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Antimitotic Activity and Brine Shrimp Lethality Test of Tectona grandis Linn. Bark

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ABSTRACT

Tectona grandis Linn.f. is a large tree commonly found throughout the tropica. It is commonly known as 'Teak tree' (Family-Verbenaceae). Bark of the plant was extracted with 70 % alcohol and water to produce respective extracts. These extracts were screened for antimitotic activity by Allium cepa method and brine shrimp lethality test using Artemia salina eggs. Results showed that 70 % alcohol extract was most effective in both the models. Further 70 % alcohol extract was subjected to column chromatography for activity guided fractionation and obtained various fractions were again screened for the same activity. Chloroform fraction of 70 % alcohol extract was found to be most active in both the models.

Key words: Tectona grandis, Verbenaceae, Teak tree, Antimitotic, Brine shrimp, lethality test.

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INTRODUCTION

Tectona grandis Linn is a large deciduous tree belonging to family Verbenaceae and also known as 'Teak tree' or 'Sagwan'. Its branchlets are quadrangle, channeled, and stellately tomentose. Leaves are elliptic, acute, opposite or whorled, large, petiolate, entire, upper surface is rough but usually glabrous. Flowers are small, white, numerous, in dichotomous cymes arranged in large terminal panicles. Fruits are enlarged long, bladder like, subglobuse, 4 lobed and the pericarp is soft with dense felted stellate hairs; endocarp is bony.

In ethnomedicine, roots are given in anuria and retention of urine. Bark is astringent, sweet, acrid and used in bronchitis. Wood of the plant is sedative, refrigerant, astringent, diuretic, used in headache, toothache and also subdues the inflammation and irritation of skin [1,2].

Root heart wood of the plant showed a high level of activity in cytotoxic tests against brine shrimps. Leaves of the plant possess wound healing and antiviral activity. Antimutagenic effect of plant was tested using the micronucleus test in mice [3]. Leaves possess antifertility activity [4].

5-hydroxylapachol, lapachol, lapachone, anthraquinone, naphtaquinone, and tectol derivatives were reported from this plant. Squalene, and methylquinizarin were identified from root heart wood of the plant [5,6,7].

Mitosis is a process of cell division. Mitosis is remarkably similar in all animals and plants The mitosis occurs in the somatic cells and it is meant for the multiplication of cell number during embryogenesis and blastogenesis of plant and animals. Antimitotic agents interrupt or stop the process of cell division so that beneficial in life threatening diseases like cancer [8].

Brine shrimp test is simple bioassay for natural product research. The procedure determines lethal concentration of active compounds in brine media. This method is rapid reliable and has been used for over 30 years in toxicological studies. A positive correlation exists between brine shrimp lethality and human carcinoma. [9, 10]
Hence, efforts have been made to determine the preliminary in-vitro anticancer activity of T. grandis bark extracts.

MATERIALS AND METHODS

Plant Material

Fresh sample of T. grandis bark was collected from Ahmednagar district (Maharashtra) and authenticated at Botanical Survey of India, Pune. A voucher specimen of plant was deposited for future reference (Voucher specimen number Gaikwad – 1).

Extraction

Dried and powdered bark material was subjected to Soxhlet extraction using 70% ethanol as solvent. Marc left was refluxed with distilled water. Both the extracts were concentrated using vacuum evaporator and dried in open air. Extracts obtained were stored in air tight container and used further for preliminary phytochemical screening [11] and in-vitro anticancer screening.

Drugs and Chemicals

The following drugs and chemicals were used.

Methotrexate (Dabur Pharma, India).
Ethanol AR, Acetic acid, HCl, Carmine stain (PCL, India).

Antimitotic Activity [12, 13, 14]

The activity was performed as described by More et al (2006) and Williams et al (1996). Antimitotic activity was evaluated by using the meristematic cells of A. cepa roots. A. cepa bulbs were sprouted in tap water at room temperature, when the roots were about 5 mm long the bulbs were placed on beakers containing the extracts (10 mg/ml) such that the roots were immersed in the extracts. The duration of extract treatments for each bulb was 1 and 3 hrs respectively. Five bulbs were used for each extract and duration of treatment. The sprouted roots were also treated with distilled water (Control group) and methotrexate (0.1 mg/ml, Standard drug). One hour later the root tips were cut and transferred to fixing solution of 45 % acetic acid and 95 % ethanol in the ratio of 1:3 v/v (10-12 hrs) followed by warming the root tips in 1 N HCl in oven at 50ºC for 15 min and then stained with carmine stain. The slide was observed under microscope to record the number of non-dividing and dividing cells. Same procedure was repeated after 3 hrs of extract treatment. Mitotic index was calculated using the following formula.

$$\textbf{Mitotic Index} = \frac{\text{Number of dividing cells}}{\text{Total number of cells}} \times 100$$

Brine Shrimp Lethality Test [9, 10, 15]

In this test, brine shrimp (Artemia salina) eggs were hatched in artificial sea water (38 g/l of sea salt). The brine shrimp test (BST) bioassay experiment was performed according to the procedure described by Meyer (1982). Various concentrations of extracts were prepared (50, 100, 200, 400 µg/ml). Methotrexate and caffeine were used as standard cytotoxic drugs. After 48 hr of incubation, 10 brine shrimps were transferred to each sample vial using Pasteur pipette and artificial sea water was added to make 5 ml. Survivors were counted after 12hr and 24 hr.

five replicates were prepared for each concentration of test drugs. Artificial sea water was used as a control group.

Column Chromatography [16]

70 % alcohol extract (5.0 g) of T. grandis bark was dissolved in small volume of ethanol and subjected to column chromatography (250 g silica gel, 60 × 4 cm) packed with silica gel G (#60-120) in Chloroform which was gradiently eluted by using different solvents like chloroform, ethyl acetate: Methanol (1:1), and Methanol. All obtained fractions were concentrated by vacuum evaporation and dried in open air.

Further, isolated fractions were again studied for antimitotic activity and brine shrimp lethality test with decreased concentration of fractions

Antimitotic Activity of Fractions

Same procedure was repeated with the fractions (1 mg/ml) and standard drugs (0.1 mg/ml).

Brine Shrimps Lethality test of Fractions

Same procedure was repeated with the fractions and standard drugs of concentration of 10, 20, 30, 40, 50 μg/ml

Statistical Analysis

Data was analyzed statistically by one-way ANOVA followed by Dunnett's multiple comparison test. $P< 0.05$ was taken as statistically significant.

RESULTS AND DISCUSSION

Preliminary Phytochemical Screening

70 % alcohol extract has shown the presence of carbohydrates, proteins, steroids anthraquinone glycosides, phenolic compounds and saponins while glycosides, steroids and proteins are absent in aqueous extracts.

Antimitotic Activity

An agent that prevents or disrupts mitosis is called as antimitotic agent. Antimitotic constituents can stop the mitosis in anywhere of the cell cycle. 70% Alcohol extract of T. grandis bark reduced mitotic index significantly after 1 and 3 hour extract treatment (10 mg/ml) which was comparable with mitotic index of methotrexate (0.1 mg/ ml) (Figure 1). Mitosis was normal

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in the control roots. Also the mitotic index of aqueous extracts treated roots were significantly lowered than the mitotic index of the control (data not shown). 70 % alcohol extract exhibited significant antimitotic activity as reduction in mitotic index (P<0.01) was observed from 94.2 % to 63.4 % and from 99.6 % to 50.4 % after 1 and 3 hour treatment of extract having concentration of 10 mg/ ml. Mitotic index of methotrexate was found to be 55 % and 53.8 % after 1 and 3 hour treatment respectively.

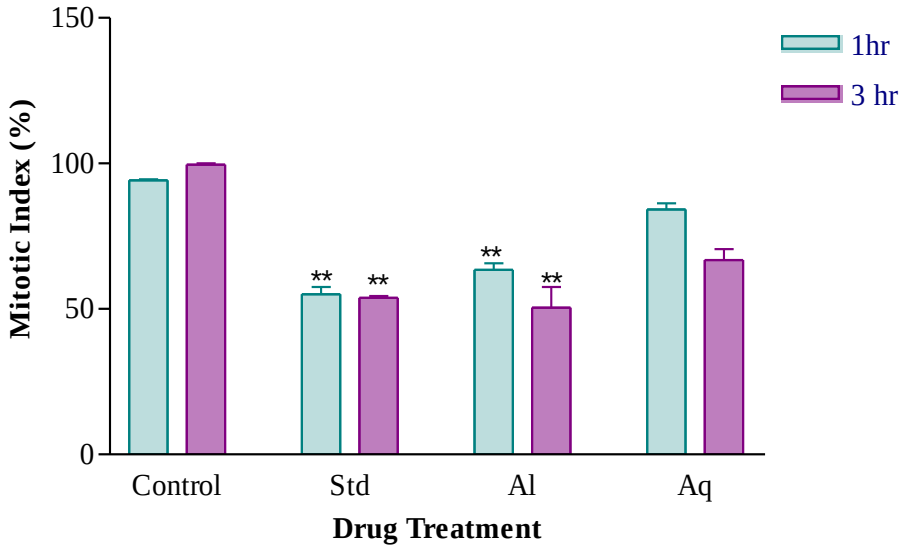

Figure 1: Effect of various extracts of T. grandis on Mitotic index by A. cepa Method.

So, 70% Alcohol extract of T. grandis bark was found to be effective against cells that are proliferating and produce cytotoxic effect either by damaging the DNA during the S-phase of the cell cycle or by blocking the formation of the mitotic spindle in M-phase. An alkylating agent, methotrexate interferes with DNA integrity and thereby exhibits strong anti-mitotic activity both in vivo and in vitro.

Brine Shrimp Lethality Test

The brine shrimp test represents a rapid, inexpensive and simple bioassay for testing plant extract lethality, which in most cases correlated reasonably well with cytotoxic and anti-tumor properties. In this test numbers of survivals were counted. Larvae were considered dead if they did not exhibit any internal or external movement during several seconds of observation. Brine shrimp can survive upto 48 hrs without food as they still feed on their yolk sac.

This test was performed by using Artemia salina eggs in different concentration of extracts and standard drug. Number of shrimps dead after 12 and 24 hours were counted. The lethal concentration of tests resulting in 50 % mortality of brine shrimps. The results shown in figure 2 indicates that the lethality shown by 70 % alcohol extract (P<0.001) of T. grandis bark (400 µg/ml) was equal to lethality shown by standard drug i.e. methotrexate at a concentration

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of 400 µg/ml (P<0.001) and was more than caffeine (400 µg/ ml) after 12 hours. The results shown in figure 3 indicates that the lethality shown by 70 % alcohol extract at concentration of 400 µg/ml was extremely significant (P<0.001) and nearly equal to lethality shown by standard drug i.e. methotrexate and caffeine at concentration of 400 µg/ ml after 24 hours.

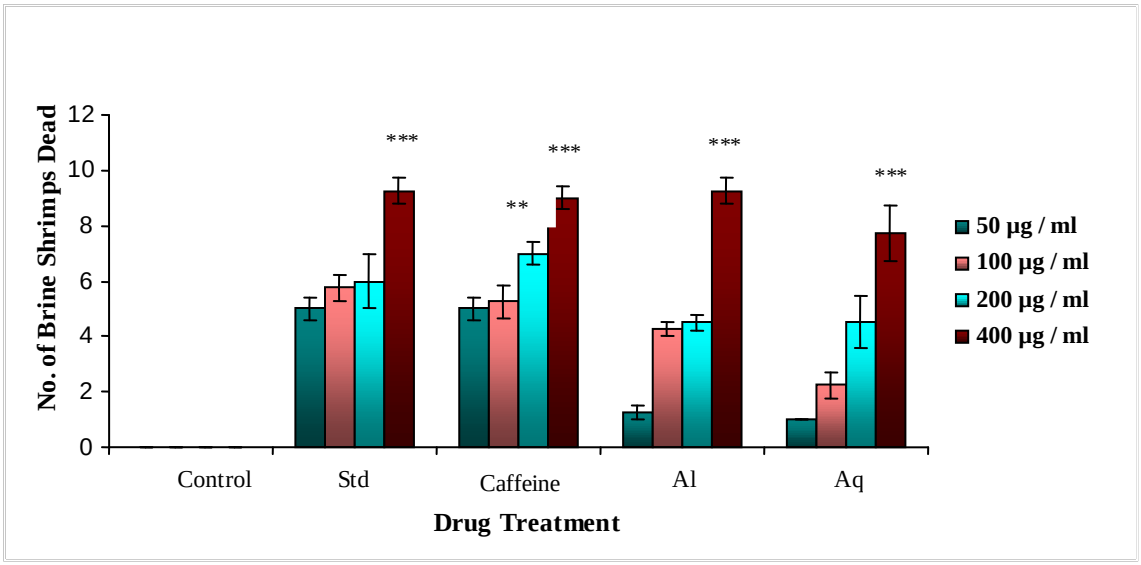

Figure 2: Number of brine shrimps dead after 12 hrs with different concentration of extracts.

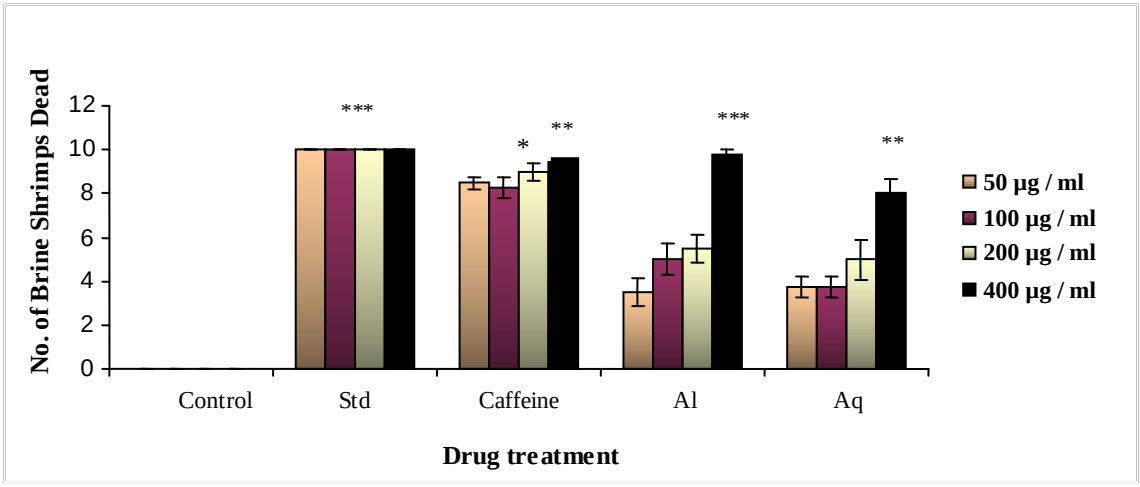

Figure 3: Number of brine shrimps dead after 24 hrs with different concentration of extracts.

Column Chromatography

70 % alcohol extract was found to be more effective in both the models used for screening of in-vitro anticancer activity. Thus it was found worthy to isolate the active constituents from 70 % alcohol extract by using column chromatography. The isolated fractions were again screened for in-vitro anticancer activity by using antimitotic activity by A. cepa method and brine shrimp lethality test.

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Antimitotic Activity of Fractions

Fractions isolated by column chromatography were tested for antimitotic activity by using A. cepa method. Out of these fractions chloroform fraction was found to be more significant (P<0.01). Chloroform fraction at a concentration of 1 mg/ml reduced mitotic index significantly (P<0.01) after 1 and 3 hrs treatment (Figure 4).

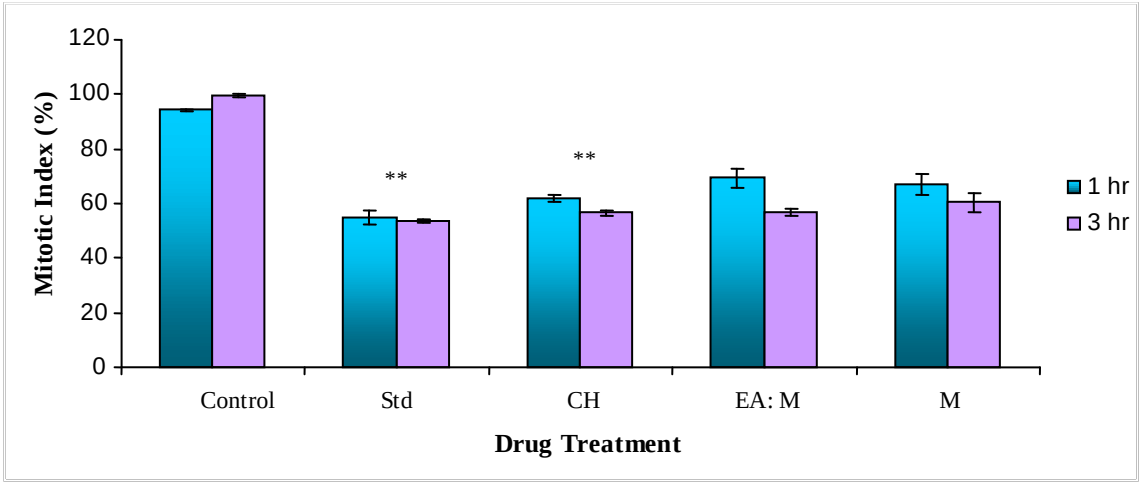

Figure 4: Effect of various fractions of T. grandis on Mitotic index by A. cepa method.

Brine Shrimp Lethality Test of Fractions

The results shown in table 1 and 2 indicate that the lethality shown by chloroform fraction at all concentrations were more than standard drug methotrexate and caffeine after 12 and 24 hrs but extremely significant at 50µg/ml. Chloroform fraction has not shown the concentration dependant lethality. While ethyl acetate: methanol and methanol fraction has shown lethality with the increasing concentrations of fractions. Amongst all the fractions chloroform fraction even at low concentration was found to be more lethal to brine shrimps.

Table1: Mean number of brine shrimps dead after 12 hrs with fractions

Drug Treatment	No. of Brine Shrimps Dead (Mean ± SEM)				
	10 µg/ ml	20 µg/ ml	30 µg/ ml	40 µg/ ml	50 µg/ ml
Control	0	0	0	0	0
Std	0.75 ± 0.28	1.25 ± 0.25	2.25 ± 0.47	3.0 ± 0.70	5.0 ± 0.40
Caffeine	0 ± 0	0.75 ± 0.25	1.0 ± 0.0	1.25± 0.25	1.5 ± 0.28
CH	9.0 ± 0.40*	9.25 ± 0.75*	9.25 ± 0.47*	9.75 ± 0.2**	10.0 ± 0.0***
EA: M	6.75 ± 0.47	6.25 ± 0.47	8.75 ± 0.40	9.25 ± 0.47*	9.25 ± 0.40*
M	4.75 ± 0.47	5.75 ± 0.40	7.75 ± 0.25	9.0 ± 0.40*	9.25 ± 0.47*

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Table 2: Mean number of brine shrimps dead after 24 hrs with fractions.

Drug Treatment	No. of Brine Shrimps Dead (Mean ± SEM)				
	10 µg/ ml	20 µg/ ml	30 µg/ ml	40 µg/ ml	50 µg/ ml
Control	0	0	0	0	0
Std	1.25 ± 0.25	2.75 ± 0.25	3.75 ± 0.47	7.0 ± 0.91	9.25 ± 0.47*
Caffeine	0.25 ± 0.0	1.0 ± 0.0	1.25 ± 0.25	1.75 ± 0.75	2.0 ± 0.40
CH	9.25 ± 0.25*	9.5 ± 0.50*	9.5 ± 0.28*	9.75 ± 0.25**	10.0 ± 0.0***
EA: M	7.75 ± 0.25	7.5 ± 0.5	9.5 ± 0.28*	9.5 ± 0.28*	9.75 ± 0.25**
M	6.5 ± 0.64	7.25 ± 0.85	8.5 ± 0.28	9.75 ± 0.25**	9.75 ± 0.20**

Where, *p<0.05 Significant, **p<0.01Very significant, ***p<0.001 extremely significant.
Values are expressed as Mean ± S.E.M.
STD: Methotrexate (0.1 mg/ml)
Al & Aq: 70 % alcohol and aqueous extracts of *T. grandis* respectively (10 mg/ml),
CH: Chloroform, EA: M-ethyl acetate: Methanol, M: Methanol (1mg/ml)
Methotrexate treatment group were compared with control.
Extract and fractions treated groups were compared with control.

CONCLUSION

From the above study, it can be concluded that cytotoxic components may be present in the bark of the plant. Alcoholic extract has shown the presence of glycoside especially anthraquinone glycosides, and phenolic compounds. So we can say that may be these compounds are responsible for antimitotic activity and brine shrimps lethality test. Chloroform fraction of alcoholic extract has shown the highest activity in both the models so may be therapeutically active components are present in chloroform fraction. In future it will be interesting not only to isolate the active chemical constituents but also to determine the mechanism of action of the same by using different in-vivo anticancer models.

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