***Original Research Article***

# Pharmacological investigation of in-vitro anti-inflammatory, antimicrobial, thrombolytic, cytotoxic and in vivo analgesic activities of *Diospyros malabarica*




## ABSTRACT

**Aims:** The objective of this study was to investigate the effects of leaf extracts from the *Diospyros malabarica* plant made in ethanol on a number of in vitro activities, including antibacterial, anti-inflammatory, cytotoxic, and thrombolytic activities, as well as in vivo analgesic activities.

**Study design:** Due to the pharmacological interest in the chemical constituents of the plant, ethanolic extract of *Diospyros malabarica* leaf (EEDML) was predominantly investigated by in vitro activities, including antimicrobial, anti-inflammatory, cytotoxic, and thrombolytic activities. Whether the variations in its in vivo analgesic efficacy demonstrated when evaluated in research animals are statistically significant.

**Place and Duration of Study:** The Laboratory of Phytochemistry and Pharmacology at the Department of Pharmacy and the Laboratory of Microbiology at Stamford University Bangladesh and the Bangladesh Council of Scientific and Industrial Research, Dhaka, conducted this investigation from January 2022 to June 2023.

**Methodology:** The study employed the Egg Albumin Protein Denaturation Assay to evaluate the anti-inflammatory effects of various doses. The Disk Diffusion Method was employed for conducting an antimicrobial assay. Different tests were conducted to evaluate the effects of thrombolytic and cytotoxic substances. These tests included the Clot Lysis assay and the Brine Shrimp Lethality Assay. In-vivo analgesic testing was conducted using the hot plate method and the acetic acid-induced writhing test.

**Results:** The experimental tests showed significant pain-relieving effects, with inhibition percentages of 46.2% and 66.61% observed at doses of 200 and 400 mg/kg, respectively. The anti-inflammatory test showed a significant inhibition of EEDML at a concentration of 1000µg/mL, with an inhibition rate of 85.45%. The antimicrobial test showed a moderate antimicrobial effect, with a zone of inhibition ranging from 07-15, depending on the microorganism and dosage. The thrombolytic test showed a clot lysis rate of 85.37%, while the cytotoxic test indicated an LC50 value of 1.47.

**Conclusion:** The plant's phytochemical displays strong pharmacological properties, suggesting its potential for drug discovery in diverse areas.


*Keywords: anti-inflammatory, antimicrobial, cytotoxic, thrombolytic, analgesic*

## 1.0 INTRODUCTION

The utilization of medicinal plants has been a significant contributor to the advancement of healthcare in contemporary times. Natural compounds with therapeutic properties have been identified as a valuable source for the development of new drugs. These compounds have been found to serve as a foundation for the creation of novel therapeutic agents. The utilization of plant-based compounds or plants as a source of prescription drugs has been a topic of interest in medicine. In a study published in Phytomedicine, it was reported that around 25% of prescription drugs worldwide are derived from plants or plant-based compounds (Cragg & Newman, 2013). The botanical kingdom has served as a valuable source of inspiration for developing a plethora of pharmaceutical drugs. Paclitaxel, a chemotherapeutic agent, has been derived from the Pacific yew tree (Taxus brevifolia) and has demonstrated efficacy against a range of cancers (Kilmer, 2010). Artemisinin, a sesquiterpene lactone, is an essential constituent in the management of malaria. It is extracted from the plant Artemisia annua, commonly known as sweet wormwood. The significance of artemisinin in the treatment of malaria has been well documented (White, 2008). The utilization of medicinal plants has been recognized as a promising avenue for drug discovery and development due to their abundant bioactive compounds. These compounds have been found to possess significant therapeutic potential, making them valuable resources for the pharmaceutical industry. The opium poppy (*Papaver somniferum*) is known to contain alkaloids that exhibit strong analgesic properties (Ramawat et al., 2009). One such alkaloid is morphine. The presence of terpenoids in various plant species, such as Panax ginseng and Curcuma longa, has been observed to demonstrate significant anti-inflammatory and antioxidant properties (Dawid-Pać, 2013). The potential of flavonoids, which are found in abundance in fruits and vegetables, has been investigated in relation to their anticancer, anti-inflammatory, and neuroprotective effects (Mhalhel et al., 2023). In recent times, the integration of traditional medicinal practices, which predominantly employ the use of medicinal plants, with conventional medicine has garnered significant recognition. The integration of traditional Chinese herbal formulations, such as Artemisia annua and other herbs, into malaria treatment protocols has been documented (Tu, 2016). The use of Ayurvedic herbs has gained significant popularity due to their various therapeutic effects. Among these herbs, Ashwagandha (*Withania somnifera*) and Turmeric have been extensively studied for their potential health benefits (Tripathi et al., 2020). The significance of medicinal plants in the contemporary era is noteworthy due to their potential to stimulate novel drug development, furnish valuable natural compounds, and amalgamate traditional medicine with conventional healthcare practices. In light of these factors, it can be concluded that medicinal plants hold a pivotal position in the field of medicine.

*Diospyros malabarica* is a botanical species classified as a flowering tree, belonging to the family Ebenaceae. It is predominantly found in the South-East region of Asia, where it is native to the local flora. The utilization of fruit extract as a therapeutic agent for the treatment of various ailments, including diabetes, dates back to ancient times (Polash et al., 2022). This evergreen tree has the potential to reach a height of 25 meters and has a crown that is densely packed with glossy, dark green leaves. *Diospyros malabarica*, commonly known as the Malabar persimmon, produces diminutive, spheroidal drupes that undergo a color transformation from green to yellow or orange upon reaching maturity. They possess the quality of edibility and are characterized by a taste profile that is both sweet and tangy. The consumption of fruits is widely accepted in the surrounding region, and they are also utilized in traditional medicine due to their diverse medicinal properties. The subject under investigation is recognized to possess antioxidant, antimicrobial, and anti-inflammatory properties (Moniruzzaman et al., 2019). The Malabar ebony tree's bark has been a subject of interest in Ayurvedic medicine due to its therapeutic properties. The traditional use of this plant in Ayurvedic medicine has been documented. According to (Kavatagimath & Jalalpure, 2016) the plant in question is purported to possess properties that exhibit anti-diabetic, anti-

inflammatory, and anti-ulcer effects. The present study focuses on the medicinal properties of *Diospyros malabarica* leaves. The leaves of this plant have been traditionally used for treating various ailments such as skin diseases, rheumatism, and digestive disorders.
In the current research, the organic soluble components of a methanol extract of the complete plant were examined for the first time for their in-vitro anti-inflammatory, antimicrobial, thrombolytic, cytotoxic and in vivo analgesic activity. These activities were investigated for the ethanolic leaf extract of the plant

## 2.0 MATERIAL AND METHODS

### 2.1 Sample Collection

In Monohorganj, Cumilla, Bangladesh, a leaf of the *Diospyros malabarica* plant was harvested in July 2022. The specimens were brought in for identification by a Scientific Officer from the University of Dhaka's Botany Department in Bangladesh. For the purpose of research, it has been preserved by submitting a voucher specimen (10815) in the Herbarium.

### 2.2 Plant extract preparation

The plant leaves were suitable for usage after three weeks of air drying at room temperature. The plant parts were dried, ground into powder, sieved, and then kept in plastic containers. A total of 874g of the dried plant was pulverized and then percolated in 1000 mL of ethanol for 24 hours. The extract was then filtered using filtration method with filter papers before being placed in a conical flask. The resultant extracts were concentrated and dried out with a rotary evaporator.

### 2.3 Reagents

Sigma Chemical Co., USA provided methanol, NaOH, diluted HCl acid, concentrated $H_2SO_4$. The sterile saline solution was purchased from Orion Infusion Ltd. Diclofenac sodium was produced by Square Pharmaceuticals Ltd. Vincristine sulphate was purchased from Polysciences, Inc. India. Streptokinase was purchased from Incepta Pharmaceuticals Ltd, Bangladesh. Vin-Cristine Sulphate was sourced from Celon Laboratories Pvt. Ltd., an Indian pharmaceutical company. Gonoshasthaya Pharmaceuticals Ltd. provided morphine sulfate.

### 2.4 In-Vitro Anti-Inflammatory Test

The experimental solution was prepared by combining 0.2 mL of egg albumin obtained from a fresh hen's egg, 2.8 mL of phosphate-buffered saline (PBS) with a pH of 6.4, and 2 mL of an extract with varying concentrations. The final concentrations of the extract were adjusted to 100, 200, 300, 400, and 500 µg/mL. The total volume of the reaction mixture was 5 mL. A control was utilized in the experiment, consisting of an equivalent volume of double-distilled water. Subsequently, the mixtures were subjected to incubation at a temperature of (37°C ± 2) within a BOD incubator manufactured by Lab-Line Technologies for a duration of 15 minutes, followed by a heating process at 70°C for a period of 5 minutes. Following the cooling process, the absorbance of the sample was assessed at a wavelength of 660 nm (utilizing a SHIMADZU UV 1800 instrument) with the vehicle serving as the blank. The reference drug, Acetyl Salicylic Acid, was utilized at varying final concentrations of 100,200, 300, 400, and 500µg/mL. The drug was subjected to a similar treatment for the purpose of absorbance determination (H M Arif Ullah, Sayera Zaman, Fatematuj Juhara, Lucky Akter, Syed Mohammed Tareq, Emranul Haque Masum, 2014). The formula for determining the proportion of protein denaturation that was prevented is as follows:

% Inhibition = $\frac{(Absorbance\ \ of\ control - Absorbance\ \ of\ sample)}{Absorbance\ \ of\ control} \times 100$

## 2.5 Antimicrobial test

### *2.5.1 Microorganisms*

From Microbiology Department of Stamford University Bangladesh and Bangladesh Council of Scientific and Industrial Research, pure culture of fungi (*Penicillium chrysogenum*, *Aspergillus niger, Mucor hiemalis* and *Yeast budding*) and pure culture of Gram-positive (*Staphylococcus aureus, Bacillus megaterium*) and Gram-negative (*Klebsiella pneumonia, Escherichia coli, Pseudomonas aeruginosa*) bacterial pathogens were obtained.

### *2.5.2 Antimicrobial susceptibility test*

In this research, sterile discs with a diameter of 6.0 mm obtained from Becton Dickinson Microbiology System in the United States were utilized to impregnate three distinct dilutions of the extracts. The extract is available in concentrations of 30, 50, and 70 mg/mL.
The discs were subjected to a storage temperature of -5°C prior to their utilization. The disc diffusion method was employed to conduct the tests. Impregnated discs were positioned on agar and subjected to incubation at either 37°C for a duration of 24-48 hours for bacterial growth or at 30°C for a period of 72 hours for fungal growth. The presence of clear zones of inhibition was utilized to determine the antibacterial or antifungal activities (Somchit et al., 2003).

## 2.6 Thrombolytic test

### *2.6.1 Blood specimen*

A specimen of 10 human volunteers who had no history of oral contraceptive or blood thinner use was selected, and 4 mL of whole blood was collected from a vein.
The aforementioned task was executed by a protocol sanctioned by the Institutional Ethics Council of Stamford University Bangladesh. A healthcare practitioner assisted in the extraction of blood from a venous source. Following the collection of the blood sample, 500 µL of the sample was dispensed into each of the 10 microcentrifuge tubes.
2.6.2 Statement on informed consent of the donors
The investigators provided the volunteer participants with a consent form that explicated the purpose of the inquiry, along with the appellation of the research project, the identities of the investigators, and their contact particulars. The research will be accompanied by a comprehensive account detailing the criteria for inclusion and exclusion of donors, the administration or non-administration of therapy to donors, the quantity of blood to be extracted, the probable discomfort of the puncture sites, and the duration of time required for blood sampling.

### *2.6.3 Thrombolysis*

The method used for clot lysis experiments was conducted in accordance with the procedures outlined in a previously published research article (Antioxidant & Mill, 2023), whereby the percentage of total clot lysis was expressed. To summarize, a total of 10 sterile microcentrifuge tubes, each pre-weighed and with a volume of 0.5 mL, were utilized to contain 2.5 mL of venous blood obtained from healthy subjects. The tubes were then subjected to incubation at a temperature of 37°C for a duration of 45 minutes. Following the formation of a clot, the serum was meticulously extracted from the tubes while ensuring that the clot remained undisturbed. The tubes were subsequently re-weighed to determine the weight of the clot, which was calculated by subtracting the weight of the tube from the weight of the tube containing the clot. To ensure proper supplementation, it is necessary to add 100

µL of EEDML to each microcentrifuge tube containing a weighted clot. The lyophilized form of streptokinase, obtained from Incepta Pharmaceutical Ltd. in Dhaka, Bangladesh, was reconstituted using 2.5 mL of phosphate-buffered saline (PBS) and thoroughly mixed. To serve as a positive control, a volume of 100 µL of the aforementioned suspension was introduced into the microcentrifuge tube. Negative control was employed using a volume of 100 µL of distilled water. Following a 90-minute incubation period at 37°C, an assessment was conducted to determine the presence of clot lysis within the tubes. Following incubation, the tubes were re-weighed in order to quantify the extent of weight alteration resulting from the disintegration of the clot. The percentage of clot lysis was determined through the measurement of weight variation prior to and post-clot lysis. The percentage of clot-lysis was calculated and the equation used for this determination:

% of Clot lysis = $\frac{A}{B} \times 100$

Here, A and B represent the weight of released clot before and after treatment.

## 2.7 Cytotoxicity test

### *2.7.1 Brine shrimp lethality test*

The brine shrimp lethality test (Shaira et al., 2023) was employed as the standard bioassay to assess the cytotoxic properties of EEDML, a method commonly used for screening bioactive compounds. The present study utilized *Artemia salina* as a zoological organism model. Initially, the procurement of brine shrimp eggs was carried out by acquiring them from a commercial establishment specializing in pet supplies located in Dhaka, Bangladesh. Following a 48-hour incubation period in a synthetic seawater medium consisting of a 3.8% sodium chloride solution, the brine shrimp successfully hatched and underwent development into larval shrimp, also known as nauplii. The methodology utilized for assessing the cytotoxicity of brine shrimp nauplii involved the application of Meyer's technique. In order to prepare the samples for testing, the EEDML was dissolved in a dimethyl sulfoxide solution with a maximum concentration of 50 µL per 5 mL. To attain concentrations of 0.98, 1.95, 3.91, 7.81, 15.625, 31.25, 62.5, 125, 250, 500µg/mL, a 3.8% NaCl solution (saltwater) was supplemented up to a volume of 5 mL. In this study, the positive control utilized as vincristine sulfate, which is a standard medication. Each test tube was populated with ten fully-grown shrimps. Following a 24-hour incubation period, the vials were subjected to visual inspection utilizing a magnifying instrument to determine the number of viable nauplii. The LC50 value was determined through the utilization of a logarithmic graph plotting the concentration levels against the corresponding mortality rates.

## 2.8 Experimental Animals for In-vivo Pharmacological Investigation

For the purpose of our investigation, a cohort of young and healthy *Swiss albino* mice, with a weight range of 22-30g, was chosen as the experimental subjects.

The mice were procured from the Saver facility of Jahangirnagar University located in Dhaka, Bangladesh. Maintaining the existing state of affairs was deemed crucial. Typical variations in the atmosphere encompass a temperature range of approximately 79°F, a relative humidity level ranging from 55% to 65%, and a consistent 24-hour light/dark cycle. The specimens are subjected to consistent environmental conditions for a period of 9 days subsequent to their collection. In order to aid in the recovery of mice from water and food deprivation incurred during transportation and to facilitate their adjustment to the laboratory setting, a diet consisting of appropriate nourishment and uncontaminated water was provided, in accordance with the guidelines prescribed by Jahangirnagar University. Following a 12-day period of recovery, the mice were deemed prepared for the commencement of the experiment.

## 2.9 Analgesic investigation

### *2.9.1 Hot plate test*

The experiment involved the utilization of a hot plate analgesia meter, specifically Eddy's Hot Plate using the method from previously described paper, to conduct the hot plate test (Nguyen et al., 2020). The mice were assigned to four groups in a random manner, with each group consisting of five mice. The groups were as follows: the model group, the Diclofenac sodium group (5 mg/kg), the low-dose EEDML group (200 mg/kg), and the high-dose EEDML group (400 mg/kg). The nociceptive latency, which refers to the time it takes to respond to a painful stimulus, was measured by observing behaviors such as jumping or paw licking in mice placed on a heated surface at a constant temperature of 55 ± 0.1℃. After administering treatments, the latencies were measured and considered the baseline value. Only mice that exhibited initial pain responses within a time range of 5 to 20 seconds were chosen for inclusion in the experiment. The reaction time of each animal after receiving EEDML or Diclofenac sodium was measured at three-time intervals: 30, 60, and 120-minutes post-treatment. The designated time limit was established at 20 seconds.
The formula for the analgesic effectiveness of treatment was as follows:
Percent Analgesic Score = $\frac{Ta-Tb}{Ta}$ X100.
Time (in seconds) to react (before medication administration): Tb; Time (in seconds) to react (after drug administration): Ta.

### *2.9.2 Acetic-acid induced writhing test*

The experiment involved conducting the acetic acid-induced abdominal writhing test on mice in order to evaluate the analgesic effects of EEDML (Wang et al., 2019). The mice were divided into five groups (n = 5) according to the hot plate test protocol. In this study, the mice were subjected to intraperitoneal administration of diclofenac sodium and EEDML one hour prior to the induction of writhing. The writhing was induced by intraperitoneal injection of 0.6% acetic acid at a dosage of 10 mL per kg of body weight. Individual mice were placed in a spacious glass enclosure, and the frequency of writhing movements was recorded during a 10-minute interval.

## 3. RESULTS AND DISCUSSION

## 3.1 Statical analysis

The bioassay readings were conducted twice, and the tabular data presented represent the mean value. The statistical analyses were performed utilizing the software program Excel.

## 3.2 Anti-inflammatory activity

In this experiment, it has been observed that both MEPLL and EEDML exhibit significant effects when compared to the conventional acetyl-salicylic acid, as demonstrated in Table 1.

**Table 1. Percentage inhibition in egg albumin denaturation of EEDML**

| Concentration | % Inhibition of Acetyl salicylic acid | % Inhibition of Extract EEDML |
|---|---|---|

| | | |
|---|---|---|
| 62.5 | 89.06 | 73.63 |
| 125 | 91.89 | 80.90 |
| 250 | 93.69 | 84.54 |
| 500 | 94.59 | 76.36 |
| 1000 | 98.19 | 85.45 |

The anti-inflammatory effects of *Diospyros malabarica* were evaluated using the anti-denaturation of egg albumin technique. Furthermore, previous studies have demonstrated that conventional nonsteroidal anti-inflammatory drugs (NSAIDs), including phenylbutazone and indomethacin, not only reduce the synthesis of endogenous prostaglandins by inhibiting the COX enzyme but also hinder protein denaturation (Yamamoto et al., 2003). Given the circumstances, the anti-denaturation assay offers a pragmatic approach to assess the presence of anti-inflammatory characteristics. The findings of the current study indicate that the extract possesses strong anti-inflammatory properties. A comparative analysis was conducted to evaluate the effectiveness of *Diospyros malabarica* in regulating auto antigen synthesis and preventing protein denaturation, in comparison to the standard medication salicylic acid.

## 3.3 Anti-microbial activity

The antibacterial and antifungal activity of various dosages of plant extract was evaluated against gram-positive and gram-negative bacteria, as well as four different types of fungi, by measuring the zone of growth inhibition in millimeters (mm). The findings indicate that there was a positive correlation between the concentration of plant extract and the size of the inhibitory zone, as observed in Table 2 and Table 3. The antibacterial activity exhibited a zone of inhibition ranging from 08 to 18 mm, while the zone of inhibition for fungi ranged from 06 to 14 mm. The results presented indicate that EEDML exhibits the highest level of antibacterial activity against Escherichia coli and Bacillus cereus. These bacteria are known to cause various hazardous diseases such as urinary tract infections, respiratory illness, pneumonia, gastrointestinal illness, and diarrhea.

**Table 2. Inhibition zone of EEDML against different bacteria**

| Diameter of Zone of Inhibition (mm) | | | | |
|---|---|---|---|---|
| Test organisms | EEDML (300 µg/disc) | EEDML (500 µg/disc) | EEDML (700 µg/disc) | Ciprofloxacin |
| Gram Positive Bacteria | | | | |
| *Bacillus cereus* | 08 | 09 | 11 | 25 |
| *Staphylococcus aureus* | 08 | 09 | 10 | 26 |
| Gram Negative Bacteria | | | | |
| *Escherichia coli* | 07 | 07 | 09 | 25 |
| *Vibrio cholerae* | 11 | 08 | 08 | 27 |
| *Klebsiella pneumonia* | 07 | 08 | 09 | 24 |

**Table 3. Inhibition zone of EEDML against different fungi**

| **Diameter of Zone of Inhibition (mm)** | | | | |
|---|---|---|---|---|
| Test organisms | MELA | MELA | MELA | Griseofulvin |

| | (300 µg/disc) | (500 µg/disc) | (700 µg/disc) | (50 µg/disk) |
|---|---|---|---|---|
| **Fungi** | | | | |
| *Penicillium chrysogenum* | 07 | 10 | 15 | 19 |
| *Aspergillus niger* | 08 | 09 | 11 | 20 |
| *Yeast budding* | 9 | 11 | 15 | 21 |
| *Mucor hiemalis* | 08 | 10 | 12 | 21 |

The assessment of antimicrobial agents' efficacy necessitates the inclusion of antibacterial and antifungal tests. Antibacterial tests are conducted to assess the capacity of a substance to hinder the proliferation of bacteria, while antifungal tests are performed to evaluate the efficacy of a substance in suppressing the growth of fungi. The agar diffusion method, also known as the Kirby-Bauer method, is widely used for testing antibacterial activity. During the experimental procedure, bacteria are cultivated on an agar plate, followed by the placement of discs containing the antimicrobial agent onto the agar's surface. The magnitude of the zone of inhibition surrounding each disc is indicative of the level of bacterial growth inhibition. The measurement of the zone of inhibition's diameter is utilized to determine the antimicrobial susceptibility of the bacteria. This measurement is then compared with a standard chart.

Antifungal tests can be conducted using the agar diffusion method, which bears resemblance to the Kirby-Bauer method employed for bacterial tests. The agar diffusion method involves the cultivation of a fungal strain on an agar medium. Subsequently, discs containing the antifungal agent are positioned on the surface of the agar. The measurement of zone of inhibition surrounding each disc is utilized to assess the antifungal activity of the substance.

In conclusion, the evaluation of antimicrobial agents' effectiveness relies heavily on conducting antibacterial and antifungal tests. The agar diffusion and broth dilution methods are frequently employed for assessing the extent of bacterial and fungal inhibition or kill rate caused by various substances. These tests play a crucial role in the development and assessment of novel antimicrobial agents, as well as in the identification of appropriate agents for the management of bacterial and fungal infections.

### 3.4 Thrombolytic activity

The efficacy of plant extracts in the dissolution of blood clots is assessed through an in vitro thrombolysis assay conducted under controlled laboratory conditions. In the event of bodily injury, the process of hemostasis is initiated, whereby blood cells and proteins aggregate to form a coagulum, thereby impeding the continuation of hemorrhaging. Nevertheless, an overabundance of clotting can lead to life-threatening conditions such as myocardial infarctions and cerebrovascular accidents. The subsequent table illustrates a notable disparity in the percentage of clot lysis between EEDML and conventional streptokinase.

**Table 4. Mean value of percent of clot lysis (N=10)**

| **Sample** | **% of clot lysis** |
|---|---|
| Negative control | 7.296 |
| Streptokinase | 91.304 |
| EEDML | 85.37 |

Various studies have been conducted to investigate the thrombolytic potential of supplements, herbs, and natural food sources in the treatment of coronary events and strokes. The present study aimed to assess the thrombolytic efficacy of EEDML. The thrombolytic efficacy of EEDML was observed to be rapid, with a value of 85.37%, as

compared to the standard value of 91.304% (Table 4). The observed value is derived from the fact that EEDML reduces the coagulation of human blood in vitro, thereby suggesting its potential as a cardioprotective agent. The EEDML exhibits considerable significance, potentially yielding significant implications for cardiovascular health. Consequently, it might facilitate the development of novel thrombolytic agents derived from *Diospyros malabarica* (Ratnasooriya et al., 2008).

### 3.5 Cytotoxic activity

The brine shrimp lethality assay is a commonly employed bioassay in the field of plant extract evaluation, serving as an initial screening method to assess the biological activity of such extracts. The purpose of this test is to evaluate the toxicity of compounds and assess the cytotoxic properties of plant extracts. The initial stage of plant screening involves the assessment of cytotoxicity, which provides valuable insights into the potential antitumor and anticancer properties of the plant extract. The cytotoxic effects of EEDML on the growth of brine shrimp were investigated.

**Table 5. Brine Shrimp Assay (Mortality %, $LC_{50}$ value)**

| Sample name | Concentrations | Mortality % | $LC_{50}$ value |
|---|---|---|---|
| Vincristine Sulphate | 7.81 | 40 | 20.57 |
| | 15.625 | 40 | |
| | 31.25 | 50 | |
| | 62.5 | 60 | |
| | 125 | 100 | |
| | 250 | 100 | |
| | 500 | 100 | |
| EEDML | 0.98 | 10 | 1.147 |
| | 1.95 | 30 | |
| | 3.91 | 40 | |
| | 7.81 | 40 | |
| | 15.625 | 50 | |
| | 31.25 | 70 | |
| | 62.5 | 70 | |
| | 125 | 80 | |
| | 250 | 100 | |
| | 500 | 100 | |

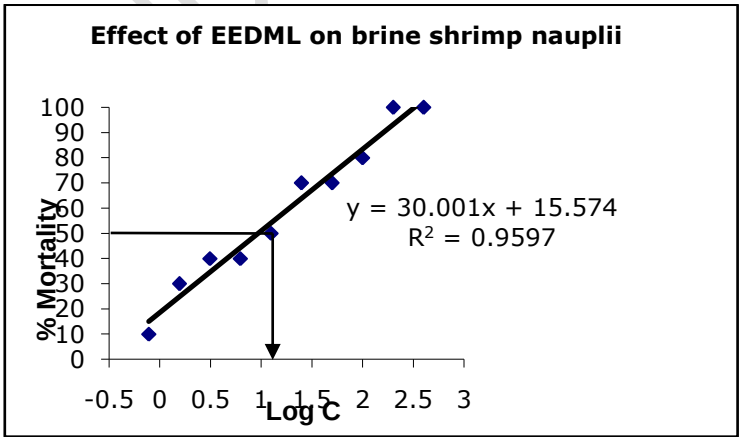

**Fig 1. Cytotoxic activity of EEDML on Brine shrimp nauplii**

The brine shrimp assay represents a cost-effective and straightforward approach to assessing the cytotoxic characteristics of plant extracts. The method employed in this study was used to assess the cytotoxic activity of EEDML. The findings obtained from this analysis have been succinctly presented in Table 4. The LC50 values for EEDML and the standard drug Vincristine Sulphate were determined to be 1.147 µg/mL and 0.608 µg/mL, respectively, as shown in Figure 1 and Figure 2. Furthermore, various concentrations of the test solution exhibited varying levels of mortality in Artemia salina. The LC50 values exhibited a range of 1.95 µg/mL (statistically significant) to 500 µg/mL (highly statistically significant), indicating a clear relationship between concentration and LC50. The highest mortality rate was observed at a concentration of 500 µg/mL, whereas the lowest mortality rate was observed at a concentration of 1.147 µg/mL. It can be posited that there exists a positive correlation between the concentration of the test samples and the percentage of mortality, whereby an increase in concentration leads to a corresponding increase in mortality, and conversely, a decrease in concentration results in a decrease in mortality. In comparison to the standard vincristine sulfate concentration of 0.608 µg/mL, the EEDML demonstrates significant cytotoxicity towards brine shrimp nauplii, as evidenced by its LC50 value of 1.147 µg/mL. The cytotoxicity of MEPSL exhibits notable differences when compared to the standard vincristine sulfate. This finding warrants further investigation to explore its potential as a compound for antitumor and pesticide applications (Suffredini et al., 2006).

## 3.6 In-vivo analgesic activity

### 3*.6.1 Hot plate test*

In this experiment, the EEDML exhibited a significant analgesic activity that varied in potency and dosage dependence when compared to the standard Morphine Sulphate. The analgesic activity of the extract was determined to be slightly more than half of that exhibited by the standard. The average time tolerance values for the extract and standard are provided in Table 6.



**Table 6. Primary Data Table for Hot Plate Test for Plant Extract of EEDML**

| **Reaction time at different time intervals (in sec)** | | | | | |
|---|---|---|---|---|---|
| **Group** | **Average wt. of mice (g)** | **30 min** | **60 min** | **90 min** | **120 min** |
| Control | | 6.4 | 7.6 | 6.0 | 5.4 |
| Morphine (5mg/kg) | | 8.6 | 9.4 | 11.0 | 6.6 |
| EEDML (200mg/kg) | 20 to 26 | 9.6 | 12.6 | 14.8 | 0 |
| EEDML (400mg/kg) | | 12.2 | 13.2 | 0 | 0 |

The hot plate test is a commonly employed experimental paradigm utilized for the identification of compounds that demonstrate centrally acting analgesic effects. Multiple animal species have demonstrated that the hot plate method exhibits selectivity towards opioid-related compounds (Menyiy et al., 2021). There exists speculation regarding the potential role of these molecules in mediating the narcotic analgesic, anti-inflammatory, and anti-diabetic effects. The findings from the experiment indicate that there is a correlation between the dosage of EEDML and the amount of time it takes for thermal pain to be perceived. This suggests that the analgesic effects of EEDML may be attributed to a similar underlying mechanism.

### *3.6.2 Results of Acetic-acid induced writhing test*

In the acetic acid-induced writhing test, the methanolic extracts of Diospyros malabarica leaf exhibited inhibitory effects of 66.61% and 46.2% respectively. The standard drug, Diclofenac-Na, demonstrated an inhibition rate of 80.36%. In comparison to established standards, the findings exhibited a noteworthy level of analgesic efficacy that is contingent upon the dosage administered.

**Table 7. Analgesic Activity of EEDML on Mice by Writhing Test**

| Administered Substance | Dose | % Writhing | % Of Inhibition |
|---|---|---|---|
| Control | 10mL/kg | 100 | 0.00 |
| Diclofenac sodium | 10mg/kg | 20.40 | 79.61 |
| EEDML | 200mg/kg | 53.80 | 46.2 |
| EEDML | 400mg/kg | 33.39 | 66.61 |

The extract of Diospyros malabarica demonstrated a significant and dose-dependent reduction in abdominal writhing during an acetic acid-induced abdominal constriction test. The efficacy of the test in assessing the effectiveness of moderate analgesic non-steroidal anti-inflammatory drugs (NSAIDs) is attributed to the indirect mechanism of acetic acid. Acetic acid is believed to induce the release of prostaglandins and lipo-oxygenase products into the peritoneum, thereby activating nociceptive neurons that are responsive to NSAIDs. The findings from the experiment involving acetic acid-induced writhing provide compelling evidence that the observed effect is partially associated with the inhibition of lipo-oxygenase and/or cyclooxygenase in the peripheral tissues. This inhibition leads to a reduction in prostaglandin synthesis, which in turn disrupts the transduction mechanism in primary afferent nociceptors.

## 4.0 CONCLUSION

The methanolic extract of *Diospyros malabarica*'s leaf has been found to possess anti-inflammatory, antimicrobial, thrombolytic, cytotoxic, and analgesic properties, leading to the inference that it may have potential therapeutic applications in these areas. The dose-dependent anti-inflammatory effect of this plant is notably significant when compared to the standard. The leaf extract demonstrated a minimal level of antimicrobial activity. The thrombolytic effect exhibited by the extract derived from this plant was found to be highly significant. The findings from the bioassay conducted on the lethality of brine shrimp indicated a significant level of cytotoxicity. Both the writhing test and the hot plate test provide evidence of the analgesic effects exhibited by this plant. Furthermore, it has been established that administering a higher dosage yields greater efficacy compared to a lower dose.

## 6.0 ETHICAL APPROVAL

The authors declare that all experiments were carried out in adherence to ethical guidelines and underwent review by a suitable ethics committee.

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