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In vitro antimicrobial, cytotoxicity, antioxidant and *In vivo* analgesic activities of methanol extract of *Dipterocarpus turbinatus* leaves

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Abstract

The study aimed to investigate the antimicrobial, cytotoxic, antioxidant, and analgesic properties of a methanol extract of Dipterocarpus turbinatus leaves. The extract shows a moderate antimicrobial effect against the tested organisms at different concentrations (10-19 mm of zone of inhibition). The percentage of mortality was observed to increase as the dose increased. The LC50 was found to be 408.50 μg/mL which proved the plant to be a good source of novel drugs with less toxicity. It exhibits antioxidant effect revealed by increase in the percentage of scavenging assay observed with increase in concentration. The presence of reductants in MEDT was also revealed. The number of writhing which is a dose dependent shows highest number at a dose of 200 mg/kg body weight i.e., 32.83±1.25 and 28.16±1.89, 15.5±2 for 400 mg/kg of MEDT and 10 mg/kg of Diclofenac-Na respectively. At a dose 0f 200 mg/kg of MEDT, 28.51±0.560 seconds were spent licking the paw while at a dose of 400 mg/kg of MEDT, 23.60±0.545 seconds were spent licking the paw.

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1. Introduction

For a long period of time, plants have been a valuable source of natural products for maintaining health, especially in the last decade, with more intensive studies for natural therapies. According to World Health Organization, medicinal plants would be the best source to obtain a variety of drugs. Antimicrobials are utilized to treat or counteract bacterial infections, and occasionally protozoan contaminations (Metronidazole is

compelling against various parasitic infections). This includes the organization of a wide range of anti-toxins in view of the signs and indications displayed and is started pending lab comes about that can take a few days. Cytotoxicity is the nature of being dangerous to cells. Cases of dangerous operators are an insusceptible cell or a few sorts of venom, e.g., from the puff snake (*Bitis arietans*) or dark colored hermit arachnid (*Loxosceles reclusa*). Treating cells with the cytotoxic compound can bring about an assortment of cell destinies. The cells can stop currently developing and separating (a decline in cell feasibility), or the cells can initiate a hereditary program of controlled cell demise (apoptosis). An analgesic or painkiller is any member of the group of drugs used to achieve analgesia, relief from pain. Analgesic drugs act in various ways on the peripheral and central nervous systems. They are distinct from anesthetics, which temporarily affect, and in some instances eliminate, sensation. Analgesics include paracetamol (known in North America as acetaminophen or simply APAP), the nonsteroidal anti-inflammatory drugs (NSAIDs) such as the salicylates, and opioid drugs such as morphine and oxycodone.

In present day medication, around a quarter of the medications recommended to patients are gotten from restorative plants, and they are thoroughly tested (Smith-Hall, Larsen, & Pouliot, 2012). However, improvement of plants or concentrates having potential restorative uses is blunted by feeble logical proof, poor practices during the time spent on medication advancement, and deficient financing (Ahn, 2017). The oleoresin of the trunk is stimulant to the mucous surfaces and diuretic; used as an external application for ulcers, ringworms, and other cutaneous affections. It has been used in gonorrhea, gleet, and rheumatism.

2. Materials and methods

2.1. Chemicals and reagents

The analytical grade chemicals and reagents available at the Department of Pharmacy, Faculty of Science and Engineering, IIUC were used as needed for the study.

2.2. Collection, identifications and extraction of plant material

Fresh leaves of *D. turbinatus* were collected from Dulahazara, Chakaria, Cox's Bazar, Chittagong, Bangladesh and identified by Professor Dr. Shaikh Bokhtear Uddin, Taxonomist and Professor of Botany at University of Chittagong, Bangladesh. The leaves were sun dried for a period of one week under shade and grind into fine powder using a blender machine. The grind leaves (500 g) were soaked in enough

methanol for two weeks at room temperature with occasional shaking and then filtered through a cotton plug followed by Whitman filter paper No.1. The solvent was evaporated under reduced pressure at room temperature and gentle heat was also applied through water bath to yield semisolid extract. The crude extract was then preserved in a refrigerator until further use. Throughout the study, the methods were used like Disk diffusion technique, Serial dilution technique, Brine shrimp lethality bioassay, DPPH Free Radical Scavenging Assay, and Ferric reducing antioxidant power assay etc.

2.3. In vitro antimicrobial screening

2.3.1. Test organisms used for the study

The test organisms used for the study were collected from pharmaceutical microbiology lab. Southern University, Chattogram (Table I).

2.3.2. Primary assay

It is a qualitative or semi-qualitative test that indicates the sensitivity or resistance of microorganisms to the test sample. But, this technique cannot be used to distinguish between bacteriostatic and bactericidal agents (Reiner, 1982). The primary assay was performed by disk diffusion assay method (Barry, Hoeprich, & Saubolle, 1976), includes- Plate diffusion test, Streak test.

The plate diffusion test utilizes different concentrations of a test compound absorbed on sterile filter paper disks on the sample plate whereas the streak test permits the determination of the antibacterial effect of a test compound on several microorganisms simultaneously and is suitable for the estimation of the spectrum activity. However, the plate diffusion method was used.

2.3.3. Secondary assay

It quantifies the relative potency such as minimum inhibitory concentration (Yano, Mizoguchi, Fukuda, Haramaki, Ogasawara, Momosaki, & Kojiro, 1994). The lowest concentration of an antimicrobial agent required to inhibit the growth of the microorganisms in vitro is referred to as minimum inhibitory concentration. It is done by serial dilution technique (Reiner, 1982). Here, the broth dilution method was used.

2.4. In vitro cytotoxicity assay

Brine shrimp eggs were hatched in simulated sea water to get nauplii. Test samples were prepared by the addition of calculated amount of distilled water for obtaining desired concentration of test sample. The nauplii were counted by visual inspection and were taken in vials containing 5 ml sea

water. The samples of different concentration were added to pre-marked vials with a micropipette. The vials were left for 24 hours and then nauplii were counted again to find out the cytotoxicity of the test agents. This data were processed in a simple program for profit analysis to estimate LC_{50} values with 95% confidence intervals for statistically significant comparisons of potencies.

2.5. In vitro antioxidant assay

2.5.1. DPPH free radical scavenging assay

The 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) has been widely used to evaluate the free radical scavenging capacity of antioxidants. DPPH free radical is reduced to the corresponding hydrazine when it reacts with hydrogen donors. DPPH can make stable free radicals in aqueous or methanol solution (Braca, Tommasi, Bari, Pizza, Politi, & Morelli, 2001). With this method, it was possible to determine the antiradical power of an antioxidant activity by measuring the decrease in the absorbance of DPPH at 517 nm. Resulting from a color change from purple to yellow the absorbance decreased when the DPPH was scavenged by an antioxidant, through donation of hydrogen to form a stable DPPH molecule (Oyedemi, Bradley, & Afolayan, 2010).

2.5.2. Reducing power capacity

In this assay, the yellow color of the test solution changes to various shades of green and blue depending on the reducing power of antioxidant sample. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The presence of reductants such as antioxidant substances in the sample causes the reduction of the Fe^{3+}-ferricyanide complex to the ferrous form by donating an electron. The amount of Fe^{2+} complex can then be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Oyaizu, 1986; Ferreira, Baptista, Vilas-Boas, & Barros, 2007).

2.6. In vivo analgesic activity

2.6.1. Experimental animals

Swiss albino mice, weighing about 25 - 30 gm, were collected from Jahangirnagar University, Savar, Bangladesh. The animals were provided with standard laboratory food and distilled *ad libitium* water and maintained at natural day-night cycle with proper ventilation in the room. All the experiments were conducted in an isolated and noiseless condition. The study protocol was approved by the P&D committee meeting, Department of Pharmacy, International Islamic University

Chittagong, Bangladesh (Pharm-P&D-37/07'12). The animals were acclimatized to laboratory condition 10 days prior to the experiment.

2.6.2. Acetic acid induced writhing test

The analgesic activity of the sample was studied using acetic acid-induced writhing model in rats test samples (200 and 400 mg/kg body weight), vehicle (1% tween-80 in water) and indomethacin (10mg/kg) were administered orally 30 minutes before intraperitoneal administration of 0.7%, 0.1 ml/10 gm acetic acid. Then the rats were observed for specific contraction of body referred to as 'writhing' for the next 20 minutes (Yasmine, 2016). Full writhing was not always accomplished by the animal, as sometimes the animals started to give writhing, but they did not complete it. This incomplete writhing was considered as half-writhing. Accordingly, two-half writhing were taken as one full writhing. The number of writhes in each treated group was compared to that of a control group while Diclofenac-Na (10 mg/kg) was used as reference substance (positive control). The percent inhibition (analgesic activity) was calculated by

$$\% \text{ inhibition} = \frac{A-B}{A} \times 100$$

Where, A= Average number of writhing control group; B=Average number of writhing of the test group.

2.6.3. Formalin test

The analgesic activity of the drugs was determined using the formalin test. Control group received 5% formalin. 20 μl of 5% formalin was injected into the dorsal surface of the right hind paw 60 minutes after administration of extract (200 and 400 mg/kg, p.o.) and Indomethacin (10 mg/kg, p.o.). The rats were observed for 30 minutes after injection of formalin, and the amount of time spent licking the injected hind paw was recorded. The first 5 min post formalin injection is referred to as the early phase and the period between 15 and 30 min as the late phase. The total time spent licking or biting the injured paw (pain behavior) was measured with a stopwatch (Sharma, Bhatia, Kharya, Gajbhiye, Ganesh, Namdeo, & Mahadik, 2010).

2.6.4. Statistical analysis

For in vivo analgesic activities, data were expressed as mean ± SEM using excel window 10. The R^2 value was obtained from the trendline in Excel for window 10 in case of cytotoxicity. To analyze the differences among IC_{50} of standard and plant extract, X, Y and R^2 values obtained from trendline in Microsoft Excel were used.

3. Result and discussion

3.1. In vitro antimicrobial activity

3.1.1. Determination of relative zone of inhibition (mm)

In this experiment the methanol crude extract was found to have prominent inhibitory property against several pathogenic microbial species related to standard drug kanamycin. This property indicates the presence of one or several chemical moieties in the crude extract having antimicrobial property.

3.1.2. Determination of Minimum Inhibitory Concentration (µg/mL).

An MIC of 750 µg/ml was observed for three out of the four tested organisms and 375 µg/mL for Escherichia coli as described in Table III. Therefore, the MIC for microbials varies on the organism's species.

3.2. Cytotoxicity study

The results show an LC_{50} value 0f 408.50 µg/mL which portrays that the plant extract has mild cytotoxic activity. It is depicted that the extract may contain antitumor, antibacterial or pesticidal compounds. However, since a broad range of phytocompounds can exhibit nonspecific cytotoxicity, plant extracts with significant cytotoxic activity should be further investigated using animal models to confirm antitumor activity.

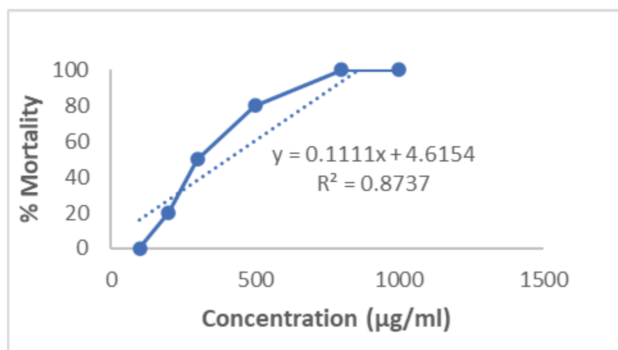

Figure 1
Cytotoxic effect of MEDT.

3.3. In vitro Antioxidant assay

3.3.1. DPPH scavenging assay

In the quantitative DPPH assay, extract displayed free radical scavenging activity of (IC50~78.83 µg/mL), which is comparable to that of ascorbic acid (IC50~166.75 µg/mL) a well-known standard antioxidant. See Table IV and Table V.

3.3.2. Reducing power capacity

In the qualitative antioxidant assay, the reducing power of the crude extract is comparable to that of known reducing agent (ascorbic acid) as shown in Figure 2 and Table VI. respectively.

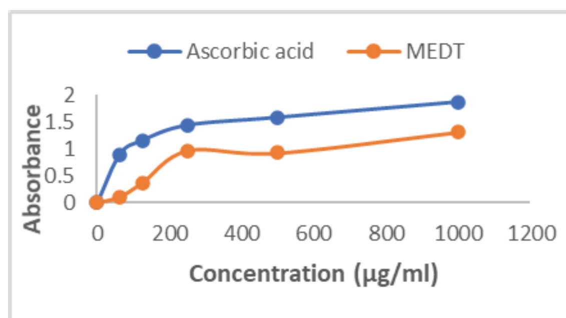

Figure 2

Reducing power of MEDT and Ascorbic acid.

3.4. In vivo analgesic study

Table VII and Table VIII displays the various readings with calculated ± SEM. The results obtained indicate that the extract possesses a moderate dose-dependent analgesic effect on the various pain models used as shown in Figure 3 and Figure 4. The methanol extract of *D. turbinatus* leaves caused a significant and dose-dependent inhibition of writhes in mice. The effect of the extract was comparable to that of Diclofenac-Na, a cyclooxygenase inhibitor. This suggests that the exact may have a peripheral analgesic action.

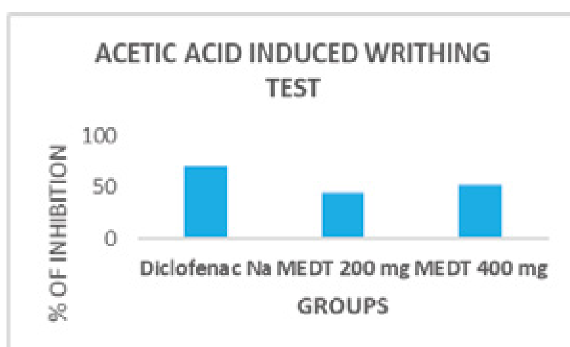

Figure 3

Relative comparative % of pain inhibition between Diclofenac-Na and MEDT

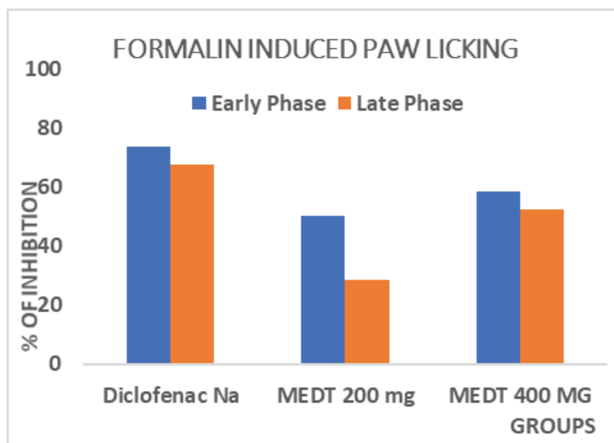

Figure 4
Relative comparative % of pain inhibition between Diclofenac-Na and MEDT

Table I
Organisms used for the study

Sl. No	Name of the test organism
1	*Bacillus cerus*
2	*Staphylococcus aureus*
3	*Escherichia coli*
4	*Bacillus azotoformans*
5	*Salmonella typhi*
6	*Pseudomonas aeruginosa*

Table II
Comparative diameter of zone of inhibition of MEDT and Kanamycin Standard

Sl. No	Name of test organism	Diameter of the zone of inhibition (in mm)			
		Meoh extract (100 µg/disc)	Meoh extract (300 µg/disc)	Meoh extract (500 µg/disc)	Kanamycin disc (30 µg/disc)
1	*Bacillus cereus*	10	15	17	35
2	*Staphylococcus aureus*	08	12	14	25
3	*Escherichia coli*	11	13	18	34
4	*Bacillus azotoformans*	12	15	19	35
5	*Salmonella typhi*	10	12	16	30

Table III
Relative minimum inhibitory concentration of MEDT

Sl. No	Name of test organism	Highest concentration which allows organism growth	Lowest concentration which inhibits organism growth	MIC
1	*Escherichia coli*	250	500	375
2	*Pseudomonas eruginosa*	500	1000	750
3	*Bacillus azotoformans*	500	1000	750
4	*Salmonella typhi*	500	1000	750

Table IV
% of DPPH radical scavenging activity of Ascorbic acid

Conc. (µg/mL)	Absorbance	% of scavenging	IC_{50} (µg/mL)
50	0.612	57.0827	
100	0.398	72.0897	
200	0.289	79.7335	166.75
400	0.268	81.2061	
800	0.198	86.1150	

Table V
% of DPPH radical scavenging activity of MEDT

Conc.(µg/mL)	Absorbance	% of scavenging	IC_{50} (µg/mL)
50	0.780	45.3015428	
100	0.498	65.0771388	
200	0.281	80.2945302	78.83
400	0.159	88.8499299	
800	0.033	97.6858345	

Table VI
Absorbance of ascorbic acid (standard) and MEDT

Conc. (µg/mL)	Ascorbic acid (absorbance)	MEDT (absorbance)
62.5	0.898	0.113
125	1.158	0.373
250	1.438	0.969
500	1.582	0.925
1000	.868	1.311

Table VII
Effect of MEDT on Acetic acid writhing test

Groups	Treatments	Dose	No. of writhing	% of inhibition
CONTROL	1% tween 80 in water	10ml/kg	59.33±1.04	
STANDARD	Diclofenac-Na	10ml/kg	15.5±2	70.51
TEST Sample (Plant Extracts)	MEDT	200mg/kg	32.83±1.25	44.66
		400mg/kg	28.16±1.89	52.53

Table VIII
Effect of MEDT on Formalin induced paw licking test.

Groups	Treatments	Dose (ml/kg)	Early phase (sec)	% of inhibition	Late phase (sec)	% of inhibition
Control	1% tween 80 in water	10	57.28±0.728	-----	40.99±0 .533	-------
Standard	Diclofenac-Na	10	14.90±0.272	73.98	13.23±0.300	67.73
TEST (Plant Extracts)	MEDT	200	28.51±0.560	50.23	29.19±0.291	28.79
		400	23.60±o.545	58.79	19.43±0.794	52.59

4. Conclusion

The overall result of this study suggests that *D. turbinatus* could be a good source of antibacterial, antioxidant, and analgesic agents. Further investigation of the plant might be beneficial for the search of other pharmacological effects. Bioassay-guided partitioning, isolation of specific bioactive compounds from the leaves, and safety assessment will be required to further investigate this species for potential new therapeutic drug leads.

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