause of mortality in the world and claims over 6 million lives each year. An extremely promising strategy

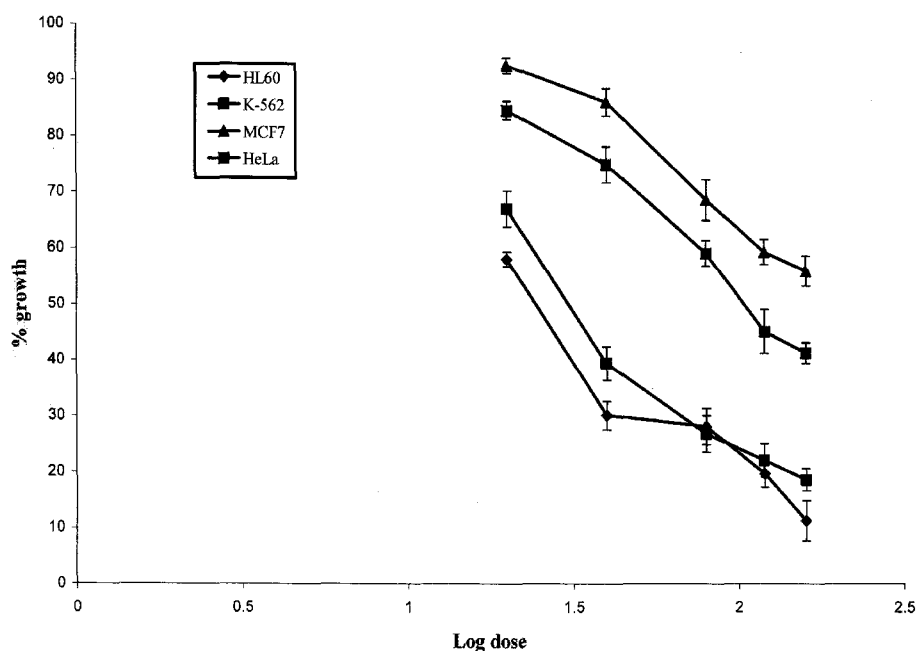


Fig. 2. Effect on cell viability of Ethyl acetate extract of *Araucaria Bidwillii* Hook in various cancer cell lines.

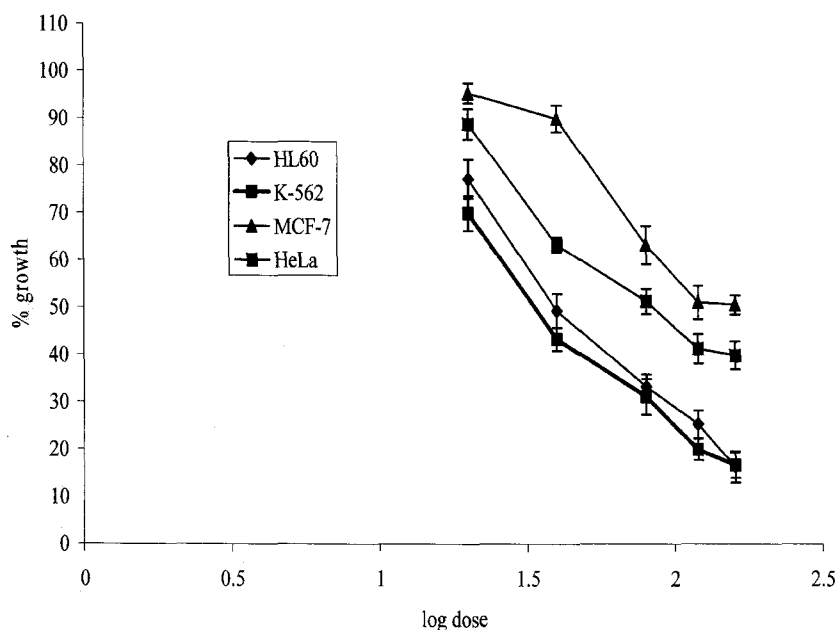


Fig. 3. Effect of methanol extract of *Araucaria bidwillii* Hook in various cancer cell lines by MTT assay.

for cancer prevention today is chemoprevention. A large and increasing number of patients in the world use medicinal plant and herbs for health purposes (Ficrat *et al.*, 2002; Mukherjee, 2003a). Considering that a bioassay is the first step necessary

for drug discovery process from ethno medicinal system, the present investigation was aimed to carry out the possible *in-vivo* brine shrimp lethality bioassay of various successive leaf extracts of *Araucaria bidwillii* Hook as a preliminary bioactive

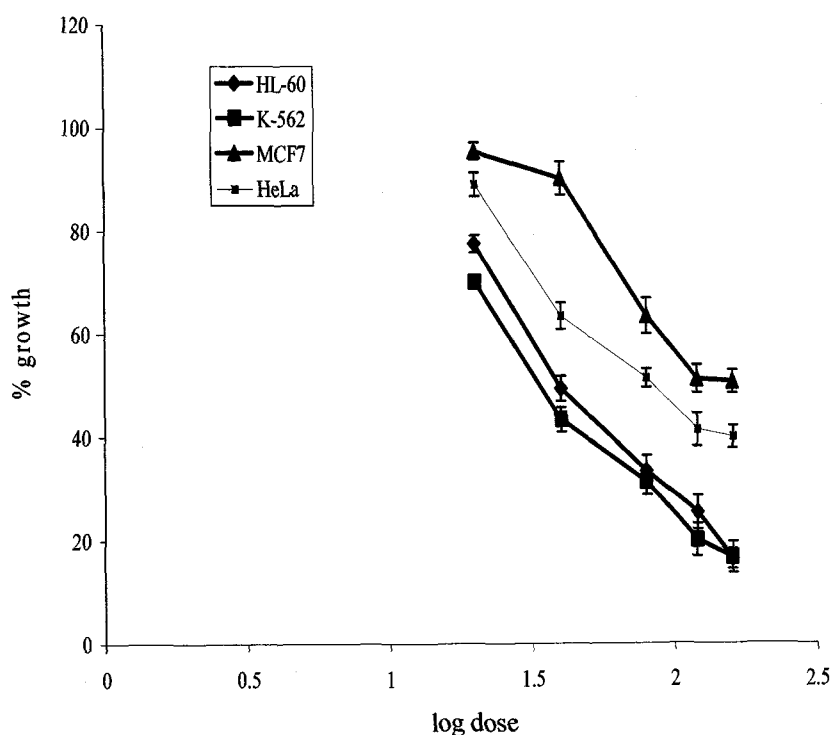


Fig. 4. Effect on cell viability of methanol extract of *Araucaria bidwillii* Hook in various cancer cell lines.

index, further the successive leaf extracts were investigated in *in-vitro* cytotoxicity in various human cancer cell lines. Therefore scientific scrutiny of their therapeutic potential, biological properties and safety profile of the plant *A. bidwillii* Hook will be useful in resuming further experiments. In the present study chloroform and methanol extracts were active in brine shrimp lethality assay between the Lethal dose ( $LC_{50}$ ) concentrations 100-1000  $\mu\text{g}/\text{ml}$ . Interestingly the ethyl acetate extract of the *Araucaria bidwillii* Hook possess the  $LC_{50}$  value in the range between 10 - 100  $\mu\text{g}/\text{ml}$ . Brine shrimp have been used for screening of pesticide, industrial toxin, opioids, anti-tumor agents and antibiotics (Grosch *et al.*, 1967; Hamill *et al.*, 1969; Granade *et al.*, 1976). Brine shrimp lethality assay is an index of bi-directional biological activity, which suggests the bioactivity of the plant extract as well as the cytotoxic activity of the plant material tested (Prashanth *et al.*, 2002). Further our work has been

directed toward to screen the cytotoxicity of the successive leaf extract of the plant *A. bidwillii* Hook. in four different carcinoma cell lines. Among all the extract, the ethyl acetate and methanol extract of the plant posses selective cytotoxicity, which was found to be more effective in leukemic cell lines and was less effective in MCF-7 and HeLa. The  $IC_{50}$  value of the ethyl acetate extract was found to be 28.18 and 34.64  $\mu\text{g}/\text{ml}$  and methanol extract was found to be 33.11 & 39.81  $\mu\text{g}/\text{ml}$ . However it doses not showed any cytotoxicity in normal cell lines (data not shown). Previous reports on this plant revealed the presence of the chemical compounds of various biflavones, which have been isolated from the leaves of *A. bidwillii* (Khan, 1970; Khan *et al.*, 1971; Khan, 1972; Ilyas *et al.*, 1978). Present study provides some evidence for use of the brine shrimp lethality assay as a non-specific bioassay to establish general pharmacological activity of successive leaf extracts of *Araucaria*

*bidwillii* Hook. This finding suggests that the successive extracts of *Araucaria bidwillii* leaf possess the definite biological activity, which has not been explored yet. Our present findings revealed that the extract of ethyl acetate possesses the significant inhibition against the brine shrimp lethality bioassay and cytotoxic effect in acute myeloblastic leukemia (HL-60), Chronic myelogenous leukemia (K-562). This activity is due to the complex interplay of the varied phytoconstituents present in successive extracts of *A. bidwillii* leaf. It can be concluded that the ethyl acetate and methanol extract from the leaves of *Araucaria bidwillii* Hooker possess anti-tumor activity, which is confirmed by brine shrimp lethality bioassay and various human carcinoma cell lines.

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