

# ***In vitro* Antibacterial and *in vivo* Antinociceptive Activities of Ethyl Acetate Fraction of *Dipterocarpus turbinatus* Leaves**

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**(Receive: April 22, 2024; Accepted: July 7, 2024; Published (web): July 30, 2024)**

## **Abstract**

The study was aimed to assess the antibacterial and analgesic properties of the ethyl acetate extract from the leaves of *Dipterocarpus turbinatus*. The antibacterial effectiveness of the extract obtained from the leaves of *D. turbinatus* was evaluated using the disc diffusion method against both positive and negative gram strains. Furthermore, the analgesic qualities were assessed by doing the formalin-incited paw licking test in mice model. The ethyl acetate fraction demonstrated significant antibacterial activity against all tested microorganisms (*Lactobacillus casei*, *Corynebacterium* sp., *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus azotoformans* and *Salmonella typhi*) at concentrations of 0.1 mg/disc, 0.3 mg/disc, and 0.5 mg/disc using the disc diffusion method. The extract's zone of inhibition measured between 12 and 25 mm, was less than the kanamycin's diameter of 32–36 mm. The analgesic activity of the substance was evaluated in swiss albino mice using the tail immersion and formalin test procedures, revealing a modest level of analgesic activity. In the formalin test, the extract had a strong dose-dependent impact at dosages of 200 mg/kg and 400 mg/kg, as shown by a larger inhibition percentage compared to the standard. This result was statistically significant (\*\*p<0.001) when compared to the control group. During the tail immersion test, the extract showed a significant impact at dose 400 mg/kg (\*\*p<0.01). The plant extract has a modest level of antibacterial and analgesic properties.

**Key words:** Antimicrobial, analgesic, pathogenic microorganisms, *Dipterocarpus turbinatus*.

## **Introduction**

Plants have been an essential reservoir of medicinal substances for thousands of years. According to estimates from the World Health Organization (WHO), 80% of individuals mostly depend on traditional remedies, such as herbal medicines, for their healthcare requirements. Furthermore, around 25% of recommended drugs include plant extracts or active components sourced from plant elements (Hossain *et al.*, 2013). Several studies have suggested that compounds from synthetic materials may have harmful effects, such as

liver damage and mutagenesis (Rani *et al.*, 2024, Ouali *et al.*, 2024). Various microorganisms may penetrate the body via the mucous membrane of the skin after injuries, or they might enter via hair follicles and result in bacterial infections. These infections first appear as localised purulent conditions, such as tonsillitis, furuncles, ulcers, phlegmons, and oropharyngeal inflammations. However, they may subsequently disseminate into the bloodstream and result in septicemia. There has been a substantial rise in local infections in recent years. In the past, antibiotics were used for therapy, but several

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**DOI:** <https://doi.org/10.3329/bpj.v27i2.75190>

infections have developed resistance to them (Mancuso *et al.*, 2021). The pharmaceutical industry has created a number of novel antibiotics during the last thirty years, but bacteria are becoming more resistant to these drugs. Generally, microbes have the potential to genetically transmit and develop resistance to drugs used as therapeutic treatments. This finding causes concern given the number of hospital patients with weakened immune systems and the rise of bacterial strains that are resistant to many antibiotics. Hospitals may therefore encounter emerging infections with a high mortality rate (MacNair *et al.*, 2024). Natural antibacterial compounds for topical application, such as compresses, ointments, gargles, and cataplasms, may be obtained from higher plant products (Nguyen and Bhattacharya, 2022). The increasing prevalence of drug-resistant human infections to conventional antibiotics, as well as the rise of new infectious diseases, have made the search for novel antimicrobial compounds from naturally occurring sources, such as plants, vital. The past several years have seen a notable increase in the continuous hunt for innovative plant-based drugs to treat this problem.

Pain is a complicated collection of feelings that usually results from a medical condition like an accident or illness. It can cause both mild and severe physical pain as well as psychological misery. About 20% of people worldwide have chronic illness diagnoses and 30% of adults globally suffer from inflammatory disorders and pain, both of which are problems that are steadily becoming more common. Due to their poor potency and adverse effects, analgesic medications such as opiates and nonsteroidal anti-inflammatory medicines (NSAIDs) are currently not very helpful. As such, it is essential to investigate alternate options. In the search for novel analgesic medications, investigating plants historically used to treat pain remains a viable and sensible research strategy.

*D. turbinatus* Gaertn. tree (also known as Garjan or Gurjan; Family: Dipterocarpacea) is endemic to western India and mainland Southeast Asia. The oleo-resin found on *D. turbinatus*' trunk is used

topically to treat ulcers, ringworms and other skin problems such as dermatitis, wounds and cuts; it also acts as a diuretic and stimulant to mucous surfaces. It has been used to treat gonorrhoea, gleet and rheumatism. (Papia *et al.*, 2016). Given the biomedical significance of *D. turbinatus*, the current study aimed to study the analgesic and antimicrobial activities of ethyl acetate fraction of *D. turbinatus* (EAEDT) leaves. To our knowledge, no recorded study has been conducted on the phytochemical analysis of this particular species utilising the ethyl acetate fraction. A prior research examined the antioxidant, antibacterial, antidiabetic, cytotoxic, anti-inflammatory, anthelmintic, thrombolytic, antidiarrheal, and antipyretic properties of *D. turbinatus* bark and leaves by the use of methanolic extracts. Current data indicates a scarcity of study on the pharmacological effects of the ethyl acetate fraction derived from *D. turbinatus* leaves. The objective of this work was to investigate the pharmacological properties of the ethyl acetate extract of *D. turbinatus* (EAEDT) leaves, with a special emphasis on its antibacterial activities in laboratory settings and its ability to reduce pain in living organisms. The objective of this research was to provide empirical information about the conventional use of this botanical species.

## Materials and Methods

The materials and catalysts, which include formalin, n-hexane, methanol and ethyl acetate were obtained from Sigma Chemical Company, which is based in St. Louis, Missouri, in the United States. The medications Nalbuphine HCl and Kanamycin were purchased from Bangladesh's Incepta Pharmaceuticals Ltd. BDH Chemicals in Leicestershire, UK, provided Tween-80, while Social Marketing Company (SMC) Ltd. in Bangladesh provided a standard saline solution (0.9% NaCl). Via Taj Scientific Ltd., Bangladesh, all other analytically grade chemicals were procured locally.

*Animals:* Swiss albino mice weighing between 22 and 30 grammes at 6-7 weeks of age were purchased from Jahangirnagar University's animal

research division in Dhaka, Bangladesh. The mice were kept in a controlled environment with a 12:12 light and dark cycle, where the humidity was kept between 55% and 65% and the room temperature was kept at  $23 \pm 2$  degree Celsius. A week prior to the study, each mouse was provided access to freshwater and a typical laboratory meal to help them adjust to their new surroundings. Furthermore, the mice were fasted for an entire night before the experiment. To conduct the *in vivo* investigation, animals were divided into four groups (Group I-IV) where each group consists of five animals ( $n = 5$ ). The treatment protocol was designed as follows: Group I, received the vehicle (Normal saline, 10 mL/kg b.w., p.o.), Group II, received the standard drug (Nalbuphine HCl/ acetyl salicylic acid 10 mg/kg b.w., p.o.), and Group III and Group IV, received the EAEDT 200 and 400 mg/kg b.w., p.o., respectively.

*Ethical considerations:* The idea was approved by the departmental planning and development committee of the Department of Pharmacy at International Islamic University in Chittagong, Bangladesh. The trials were carried out at the Department of Pharmacy at International Islamic University in Chittagong, Bangladesh. These experiments did not include any surgical procedures or scarification. The animal research was based on observation or behaviour. The 'National Animal Care Laws' and the 'Principles of Laboratory Animal Care' (NIH publication no. 85-23, amended 1985) were followed while handling the experimental animals.

*Acute toxicity analysis:* Mice were maintained in good physical condition throughout adaptation in the lab settings prior to beginning the toxicity investigation. The investigation was conducted with minimal deviations from the prescribed methodology, according to OECD requirements (Al-Araby *et al.*, 2020, Uddin *et al.*, 2020). The male Swiss albino mice were split into seven groups; comprised of six animals in each group. The test groups were given various doses (200, 400, 800, 1600, 3000, and 4000 mg/kg, p.o.), whereas the control group received tween-80 (1%) in saline water. Over up to 14 days straight, the animals were carefully examined over

the following 48 hours to detect any acute toxicological clues, such as mortality, allergic responses as well as behavioral abnormalities.

*Plant materials:* The *D. turbinatus* leaves were gathered in Chakaria, Chittagong, near the Dulahazara Safari Park. The leaves were then identified by the Taxonomist Dr. Sheikh Bokhtear Uddin, Professor, Department of Botany, University of Chittagong. The leaves were shade-dried and powdered using a mechanical grinder (Sieve No. 10/44). A voucher specimen has been deposited at the Department of Pharmacy, International Islamic University Chittagong, Bangladesh.

*Plant extraction and fraction preparation:* After the leaves were allowed to air dry in the shade, a mechanical grinder was used to crush them into a coarse powder. A mass of 351.82 grammes of powder was soaked in 1000 millilitres of methanol and allowed to stand at room temperature for two weeks, stirring occasionally. After that, the extract was divided by running it through a funnel filled with Whatman No. 1 filter paper. A rotary evaporator was used to evaporate the filtrate at a temperature of  $40^{\circ}\text{C} \pm 5^{\circ}\text{C}$ . This produced 16.87 grammes of extract with a 4.795% w/w yield. Solvent-solvent partitioning of crude extracts was carried out using the Kupchan partitioning method (Kupchan *et al.*, 1973). To make the preliminary solution, the crude extract (5 g) was dissolved in a 10% methanol in water (9:1 v/v) solution. The solvents distilled water, ethyl acetate and chloroform were subsequently used in order to split this solution into three portions. A rotary evaporator was used to evaporate the solvent, resulting in the drying of the produced fractions. The extracts were stored in a firmly sealed jar at  $4^{\circ}\text{C}$  until they were required for further use. The portion containing ethyl acetate weighed 1.125 grammes. The formula below was used to get the yield.

$$\% \text{ of yield} = \frac{\text{Weight of particular extract}}{\text{Total amount of coarse powder}} \times 100$$

*Antimicrobial activity test by disk diffusion technique:* The strains of bacteria that were used in

this investigation were *Salmonella typhi*, *Bacillus azotoformans*, *Escherichia coli*, *Lactobacillus casei*, *Corynebacterium sp.*, and *Bacillus cereus*. We performed an in vitro antibacterial investigation using the disc diffusion approach. Before making the inoculum, the bacteria were subcultured for one day. An autoclave was used to generate and sterilise a nutrient-agar medium while maintaining sterility. The medium was then poured into the required number of sterile test tubes, 5 ml at a time, to form scones. Transferring the test organisms from the pure culture to the agar slants was done using a sterile platinum loop. To guarantee that the test organisms grew, the slants were then incubated for 24 hours at 37 °C (Sayeed *et al.*, 2017). The solution was made by dissolving the extract in methanol (MeOH) at 10 µg/ml concentration. Discs of filter paper measuring 5 mm in diameter were created and the moisture was removed. The extract solution was applied separately to the discs using a micropipette, with concentrations of 100 µg/disc, 300 µg/disc and 500 µg/disc. The discs were left momentarily to facilitate complete solvent evaporation. The method used included the use of a Kanamycin disc of 30 µg in diameter. The test organisms were transferred from the sub-culture to the test tube using a sterilised platinum loop and deposited in 20 ml of medium. The test tubes were vigorously shaken to produce a uniform mixture of the organisms. The bacterial suspensions were immediately transferred to the sterile petri dish and centrifuged many times, in both clockwise and anticlockwise directions, to achieve a homogeneous dispersion of the test organisms. The test material was administered to the sample discs separately, while standard antibiotic discs were meticulously placed on agar plates that had been freshly inoculated with the test organisms. The task was accomplished by using aseptic forceps to guarantee complete contact with the surface of the medium. The arrangement of the discs guaranteed a minimum separation of 15 mm from the margins of the plates and a suitable distance to prevent any overlap with the zone of inhibition. Subsequently, the plates were inverted and put in an incubator maintained at a

temperature of 37°C for a period of 24 hrs (Chowdhury *et al.*, 2014).

*Formalin-induced paw licking test:* After making some modifications, the previously published protocol was used to evaluate the test on Swiss albino mice. As stated in the section on experimental design, the animals received their prescribed medications thirty minutes prior to the trial's commencement (Okokon *et al.*, 2017). Following a 30-minute interval, a microsyringe was used to administer a 20 µl dose of a 2.5% formalin solution into the sub-plantar area of the mice's right hind paw. Subsequently, the animals were placed in a glass enclosure for the purpose of observation. The licking and biting of the injected paw were responses to pain. The first 5 minutes were identified as the neurogenic phase, and the next 15–30 minutes were classified as the inflammatory period. The proportion of inhibition of licking was determined by using the following equation:

$$\% \text{ of inhibition of licking} = \frac{M_c - M_t}{M_c} \times 100$$

Where  $M_t$  = mean test and  $M_c$  = mean control.

Most of the research was observational or behavioral in nature, and this trial did not involve any surgical procedures or scarification. The tail immersion method is a technique used to measure the response of an animal's tail to a stimulus. The mice used for this experiment were separated into four groups, each consisting of five animals. Groups 3 and 4 were given a pre-treatment of the extract orally, with doses of 200 and 400 mg/kg, respectively. Group 1, on the other hand, got a standard saline solution of 10 mL/kg. Group 2 was administered Nalbuphine HCl at a dosage of 10 mg per kg which served as the reference standard. At the time intervals of 30, 60, and 90 minutes after drug delivery, each mouse was securely kept in an appropriate restraint, with its tail projecting outward. A 3 cm section of the tail was identified, tagged, and placed into a water bath that was kept at a constant temperature of 55 °C. Almost instantaneously, the mouse responded by retracting its tail. The length of time it took for the mouse to withdraw its tail from the water was monitored. The time was recorded as 0 minutes when

the extract was not administered orally (Zhang *et al.*, 2024).

**Statistical study:** The data was analysed using GraphPad Prism Version 5.0 (GraphPad Software Inc., San Diego, CA) and presented as the mean  $\pm$  standard error mean (SEM). Dunnett's test and one-way analysis of variance (ANOVA) were used to observe the statistical variance. p-values are classified as statistically significant if they are less than 0.05, moderately significant if they are less than 0.01, and very significant if they are less than 0.001.

## Results and Discussion

**Acute toxicity analysis:** The experimental animals were safe and able to sustain EAEDT

dosages up to 4000 mg/kg after prolonged monitoring. Throughout the trial, we observed no discernible changes in body weight, behavior, frequency of stools, food and water consumption, toxicity or death.

**Antimicrobial activity:** The antibacterial efficacy of the EAEDT fraction was assessed against seven infectious microorganisms. The extract was evaluated at doses of 100  $\mu$ g/disc, 300  $\mu$ g/disc and 500  $\mu$ g/disc. The results were compared to the conventional antibiotic Kanamycin (30  $\mu$ g/disc) by measuring the diameter of the zone of inhibition (Figure 1) in millimetres (mm). The findings demonstrate that the extract has significant antibacterial activity presented in table 1.

**Table 1. Antimicrobial effect of ethyl acetate extract of *D. turbinarius* leaves (EAEDT).**

Groups	Test organisms	Diameter of zone of inhibition (mm)			
		EAEDT-100 $\mu$ g/disc	EAEDT-300 $\mu$ g/disc	EAEDT-500 $\mu$ g/disc	Kanamycin-30 $\mu$ g/disc
Gram positive	<i>Lactobacillus casei</i>	16	18	24	33
	<i>Corynebacterium sp.</i>	14	19	25	34
	<i>Bacillus cereus</i>	14	18	24	34
Gram negative	<i>Staphylococcus aureus</i>	16	18	22	36
	<i>Escherichia coli</i>	15	18	23	32
	<i>Bacillus azotoformans</i>	16	19	25	35
	<i>Salmonella typhi</i>	12	18	24	34

**Anti-nociceptive activity: Formalin-induced paw licking test:** Table 2 displays the results of analgesic action as assessed by formalin-induced hind paw licking in mice. Mice receiving *D. turbinatus* ethyl acetate extract (200 and 400 mg/kg) showed a dose-dependent decrease in the amount of formalin-induced hind paw licking. Compared to the control, the zone of inhibition's width (mm) showed statistically significant (\*\*p<0.01) inhibition of the late phase in comparison to the standard.

**Tail immersion method:** The analgesic activity result is determined by tail immersion method. In this method, the extract at 400 dose showed significant (\*p<0.05) response compared to control (Table 3).

In contrast to synthetic pharmaceuticals, which are always a worry in cases of chronic sickness, plant-derived therapies have shown over time to be somewhat more dependable and safe (Dubale *et al.*, 2023). Traditional medicine uses many compounds from plants to treat infectious and chronic illnesses (El Hachlafi *et al.*, 2023). In an effort to identify novel sources of antimicrobial lead compounds, the extracts' antibacterial activity was assessed. This is due to the fact that almost all conventional antibiotics are developing resistance against various bacterial strains and showing more side effects than the standard therapies (Uddin *et al.*, 2021). Antimicrobial-resistant bacteria are responsible for around 700,000 fatalities worldwide each year. The

disc diffusion technique is frequently employed in busy clinical microbiology laboratories due to its simplicity and ability to test many antimicrobial medicines on each bacterial isolate (Erhonyota *et al.*, 2023). The antibacterial action measured using the disc diffusion technique revealed a higher value that was more in line with the conventional medication (kanamycin). In this new study, the *D. turbinatus* leaf extract effectively stopped the growth of *Salmonella*

*typhi*, *Lactobacillus casei*, *Corynebacterium* sp., *Bacillus cereus*, *Staphylococcus aureus* and *Escherichia coli*. The antibacterial activity of the extract or its fractions may be attributed to the inhibition of bacterial cell wall construction, suppression of bacterial protein biosynthesis, blockage of bacterial DNA replication and inhibition of bacterial folic acid metabolism (Rashid *et al.*, 2023).

**Table 2. Effect of ethyl acetate extract of *D. turbinatus* on formalin induced pain in Swiss Albino mice.**

Treatment	Dose (ml/kg)	0-5 Min	% Inhibition	15-30 Min	% Inhibition
Normal saline	10	35.46 ± 0.19	-	26.67 ± 1.91	
Acetyl salicylic acid	10	8.83 ± 0.43	75.098	3.32 ± 0.62**	87.55
EAEDT	200	20.49 ± 4.41	42.22	2.73 ± 1.51**	89.76
EAEDT	400	26.05 ± 5.29	26.54	1.69 ± 1.64**	93.66

Values implies as mean ± SEM; (n = 5). \*\*p<0.001 as compared with control (One way ANOVA followed by Dunnett's t test).

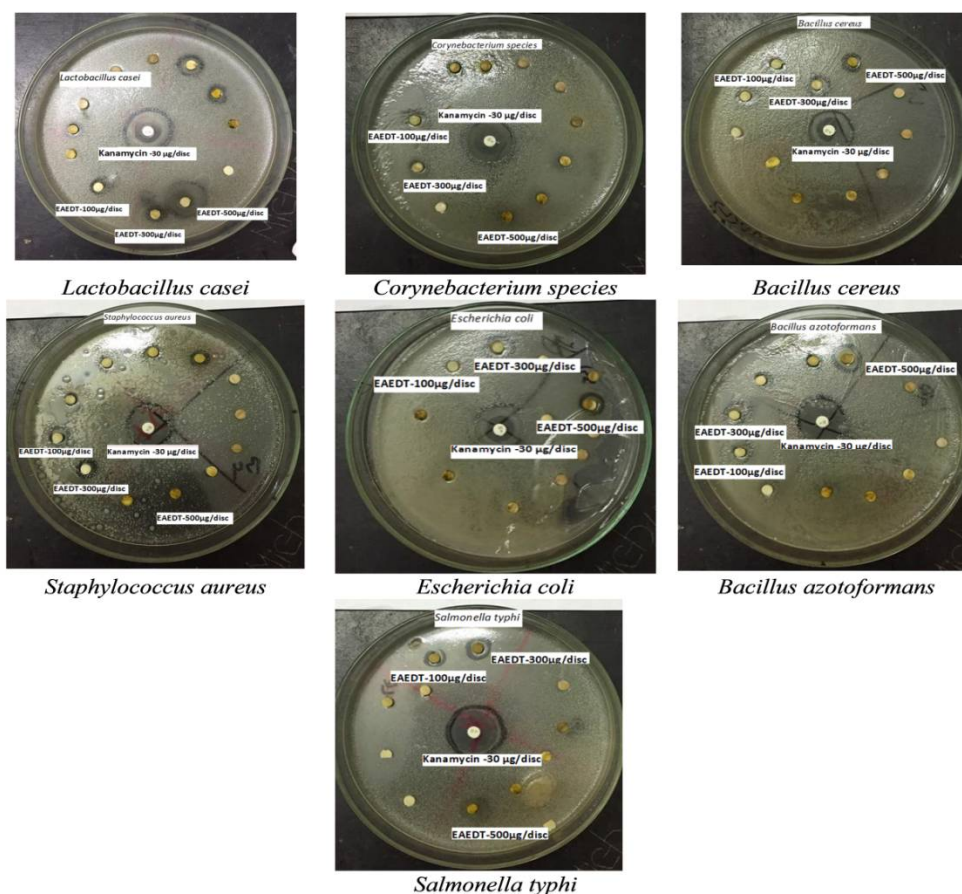


Figure 1. Antibacterial effects of EAEDT and standard drug against different microorganisms.

**Table 3. Effect of EAEDT on tail immersion in Swiss Albino mice.**

Group	Treatment	Dose	Reaction time in seconds at time (minutes)			
			0 min.	30 min.	60 min.	90 min.
Control	Normal saline	10 ml/kg	2.21 ± 0.05	2.5 ± 0.09	2.9 ± 0.18	2.39 ± 0.09
Standard	Nalbuphine HCl	10 mg/kg	2.27 ± 0.07	6.05 ± 0.24**	9.23 ± 0.20**	11.23 ± 0.52**
Extract	Ethyl acetate	200 mg/kg	1.34 ± 0.28	1.49 ± 0.29	1.47 ± 0.33	1.01 ± 0.24
Extract	Ethyl acetate	400 mg/kg	0.81 ± 0.06*	1.07 ± 0.18*	1.44 ± 0.24	2.39 ± 0.61

Values are indicated as mean ± SEM; (n = 5). \*p<0.05, \*\*p<0.01 as compared with control (One way ANOVA followed by Dunnett's t test).

NSAIDs are extensively used globally for the treatment of pain and inflammation. Nevertheless, these medications have a detrimental impact on the biological system. Opiate analgesics cause tolerance and dependency, affecting the gastric mucosa, renal, cardiovascular, hepatic and hematologic systems (Day and Graham, 2013). The investigation into the effectiveness of plants used as analgesics in traditional medicine has garnered significant interest due to their potential for reduced adverse effects. The use of animals in studies to learn more about the processes underlying pain and analgesia is growing. The formalin test is a valuable technique for inducing and measuring pain in cats and rats (Dubuisson and Dennis, 1977). Pain intensity is conditional on objective behavioural categories, which are distinctive and displayed constantly by individual animals (Sherif *et al.*, 2024). There are two stages to the exam, and each one may stand for a distinct kind of suffering. Centrally acting analgesics like morphine may suppress the early phase due to their direct actions on nociceptors. On the other hand, medicines that reduce inflammation, steroids and drugs that work centrally help slow down the late phase, which may be caused in part by prostaglandins and an inflammatory response (Emamghoreishi *et al.*, 2022). In our investigation, we observed a substantial inhibitory impact in the late phase compared to the conventional medication (acetylsalicylic acid), suggesting a stronger central nociceptive action of the extract.

The tail immersion test is very subtle to centrally acting drugs (Polomano *et al.*, 2001). The tail immersion test assesses the reaction to a non-inflammatory, acute nociceptive stimulus. During this experiment, the end of the creature's tail is submerged

in hot water to elicit a distressing response. Following the administration of extract at both doses (200 and 400 mg/kg), the reaction time depict a continuous rise for a duration of 90 minutes. This suggests a notable central analgesic effect when compared to the control group. The study demonstrated that the method of animal restraint during the test significantly influenced the results, as a biologist would observe. Factors such as the animal's restraint method and the temperature of the water bath can influence the intensity and duration of a test drug's analgesic effect. Researchers often use the tail immersion test due to its simplicity and lower technical requirements. Researchers also discovered that the immobilisation method and stimulus temperature influence the test's qualitative and quantitative results. Thus, it is reasonable to assume that the EAEDT likely contains secondary metabolites, such as flavonoids, alkaloids, terpenoids, glycosides, and others, which may effectively reduce pain feeling by blocking the production of endogenous chemicals in both the central and peripheral nerve systems (Sahu and Ahmad, 2015).

### Conclusions

The study examined the antimicrobial and analgesic effects of EAEDT using *in vitro* and *in vivo* approaches, respectively. The data suggests that EAEDT may have antimicrobial and analgesic properties, potentially associated with the plant's secondary metabolites. However, further analysis is necessary to identify the relevant chemical components and understand the underlying mechanism of these activities. For these purposes, we can utilise various instrumental techniques such as mass spectrometry, nuclear magnetic resonance, and high-performance liquid chromatography.

## Acknowledgments

This study was supported by the Department of Pharmacy at International Islamic University Chittagong, Chittagong-4318, Bangladesh.

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