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Antitumor and cytotoxic effect of different partitionates of methanol extract of *Trema orientalis* : A preliminary *in-vitro* study

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ABSTRACT

Herbal drugs are widely used in the primary health care system now-a-days and the use is increasing day by day. Thus extensive research to confirm the bioactivity of plant extract is very essential. The aim of this research was to determine the antitumor and cytotoxic actions of solvent-solvent partitioned fraction (n-hexane, ethyl acetate and hydro-methanol) of the methanol extract of root of *Trema orientalis*. The antitumor activity of different fractions of plant extract was evaluated by potato disc bioassay method where *Agrobacterium tumefaciens* was used to induce tumor on potato disc. On the other hand, the cytotoxic effect was determined by brine shrimp lethality bioassay technique. The ethyl acetate fraction of the plant extract showed 35.51% and 62.89% inhibition of tumor formation in potato disc bioassay method at 50 µg/disc and 100 µg/disc respectively. The hydro-methanol extract showed 37.51% inhibition of tumor formation at 100 µg/disc while at 50 µg/disc no significant inhibition was observed. In contrast, n-hexane fraction did not show any antitumor activity. In cytotoxic study by brine shrimp lethality bioassay, the lethal concentration-50 (LC_{50}) value of the n-hexane, ethyl acetate and hydro-methanol fraction was 1377.03 µg/ml, 11.67 µg/ml and 48.62 µg/ml respectively. Ethyl acetate and hydro-methanol fractions of the solvent-solvent partitioned methanol extract of *T. orientalis* showed high antitumor and cytotoxic effect. These fractions are highly promising for further exploration to identify the bioactive compounds.

Key words: *Trema orientalis, Potatodisc Bioassay, Brine Shrimp Lethality Bioassay.*

INTRODUCTION

Starting from the stone-age, medicinal plants are vital source of bioactive compounds with numerous pharmacological activities. According to report of World Health Organization, 70 - 95% people of the developing countries rely on non-conventional plant derived herbal drugs for their primary healthcare.[1,2] The use of plant derived herbal drugs become more popular day by day even in developed country due to low cost, less side effect and high efficacy. The biological activity of the herbal drugs might be due to presence of one or more compounds with synergistic effect in the plants. For this, screening plant extract to evaluate specific biological activity is essential primarily.

Trema orientalis (Bengali name - *Jibon* or *Chikon*), a widely distributed plant in Bangladesh, belongs to the Ulmaceae family. In traditional medicine the plant is used by the rural people in the treatment of asthma, diarrhea, muscular pain and epilepsy.[3]

Reports showed that different parts of the plant exhibit various pharmacological activities including hypoglycemic, analgesic, anti-inflammatory, anti-plasmodial, diuretic, laxative, anti-convulsant, anti-

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helmintic, anti-sickling, antioxidant and antibacterial activity.[4] These effects might be due to the presence of biologically active compounds such as polyphenols, saponins, flavanoids, triterpenoid which have been isolated from this plant.[4] Polyphenols are important class of compounds that act as antioxidant and anticancer agents.[5-7]

Though the antioxidant activity of the plant has been reported but its antitumor activity and cytotoxicity have not been explored. For this, we have investigated the antitumor activity of different solvent partitioned fractions of methanol extract by potato disc bioassay method and cytotoxic effect by brine shrimp lethality bioassay method.

Materials and Methods

Plant collection and identification

The roots of the plant *Trema orientalis* (Family - Ulmaceae) collected from Mymensingh, Bangladesh, were taxonomically identified by Bangladesh National Herberium (Accession No. 9521).

Extract preparation and solvent-solvent partitioning

The roots of the plant were sun dried for 5 days and cut into small pieces and then kept in an oven at 45°C for 24 h. The dried roots were grounded into powder with the help of a grinder. About 300 gm of powdered materials were soaked in 1.5 L of methanol for 7 days at RT (25 ± 0.5°C) with occasional stirring. The mixture was filtered through cheese cloth and then Whatman filter paper No. 1. The solvent of the filtrate was evaporated by rotary evaporator (Buchi Rotavapor R-200, Germany). The yield of the crude extract was 3.5% w/w on dry basis.

Crude extract was subjected to solvent-solvent partitioning using the protocol designed by Kupchan and Tsou[8] with some modification. The crude extract (5 g) was triturated by dissolving in 10% aqueous methanol (methanol : water; 9:1 v/v) to make the mother solution which was successively partitioned by three solvents such as n-hexane, ethyl acetate and hydro-methanol in order of increasing polarity by using separating funnel. The fractions of each partition were dried by evaporating respective solvent using rotary evaporator as earlier. All extracts were stored at 4°C in air tight containers till further analysis.

Antitumor activity of different fractions of *T. orientalis*

For antitumor activity, the procedure described by Galasky[9] with slight modification was followed. In brief, *Agrobacterium tumefaciens* virulent strain (Cambia SR 009 EHA-105) was grown for 48 hours in Luria Bertanin (LB) medium containing chloramphenicol (1 µg/ml). 1% inoculum from above culture was inoculated to 25 ml broth medium for 18 hrs for subsequent infection. Covers of two small size potatoes were peeled up followed by washing with detergent (Clorox) for 30 min. The potatoes were washed with sterilized distilled water to remove detergent and cut to form disc (5 mm x 5 mm). To impregnate, the potato discs were soaked with 50 ml of sterilized distilled water containing 20 µl of *A. tumefaciens* in a beaker for 10 min. Then water was removed and the discs were made dry by rubbing with sterilized filter paper. The impregnated potato discs were then treated with or without extract at dose of 50 µg/disc and 100 µg/disc (n=3) and transferred to petri dish containing induction medium containing 5% macronutrients, 0.5% organic stock, 0.5% EDTA iron stock, 0.1% inositol, 0.03% casein enzymatic hydrolysate and 0.3% agar. The petri dishes were incubated at 37°C for 10 days followed by staining the potato discs with Lugol's solution. The tumor number on the potato discs were observed with the aid of Olympus Microscope and percent inhibition for each concentration was determined by the formula given below.

Percent Inhibition = 100 – (Average number of tumor in sample X 100)/Average number of tumor in control.

Brine shrimp lethality assay (BSLA)

The cytotoxic effect of bioactive plant extract was tested against *Artemiasalina* nauplii followed by Meyer[10] with minor modifications. Briefly, dried cysts of *A. salina* were hatched in filtered sea water (3.8 gm/L NaCl) at 27°C under continuous illumination and aeration. After 48 hr incubation, the *A. salina* nauplii

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were collected and 10 individuals were transferred to each test tube containing 5 ml sea water. The concentration of each extract was maintained at 400.0, 200.0, 100.0, 50.0, 25.0, 12.5, 6.25, 3.125, 1.5625, 0.78125 and 0.00 µg/ml. Three samples (n=3) were taken for each concentration. The culture tube were incubated at 27°C in darkness and the number of dead larvae in each tube was counted after 24 hr (chronic toxicity). Potassium dichromate ($K_2Cr_2O_7$) and DMSO were used as a positive and negative control, respectively.The percentage mortality of brine shrimp nauplii was determined from the number of dead nauplii and LC_{50} value was estimated using the Probits statistical method.

RESULTS

The physical appearances of the three extracts obtained from solvent-solvent partitioning and their quantity have been shown in Table 1.

The formation of tumor on potato disc by *A. tumefaciens* was significantly inhibited by different fractions of extract of *T. orientalis* (Figure 1). The low polar n-hexane fraction did not show any antitumor activity. In contrast, the ethyl acetate fraction at 50 µg/disc and 100 µg/disc inhibited tumor formation by 31.51% and 62.89%, respectively. On the other hand, the polar hydro-methanol fraction showed significant inhibition (37.5%) of tumor formation at dose 100 µg/disc while it showed little inhibition at dose 50 µg/disc (Table 2).

In brine shrimp lethality bioassay, the n-hexane fraction showed no cytotoxic effect as the lethal concentration-50 (LC_{50}) value is very high while the ethyl acetate fraction showed strong effect with a LC_{50} value 11.67 µg/ml. The cytotoxic effect of hydro-methanol was found to be moderately active with a LC_{50} value 48.62 µg/ml (Table 3)

DISCUSSION

Crown gall is a neoplastic disease of plants induced by specific strains of *A. tumefaciens*. The bacteria contain tumor-inducing plasmids which transforms plant cells into autonomous tumor cells. The mechanism by which *A. tumefaciens* induces tumor to plant cell is very similar to that of human pathogens to induce tumor.[11] Galsky[9,12] demonstrated a strong correlation between the inhibition of crown gall tumors on potato and the antitumor effect of compounds and plant extracts in 3PS (*in vivo*, murine leukemia) system. Moreover, the simplicity, reliability, rapidity and inexpensiveness of the method make it more popular for reliable pre-screen 3PS antitumor activity. Consequently, several groups used potato disc bioassay for testing the antitumor properties of biological compounds.[13-15] Some compounds camptothecin, palitaxel, podophyllin, vinblastine and vincristine showed significant inhibitory effect on the crown-gall tumor.[16] So it can be said that the potato disc bioassay that has been used in the present study could be applied effectively for screening antitumor activity of plant extract. In the present study we found statistically significant inhibition of tumor formation by ethyl acetate fraction both at 50 µg/disc and 100 µg/disc.

On the other hand, the brine shrimp lethality bioassay represents a rapid, inexpensive and simple bioassay for evaluating the cytotoxicity of plant extract. It has been proved to be a convenient system for monitoring biological activities of natural products.[17] Studies showed that there is a positive correlation between the brine shrimp lethality and oral lethality test in mice in medicinal plant research. A good correlation between the *in vivo* and the *in vitro* tests ($r = 0.85$, $p < 0.05$) makes this method useful tool for predicting oral acute toxicity of plant extracts.[18] The LC_{50} - value less than 20-30 mg/ml is considered as significant cytotoxic effect.[19] In the present study, the LC_{50} of the ethyl acetate extract is 11.67 µg/ml indicating significant cytotoxic effect of the extract. Moreover, the LC_{50} value of the positive control $K_2Cr_2O_7$ was found to be 289.19 µg/ml (Table 3) which is very close to that of found by other researchers.[20]

The proposed antitumor and cytotoxic property of plant *T. orientalis* is the first report and a preliminary work and hence further phytochemical analysis is essential to isolate and elucidate structure of the bioactive compounds and confirm the antitumor and cytotoxic activity by cell line study.

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Figure 1: Image of potato disc bioassay

Fig 1.1: Control

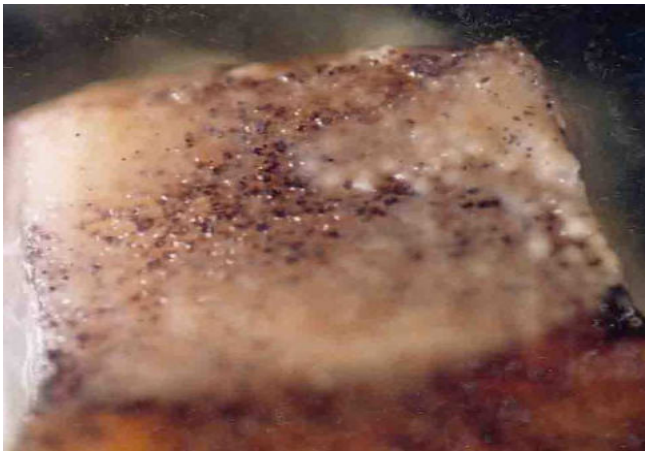

Fig 1.2: n-hexane fraction

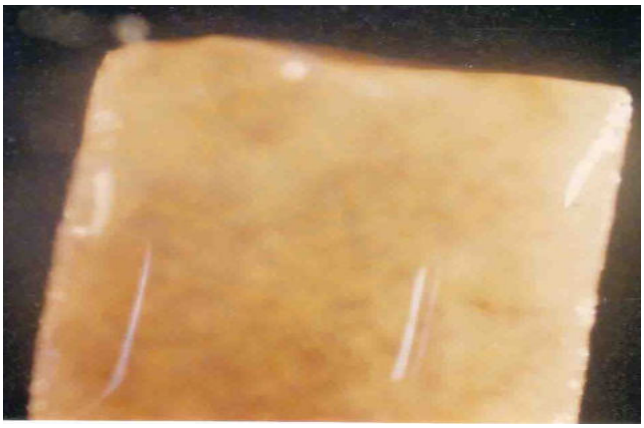

Fig 1.3: Ethyl acetate fraction

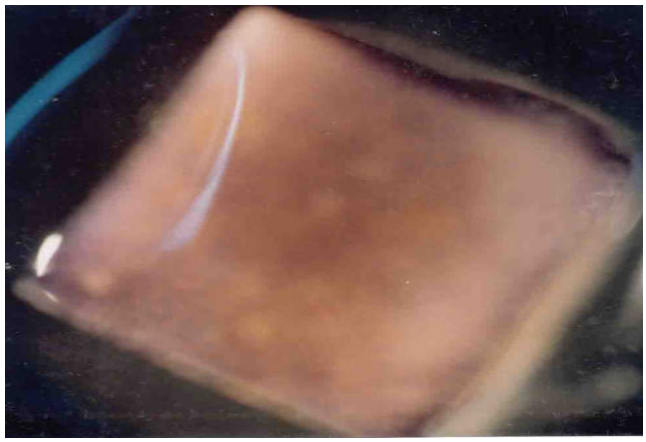

Fig 1.4: Hydro-methanol fraction

CONCLUSION

In the present research work, we have assessed the antitumor and cytotoxic property of different solvent-solvent partitioned fractions of methanol extract of roots of *T. orientalis*. The ethyl acetate and hydro-methanol fraction of the extract showed promising cytotoxic and anti-tumor effect. For this, further evaluation is required to isolate the bioactive compounds.

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Table 1: Different fractions of *T. orientalis* obtained after Kupchan-partitioning of the crude methanol extract

Plants	Extract/Fractions	Amount (g)	Yield (% w/w)	Physical appearance
T. orientalis	n-hexane	0.375	0.1250	Deep green, gummy mass
	Ethyl acetate	0.638	0.2125	Red muddy, sticky mass
	Hydro-methanol	1.688	0.5625	Ash like, sticky mass

Table 2: Antitumor activity solvent-solvent partition fractions of methanol extract of *T. orientalis*

Test Sample	No. of Tumor on potato							
	Control (n=3)	Average±SD	Sample 50µg/	Average±SD	% Inhibition	Sample 100µg/	Average±SD	% Inhibition
n-Hexane Fraction	21 20 18	19.67±1.53	19 20 20	19.67±0.58	0.00	16 19 19	18.00±1.73	8.49
n-Ethyl Acetate	22 21 19	20.67±1.52	15 13 12	13.33±1.53	35.51*	9 7 7	7.67±1.15	62.89*
Methanol	23 21 20	21.33±1.53	19 18 20	19.00±1.00	10.92	14 13 13	13.33±0.58	37.51*

*P<0.05 is considered as statistically significant

Table 3: Brine shrimp lethality bioassay of solvent-solvent partition fractions of methanol extract of *T. orientalis*

Sample Conc. (µg/ml)	LogC	Average No. of viable shrimp (out of 10), n=3				% Mortality				LC_{50} (µg/ml)			
		HE	EE	ME	$K_2Cr_2O_7$	HE	EE	ME	$K_2Cr_2O_7$	HE	EE	ME	$K_2Cr_2O_7$
400	2.60206	7	0	1	3.67	30	100	90	63.33	1377.03	11.67	48.62	289.19
200	2.30103	7.67	0	1.67	6.33	23.3	100	83.33	36.67				
100	2.0	9.33	0.67	3.67	7.67	6.7	93.33	63.33	23.33				
50	1.69897	10	2.67	5.33	9.33	0	73.33	46.67	6.67				
25	1.39794	10	4.33	6.33	10	0	56.67	36.67	0				

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12.5	1.09691	10	5.33	7.67	10	0	46.67	23.33	0				
6.25	0.79588	10	6.0	9.33	10	0	40.00	6.67	0				
3.125	0.49485	10	7.33	10	10	0	26.67	0	0				
1.5625	0.19382	10	8.67	10	10	0	13.33	0	0				
0.7813	-0.1072	10	9.67	10	10	0	3.33	0	0				
0.3906	-0.4083	10	10	10	10	0	0	0	0				
0.195	-0.7099	10	10	10	10	0	0	0	0				

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