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ANTIBACTERIAL, INSECTICIDAL AND *IN VIVO* CYTOTOXICITY ACTIVITIES OF *SALIX TETRASPERMA*

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ABSTRACT

The present study was designed to investigate antibacterial, insecticidal and in vivo cytotoxic activities of the methanol: ethylacetate (1:9) extract of leaves, barks and roots of *Salix tetrasperma*. Antibacterial test were done against five Gram-positive and four Gram-negative bacteria. Bark extract was active against all tested microbial species and the highest activity was shown against Escherichia coli with a zone of inhibition 12 ± 0.02 mm. The leaves extract was active against all the Gram positive bacterial strain whereas inactive against all the Gram negative bacterial strain except *Shegella flexneri* while root extract showed insignificant activity. In insecticidal study, the root and leaves extract of *Salix tetrasperma* showed moderate activity with 26% and 20% mortality rate of *Tribolium castaneum* at a dose of 50mg/ml in 48 hours, respectively and bark extract had no activity. In respect of in vivo cytotoxicity, Root and bark extract showed moderate cell growth inhibition, whereas leaves extract had no activity at all.

INTRODUCTION: The history of plants being used for medicinal purpose is probably as old as the history of mankind. Simultaneous with population explosion, virulent strains of microorganisms become more common and their increased attack accounts for increased mortality [1].

Bangladesh, being a country with high density of population, infectious diseases becomes a great challenge in the health and economic sector. Despite the large numbers of synthetic antibiotics having different chemical nature have been developed in the last few decades, inappropriate and injudicious uses of antibiotics or self treatment practices developed resistance to microbes [2]. So the developments of new antibacterial agents are necessary to combat the problem of microbial resistance and for substitution with ineffective ones. Moreover, higher plants are rich source of novel natural substances that can be used to develop new antibacterial agent as well as environmental safe methods for insect control [3].

Not only the world wide annual losses of food grains storage caused by insects have been estimated to be about 10% of the world's production, but losses of 25% or more may also occur in tropical countries through

insect attack after harvest [4]. Control of these insects relies heavily on the use of synthetic insecticides and fumigants, which has led to problem such as disturbances of the environment, increasing cost of application, pest resurgence, resistant to pesticides and lethal effects on non-target organism in addition to direct toxicity to users [5]. So there is an immediate need to develop safe alternatives with low cost, easy to use and friendly to the environment.

Cancer, the second leading cause of death worldwide next to cardiovascular diseases, is a group of more than 100 different diseases, characterized by uncontrolled cellular growth, local tissue invasion, and distant metastases [6] and can be treated with surgery, radiation, chemotherapy, hormone therapy and biological therapy. Chemotherapy is still a major challenge to the cancer patients because less than 1% of injected drug molecules can reach their target cells, whereas the rest may damage healthy cells and tissue [7].

Plants have a long history of use in the treatment of cancer. It is noteworthy that a number of plants are known to be the source of useful drugs in modern medicine[8] and have been accepted currently as one of the main source of cancer chemoprevention drug discovery and development [9] due to their diverse pharmacological properties including cytotoxic and cancer chemopreventive effects [10]. *Salix tetrasperma* Roxburgh. (Family: Salicaceae), generally called Indian Willow [11]. Phytochemical studies of this plant resulted in the isolation of several tannins, triterpenes, viz. □-amyrin, lupeol [12] and chalcinasterol [13], steroids viz. □-sitosterol and stigmasterol [14].

Various types of sapogenins such as quinovic acid, salicortin, saligenin, phenolic glycosides and pyrocatechol were isolated from the barks and leaves [15]. The active extract of the bark, called salicin [16] was isolated to its crystalline form. Aqueous extract of dried leaf reported to possess cardiotonic activity and the methanol extract of the dried leaf possess reverse transcriptase inhibition effect [17]. The aqueous extract of the stem bark has been reported to increase testosterone level in rats at 500.0 mg/kg, p.o. [18] and also accelerates semen coagulation in rats at a concentration of 2%w/v [19]. A dose of 0.094 mg/kg of aerial parts shows hypothermic activity in mice [20]. The paste of both leaf and root is applied externally in scorpion stings, bug bites, for sores and warts and the decoction of the dried root is taken orally for the treatment of hepatitis [21] and whooping cough in children [22]. Based on these reports our studies have been designed to examine whether the methanol: ethyl acetate (1:9) extract of *Salix tetrasperma* exerts antibacterial, insecticidal and *in vivo* cytotoxicity activity.

MATERIALS AND METHODS:

Plant materials: Different parts (roots, leaves and barks) of *S. tetrasperma* were collected from the adjoining area of Rajshahi University Campus, Bangladesh during the month of April 2009 and were identified by Taxonomist, Department of Botany and University of Rajshahi, Bangladesh where a voucher specimen (Voucher No # 35) has been deposited for future references.

Preparation of extracts: The roots, leaves and barks of *S. tetrasperma* were dried in an oven at 37^0C and then pulverized into course powder with a mechanical grinder, passing through sieve #40, and stored separately in an air tight container. The dried powdered materials (1.0 kg) each, extracted three times by sonication for 30 min with MeOH: EtOAc (1:9) mixture (1000ml) and then filtered. Each of the filtrate was concentrated to dryness, in vacuum at 40^0C to render the crude extracts 300 g, 240 g and 320 g respectively.

Antibacterial assay: Sterile 6.0 mm diameter blank discs (BBL, Cocksville, USA) were impregnated with test substances at the dose of 200 µg/disc. This disc, along with standard discs (Ciprofloxacin, Oxoid Ltd, UK) and control discs were placed in petri dishes containing a suitable agar medium seeded with the test organisms using sterile transfer loop and kept at 4^0C to facilitate maximum diffusion. The plates then kept in an incubator (37^oC) to allow the growth of the bacteria. The antibacterial activities of the test agents were determined by measuring the diameter of the zone of inhibition in terms of millimeter. Antimicrobial activity was tested against *Bacillus anthracis***,** *Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonus aeruginosa*, *Shigella flexneri***,** and *Shigella boydii* were obtained

from International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) [23].

Determination of Relative Percentage Inhibition: The relative percentage inhibition with respect to positive control was calculated by using the following formula[24]. Relative percentage inhibition of the test extract = [{100 x (a - b)}/(c - b)]. Where, a: total area of inhibition of the test extract; b: total area of inhibition of the solvent; c: total area of inhibition of the standard drug. The total area of the inhibition was calculated by using area = πr^2; where, r = radius of the zone of inhibition.

Screening of Insecticidal Activity: Insect's *Tribolium castaneum* used in the present experiment were taken from the stock cultures of the Crop Protection and Toxicology Laboratory, University of Rajshahi, Bangladesh. To conduct surface film activity test 60mm petri dishes were taken for all extracts and their replication. Each extract (50mg) was dissolved into 1ml respective solvent. Then they were poured into the lower part of the petri dish and allowed them to dry out. Then insects were released in each of the treated petri dish.

A control experiment by applying the only solvent into the petri dish was also set at the same time under the same condition[25]. After completing all the arrangements treated petri dishes were placed in a secured place at room temperature. The whole experiment was observed from time to time and mortality was observed first 30 minutes (after starting the experiment) and then 48hs of exposure. The data was recorded. A simple microscope was used to check each and every beetle by tracing natural movement of its organs. In some cases hot needle was taken closer to the bodies (without movement) to confirm death. Attention was also paid to recovery of the insects if occurred. The mortality records of the *Tribolium Castaneum* adults were corrected by the Abbott's formula [26];

$$P_r = (P_0 – P_c \setminus 100 - P_c) \times 100$$

Where, P_r = Corrected mortality (%); P_o = Observed mortality (%); P_c = Control mortality (%), sometimes called natural mortality (%)

Screening of *in vivo* anticancer activity:

Animal: Albino mice (25-30g) and Wistar rats (175-250 g) of both sexes were used for assessing biological activity. The animals were maintained under standard laboratory conditions and had free access to food and water *ad libitum.* The animals were allowed to acclimatize to the environment for 7 days prior to experimental session. The animals were divided into different groups, each consisting of five animals which were fasted overnight prior to the experiments. Experiments on animals were performed in accordance with guidelines of the Institutional Animal Ethics Committee, Atish Dipankar University of Science & Technology, Dhaka, Bangladesh.

Transplantation of tumor: Ehrlich ascites carcinoma (EAC) cells were obtained from Indian Institute of Chemical Biology (IICB), Calcutta, India. The EAC cells were maintained *in vivo* in Swiss albino mice by intraperitoneal transplantation of 2×10^6 cells per mouse after every 10 days. Ascitic fluid was drawn out from EAC tumor bearing mouse at the log phase (days 7-8 of tumor bearing) of the tumor cells. Each animal received 0.1 ml of tumor cell suspension containing 2×10^6 tumor cells intraperitoneally.

Treatment schedule: 60 Swiss albino mice were divided into six groups (n = 10) and given food and water *ad libitum*. All the animals in each groups except Group-I received EAC cells (2×10^6 cells/mouse i.p.). This was taken as day '0'. Group-I served as normal saline control (5 ml/kg i.p.) and Group-II served as EAC control. 24-h after EAC transplantation, Group-III, Group-IV and Group-V received crude extract of roots, leaves and barks of *S. tetrasperma* at a dose of 50 mg/kg i.p. for nine consecutive days, respectively. Group-VI received reference drug Bleomycin (0.3 mg/kg i.p) for nine consecutive days[27]. Twenty-four hours of last dose and 18 h of fasting, 6 animals of each group were sacrificed by cervical dislocation to measure antitumor activity. The antitumor activity of the extracts of *S. tetrasperma* was measured in EAC animals with respect to the following parameters.

Statistical analysis: All assays were performed in triplicate under strict aseptic conditions to ensure consistency of all findings. Data of all experiments were statistically analyzed and expressed as the mean ± standard deviation of three replicate experiments.

RESULT AND DISCUSSION: Table 1 expressed the antibacterial activity (zone of inhibitions) of the roots, leaves and barks of the *S. tetrasperma*. Bark extract showed prominent activity against all the tested bacteria with the zone of inhibition range was found to be 9 to 12 mm. The highest zone of inhibition was found against *Escherichia coli* (zone of inhibition 12 mm) while the least activity was shown against *Bacillus megatherium,* (zone of inhibition was 9 mm).

The leaves extract was active against all the Gram positive bacterial strain whereas inactive against all the Gram negative bacterial strain except *Shegella flexneri.* On the other hand, root extract was active against *Bacillus subtilis, Bacillus cereus, Pseudomonas aeruginosa* and *Escherichia coli* while *Staphylococcus aureus, Bacillus megatherium, Bacillus anthracis, Shigella flexneri and Shigella boydii* showed resistance to the roots extracts. Bark extract of *S. tetrasperma* showed the maximum relative percentage inhibition against *Shigella flexneri* (25.00%) followed by *E. coli* (21.30%) and *Bacillus cereus* (21.00%).

However, the relative percentage inhibition ranges was 4.72% to 11.11% for leaves extract while no relative percentage inhibition was found against *Pseudomonas aeruginosa*, *Escherichia coli* and *Shigella boydii*. On the other hand, root extract showed relative percentage inhibition only for *Pseudomonas aeruginosa*, *Escherichia coli, Bacillus subtilis* and *Bacillus cereus* and was found to be 4%, 5.32%, 4% and 4.34%, respectively (**Table 1**).

TABLE 1: *IN VITRO* ANTIMICROBIAL ACTIVITY OF *SALIX TETRASPERMA* (ROOT, LEAVES AND BARKS EXTRACT)

Bacterial strain	Diameter of zone of inhibition (mm)			
	[b]Std. K (30µg/disc)	[a]Root (200µg/disc)	[a]Leaf (200µg/disc)	[a]Bark (200µg/disc)
Gram positive				
Staphylococcus aureus	27 ± 0.12	NA	6 ± 0.11 (4.93%)	10 ± 0.09 (13.66%)
Bacillus cereus	24 ± 0.02	5 ± 0.12 (4.34%)	8 ± 0.32 (11.11%)	11 ± 0.19 (21.00%)
Bacillus megatherium	22 ± 0.17	NA	7 ± 0.02 (10.12%)	10 ± 0.05 (20.66%)
Bacillus subtilis	25 ± 0.12	5 ± 0.19 (4.00%)	6 ± 0.12 (5.75%)	11 ± 0.01 (19.36%)
Bacillus anthracis	23 ± 0.22	NA	5 ± 0.19 (4.72%)	10 ± 0.11 (18.90%)
Gram negative				
Pseudomonas aeruginosa	25 ± 0.11	5 ± 0.09 (4.00%)	NA	10 ± 0.19 (16.00%)
Shigella flexneri	22 ± 0.07	NA	5 ± 0.11 (5.16%)	11 ± 0.05 (25.00%)
Shigella boydii	24 ± 0.02	NA	NA	10 ± 0.03 (17.36%)
Escherichia coli	26 ± 0.04	6 ± 0.19 (5.32%)	NA	12 ± 0.02 (21.30%)

[a] Values of the observed diameter zone of inhibition (mm). Incubation conditions for bacteria- 24 hours at 37^{o}C. Assay was performed in triplicate and results are the mean of three values ± Standard Deviation. [b] Reference standard; Kanamycin. NA- Zone of inhibition < 5 mm consider as no activity. Parenthesis indicate the relative percentage of inhibition

Antimicrobial activities of tannins [28], flavonoids [29], saponins [30], terpenoids [31], and alkaloids [32] have been documented. Previous Phytochemical studies of this plant revealed that tannins, flavonoids, triterpenes, as well as steroids [12-15] are the main chemical constituents of leaves and bark of *S. tetrasperma*. So, the highest antimicrobial activity showed by extracts (leaves and bark) than roots of *S. tetrasperma* may be due to presence of such type of phytoconstituent.

For insecticidal activity root and leaf extract have shown 26% and 20% mortality rate of *Tribolium castaneum* at a dose of 50mg/ml in 48 hours while bark extract had no activity (**Figure 1**). It is noteworthy that carbohydrates, saponins, phytosterol, phenol, flavonoids and tannins have possessed larvicidal activity [33]. Therefore, tannin, terpenoids as well as other secondary metabolites of this investigated plant may explain the toxic effect in the studied insects.

In vivo cytotoxicity activity of extract against EAC tumor bearing mice was assessed by the parameter % of cell growth inhibition. Root and bark extract showed

moderate cell growth inhibition and was found to be 23% and 26%, respectively, whereas leaves extract had no activity at all (**Figure 2**).

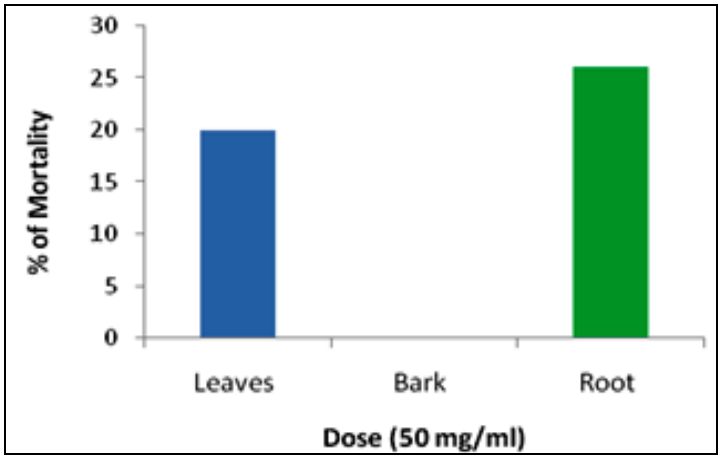

FIG. 1: INSECTICIDAL ACTIVITY OF ROOT, LEAF AND BARK EXTRACT OF *S. TETRASPERMA* ON *TRIBOLIUM CASTANEUM* (HERBST) BY SURFACE FILM TREATMENT

A number of scientific reports indicate certain terpenoids, steroids and phenolic compounds such as tannins, caumarins and flavonoids have a chemo preventive role in cancer through their effects on signal transduction in cell proliferation and angiogenesis [34]. Furthermore, flavonoids such as quercetin, kaemferol and their glycosides have been shown to possess antimutagenic and antiproliferative effect in various cancer cell line [35]. The in vivo cytotoxicity activities of *S. tetrasperma* are probably due to the presence of tannin, terpenoids, steroids as well as other bioactive compound.

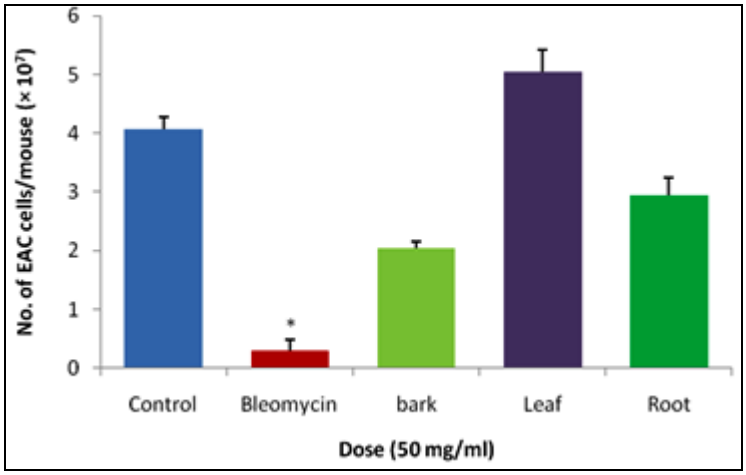

FIG. 2: *IN VIVO* CYTOTOXICITY ACTIVITY OF *S. TETRASPERMA* ON EHRLICH ASCITES CARCINOMA (EAC) CELLS.

CONCLUSION: In conclusion, the results of the present study indicate that *S. tetrasperma* exhibits interesting antimicrobial, insecticidal and *in vivo* cytotoxic properties. These results of the investigation do not reveal that which chemical compound is responsible for aforementioned activity.

Now our next aim is to investigate the isolation and structure determination of the lead compound liable for aforementioned activity from this plant and work is going on in this respect in our laboratory.

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