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Phytochemical Screening and Evaluation of *In Vitro* Thrombolytic, Anthelmintic and *In Vivo* Analgesic Activities of Ethanolic Extract of *Leea Aequata*L.

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Abstract:
Objectives: *The purpose of this study was to determine the in vitro anthelmintic, thrombolytic, and in vivo anti-nociceptive capability of crude ethanolic extract of L. aequata leaves, as well as their conceivable phytochemical composition (group determinant of plant extract).*
Materials & Methods: *A series of qualitative tests screened the extracted material for chemicals. Human blood clot lysis was employed to investigate thrombolytic activity in vitro. Roundworm paralysis and mortality rates were compared to albendazole to establish anthelmintic effectiveness. Anti-nociceptive effectiveness was assessed in vivo using acetic acid-induced writhing and formalin-induced paw licking.*
Results:*Phytochemical analysis of the crude extract revealed reducing sugars, alkaloids, carbohydrates, glycosides, phenol, tannin, and proteins. Significant thrombolytic characteristics were observed in the plant compared to the negative control ($p<0.05$) (water). The plant showed substantial clot lysis (89.69 ± 2.31%). The paralysis times for 5, 8, and 10 mg/ml were 21.33, 18.67, and 11.67 minutes. The following concentrations died in 42.33, 37.33, and 31.67 minutes. Compared to the control group, the mean writhing and inhibition rates for 200 and 400 mg/kg doses were 9.67±1.76 (84.15%) and 11.33±0.88 (81.42%), respectively. EELA at 200 and 400 mg/kg significantly reduced formalin-induced pain response in both neurogenic (5 min) and inflammatory (15-30 min) phases ($p < 0.05$).*
Conclusion:*The current study suggests this herb may be an anthelmintic, thrombolytic, and anti-nociceptive, supporting its traditional applications.*
Keywords:*Phytochemical Screening, Anthelmintic, Thrombolytic, Anti-nociceptive activity.*

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I. INTRODUCTION

In modern times, medicinal plants are thought to be potential sources of novel medications. In order to discover new medications for various disorders, scientists are screening medicinal plants. Folk medicine practitioners in Bangladesh directly administer medicinal herbs as first-line treatments[1] .To serve as an alternative to the plant's natural constituents, several chemicals have been extracted from a variety of medicinal plants. These chemicals exhibit various actions for producing drugs that are more potent and have fewer negative side effects than the aberrant health of humans or other animals that currently exists[2]. When homeostasis is lost, thrombus that forms in the circulatory system results in vascular blockage, atherothrombotic diseases, myocardial or brain-localized necrosis, and eventually death. Because there is a significant danger of bleeding inside the brain, a severe case lacks specificity and exhibits an allergic reaction, gastrointestinal hemorrhage, or hypertension. There have been significant attempts made to find and create natural products in addition to Streptokinase and Urokinase. Warfarin and other oral anticoagulants are made from coumarin, which is present in a variety of plants[3]. The idea of testing medicinal herbs for their anthelmintic activity resulted from growing issues with the development of resistance in helminths to anthelmintics[4]. WHO has set a goal of providing anthelminthic treatment to at least 75% of school-age children in endemic areas on a regular basis[5]. As a sensory modality, pain frequently serves as the sole symptom used to diagnose a number of disorders[6]. In addition to interfering with our regular activities, pain increases absenteeism from work, underemployment, and unemployment, which causes a significant economic loss for the person[7]. While steroidal and opioid

medications are used to treat acute and chronic pain, non-steroidal anti-inflammatory drugs are advised for the management of mild to moderate pain[8]. Leea is a member of the Vitaceae family. Leea species range from Africa to Asia, including northern Australia, New Guinea, and the Pacific islands (Fiji, the Solomon Islands, and the Caroline Islands)[9]. Certain species are employed as conventional folk remedies. For instance, *L. thoreliiGagnep.* roots are used as a tonic in Thailand[10], *L. guineense* G. Don leaves are used to treat cancer in Guinea[11], and *L. asiatica* (L.) Ridsdale roots are used to treat icteric hepatitis in China[12]. *Leea aequata* is a tiny tree that grows in Bangladesh, Bhutan, Cambodia, China, India, Malaysia, Myanmar, Nepal, the Philippines, Thailand, and Vietnam[12]. Leea aequata is extensively spread in Bangladesh's peripheral woods, including Chittagong, Cox's Bazar, Moulvibazar, and Sylhet. Previous studies found antibacterial activity in the seeds, stems, and roots of *L. aequata*[13].

Hence, the current study aims to investigate the in vivo analgesic effect of an ethanolic extract of *Leea aequata* leaves in Swiss albino mice, as well as the in vitro anthelmintic and thrombolytic activities.

II. MATERIALS & METHODS

Preparation of plant extract

The *Leea aequata* leaves were authenticated by Professor Dr. Sheikh Bokhtear Uddin, Department of Botany, University of Chittagong, (Specimen No: MS-020422-9174) after they were collected from the Hazarikhil Wildlife Sanctuary, a wildlife sanctuary in the Ramgarh-Sitakunda forests 45 km north of the Chittagong port in south-east Bangladesh. We only used newly harvested *L. aequata* leaves in our experiments. They were then broken down into smaller pieces so that they could be ground. Then the crude portions were rubbed for 10 days and then grounded by utilizing a mechanical grinder (NOWAKE, Japan). For 14 days at room temperature, the powder (180 g) was submerged in 500 ml of 96% ethanol while being periodically stirred and shaken using a shaker machine. Afterward, it went through a cotton plug and Whatman filter paper. The ethanolic extract of *L. aequata* (EELA) (6.7 g) was obtained by filtering the filtrate and then evaporating it at 50°C under decreased pressure in a water bath machine (Labline, India). The extract was stored in the fridge at 4 degrees Celsius until needed.

Experimental animals

From the International Center for Diarrheal Diseases Research (ICDDRB) in Dhaka, Bangladesh, we obtained (six- to seven-week-old) Swiss Albino mice of both sexes. For seven days prior to the experiment, the animals were accustomed to the laboratory setting (25±2°C with a light/dark cycle of 12 h). Research was carried out with permission from the Institutional Animal Ethical Committee of the School of Pharmacy at the International Islamic University Chittagong in Bangladesh, reference number [P&D-147/13-18].

Worm sample

The earthworms (*Pheretimaposthuma*) were taken from an aquarium shop in Jamal Khan, Chittagong and cleansed with normal saline to eliminate all the dirt. All of the procedures relied on earthworms measuring between four and six centimeters in length, one-tenth of a centimeter in width, and weighing between eight and four grams. For this reason, anthelmintic activity was studied using earthworms, which are anatomically and physiologically similar to the worm parasites that live in human intestines.

Phytochemical screening

Several qualitative tests were used to determine the presence of chemical ingredients through a phytochemical screening of the produced extract. The qualitative phytochemical screening of the ethanol extract of *L. aequata* leaves was performed using a standard technique in order to determine the alkaloids, carbohydrates, glycosides, proteins, oxalate, cholesterol, steroid, reducing sugar, tannin, flavonoids, saponins, and phenolic compound[14]. See Table (1).

Thrombolytic activity

Streptokinase was used as a reference standard to determine the thrombolytic activity of thisextraction.The 10 mg of dry crude extract was mixed with 10 ml of distilled water and allowed to sit overnight.The filtrate of the soluble supernatant was then decanted. Blood was taken from the veins of healthyvolunteers and then divided into five equal aliquots (5 ml each), with each aliquot placed in aseparate sterile microcentrifuge tube (1 ml each tube), and incubated at 37 °C for 45 minutes. Eachtube with a clot was weighed again after the serum was carefully drained off without disturbing theclot to get the clot weight. The clot was preweighed, and then 100 µl of aqueous solutions of variouspartitionates and the crude extract were added to each microcentrifuge tube. To serve as a positiveand negative non-thrombolytic control, 100 ul of streptokinase (SK) and 100 ul of distilled waterwere added to the respective control tubes. After incubating the

tubes at 37 degrees Celsius for 90minutes, we checked for clot lysis. After the clots had been disrupted and the fluid had beenevacuated, the tubes were weighed again to see if there had been any change in weight[15].

$$Clot\ lysis\ \% = \frac{Weight\ of\ lysate}{Weight\ of\ clot\ before\ lysis} \times 100$$

The weight of blood clot was calculated using the equation as followed:
Weight of clot (g) =Weight of tube containing blood clot – Weight of empty tube

Anthelmintic activity

An evaluation of anthelmintic activity was performed on roundworms. As a standard, we added albendazole solution (10 mg/mL). Petri dishes of 5 cm^3 volume were filled with 10 mL of each concentration, and twelve worms were added to each plate. Both the time it took for the worms to become paralyzed (P) and die (D) were recorded. In order to confirm the worms were dead, we dipped them in hot water at 50º Celsius, which would have caused them to wiggle if they were still alive. The placebos (control solution) were 10 mL of distilled water. Distilled water was used for all solutions, and for each concentration of sample, reference, and control, two separate plates were prepared. Means and standard errors were used to describe the data[16].

Anti-nociceptive Activity

Acetic acid induced writhing test:

Acetic acid-induced writhing in mice was used to evaluate the analgesic properties of EELA followed by the method of Adnan et al[17]. Four sets of experimental animals, each consisting of three mice, The first group (Control) received 1% Tween-80 in distilled water, the second group (Standard) received 10 mg/kg of diclofenac sodium, and the third and fourth groups (the EELA groups) received 200 and 400 mg/kg of EELA, respectively. Both the test and diclofenac groups were given oral medication for 30 and 15 minutes, respectively, before receiving an intraperitoneal administration of acetic acid (0.6% v/v) to induce abdominal contractions. After injecting acetic acid, we watched for 25 minutes to see how many times each animal contracted and then extended its trunk and hind limbs, a behavior known as "writhing." For full writhing, two half-writhes were added together, while a single half-writhe was recorded for partial writhing.

Writhing's in the control group were considered to be 100%, and the percentage of inhibition was calculated as follows:

$$\%\ of\ Inhibition = \frac{\text{Total number of writhing (control} - \text{test group)}}{\text{Total writhing number of the control}} \times 100$$

Formalin induced paw-licking test

All mice received 20 µl of formalin solution (2.5% v/v) subcutaneously in their right hind paws to produce discomfort. Two test groups (n = 3) received EELA (200 and 400 mg/kg,); the control group got vehicle (1% Tween-80 in distilled water); and the reference group received morphine (5 mg/kg body weight,). The pain response is assessed in seconds of licking and biting following formalin treatment during the first 5 minutes (neurogenic pain) and afterwards, at 15 to 30 minutes (the second phase, inflammatory pain). The percentage% inhibition of licking time measured anti-nociceptive efficacy[17].

Statistical analysis

The study data were presented as mean ± standard error, with $p < 0.05$, $p < 0.01$, and $p < 0.001$ indicating statistical significance. Using GraphPad Prism version 8.4 (GraphPad Software Inc., San Diego, CA, USA), one-way analysis of variance (ANOVA) (Dunnett's test) was used to compare the test groups to the negative control (1% Tween-80).

III. RESULTS

Phytochemical screening

Some Phyto constituents such as Alkaloid, reducing sugar, Phenols were detected in an ethanolic extract of Leea*aequata*.

Serial	Phytochemicals	Name of the test	Observation
1	Protein	Millon's Test	++
		Xanthoproteinic Test	++

2	Reducing Sugar	Benedict Test	++
		Fehling Test	+
3	Alkaloid	Wagner's Test	++
		Mayer's Test	++
4	Phenol	Lead Acetate Test	++
		Iodine Test	++
5	Phlobatanins	HCL Test	++
6	Anthocyanin	HCL Test	+
7	Resin	Acetic Anhydrous Test	-
8	Tannin	Phlonatannin Test	-
		General Test	-
9	Glycoside	H_2SO_4 Test	-
10	Saponin	Foam Test	-
11	Carbohydrate	Barfoed Test	+

Table 1: Phytochemical constituents found in *Leea aequata.*

(+ +) = Abundantly Present, (+) = Present, (-) = Absent

Thrombolytic activity

Clot lysis activity measured at (89.67 ± 2.31%) was significantly high for the *L. aequata* ethanolic extract (EELA). Streptokinase, the positive control, caused (48.86 ± 2.79%) clot lysis[15], while the non-thrombolytic control, pure water, only caused (14.43 ± 3.97%) clot lysis (Figure 1).

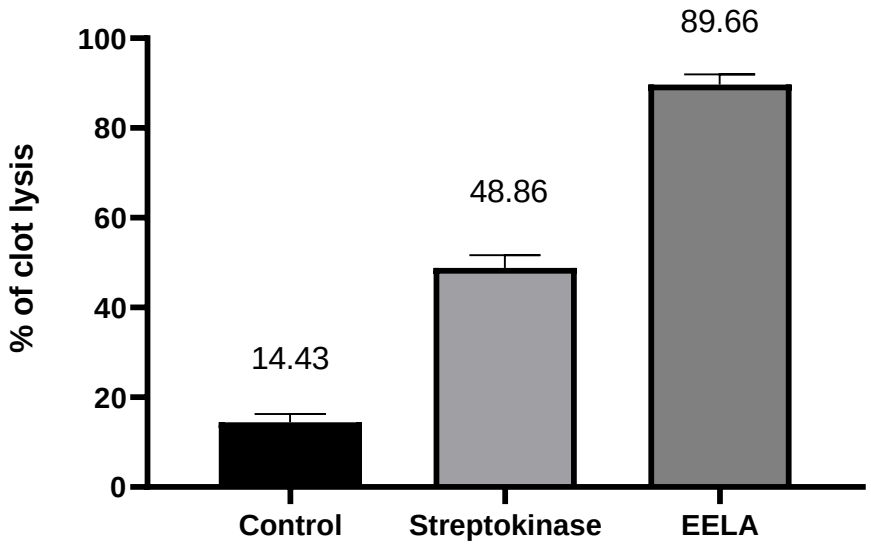


Figure 01: **Graphical representation of the thrombolytic activity of EELA. The values are displayed as the Mean ± SEM.**

Anthelmintic activity

When compared to the conventional albendazole (10 mg/ml), the ethanol extract (conc. 5, 8 and 10 mg/ml) of leaves of *L. aequata* displayed a substantial impact on paralyzing the worms in terms of paralysis period at all concentrations (Figure 2).

Table 2: Anthelmintic activity of EELA (n=12).

Group	Paralyze time (min)	Death time (min)
Control	0	0
Standard 10 mg/ml	55.3	76.3
EELA-5 mg/ml	21.33	42.33
EELA-8 mg/ml	18	37.33
EELA-10 mg/ml	11.67	31.67

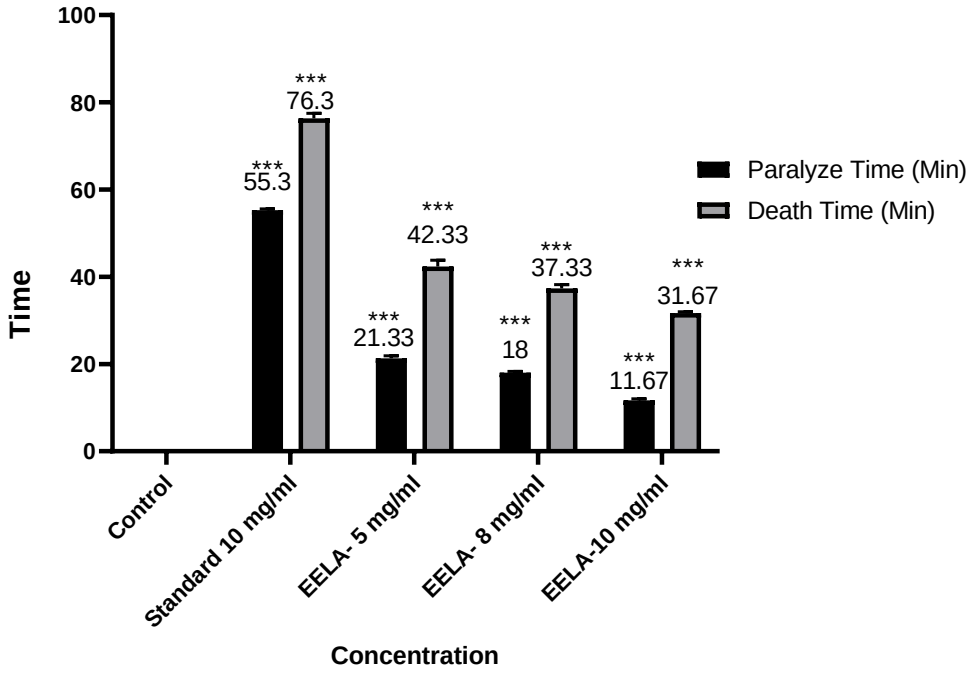


Figure 02: Graphical representation of Anthelmintic activity of *L. aequata*. ***P<0.05 considered as significantly different from the control (Dunnett's test).

Anti-nociceptive Activity

Acetic acid induced writhing test

Treatment with oral EELA (200 and 400 mg/kg) decreased acetic acid-induced writhing in a dose-dependent manner ($p<0.05$). When compared to the control group, the mean number of writhing and percentage inhibition for dosages of 200 and 400 mg/kg were 9.67±1.76 (84.15%) and 11.33±0.88 (81.42%), respectively. Reference medication diclofenac sodium 10 mg/kg demonstrated high ($p<0.05$) antinociceptive efficacy with an inhibitory rate of 66.67% (Figure 3).

Table 3: Acetic induced writhing test of EELA. The values are displayed as the Mean ± SEM (n = 3).

Group	No. of writhing (Mean±SEM)	% of inhibition
Control	61 ± 0.57	0
Standard	20.33 ± 0.88	66.67
EELA-200	9.67 ± 1.76	84.15
EELA-400	11.33 ± 0.88	81.42

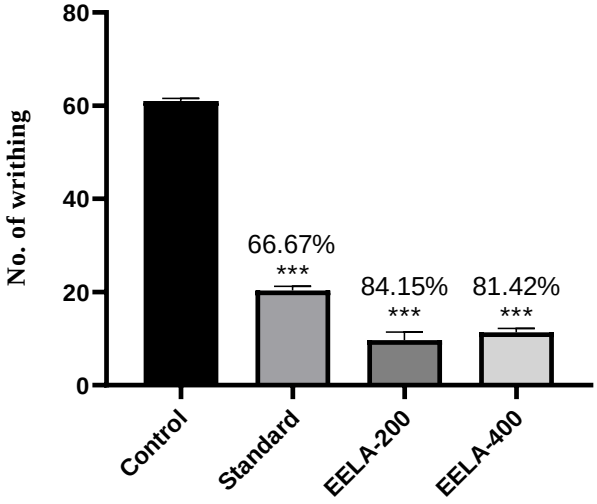


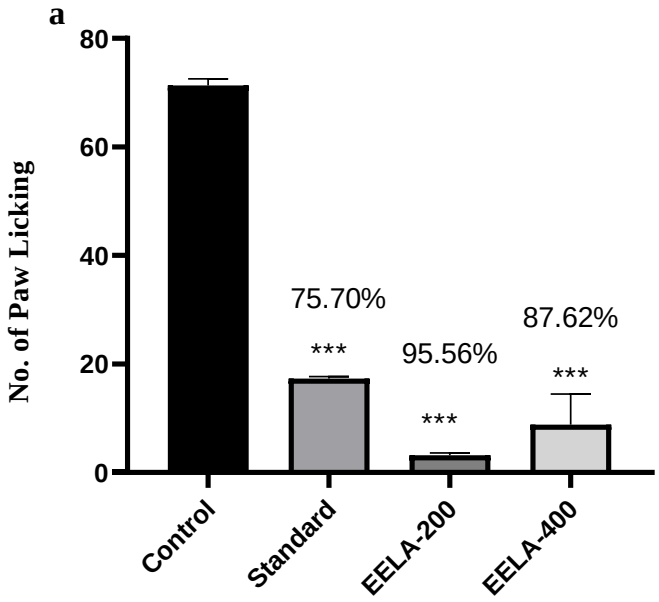
Figure 03: Graphical representation of Analgesic activity by writhing test. ***P<0.05 considered as significantly different from the control (Dunnett'stest).

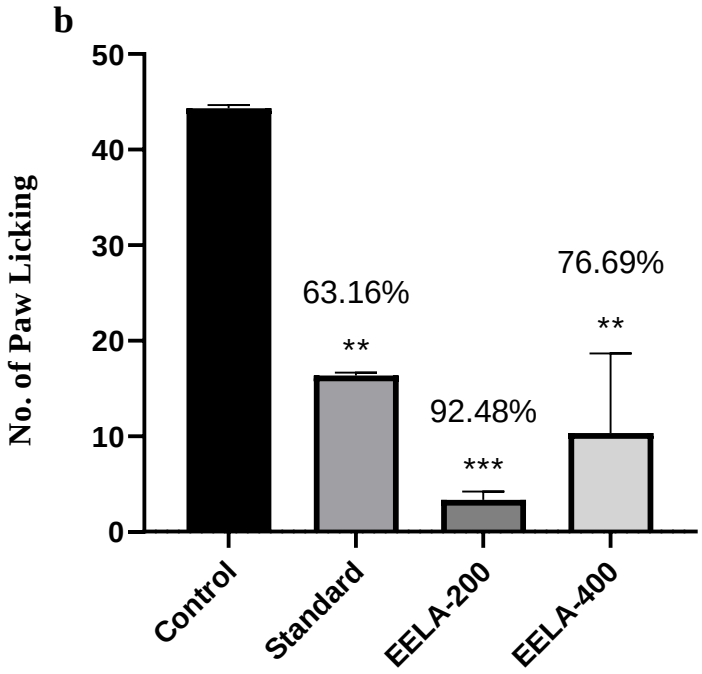
Formalin induced paw-licking test

EELA at different dosages (200 and 400 mg/kg) showed significant ($p < 0.05$) decreases in both the neurogenic (5 min) and inflammatory (15–30 min) stages of the formalin-induced pain response. In the early phase (neurogenic), the length of licking for 400 mg/kg was recorded as (8.43 ± 5.62 sec), with an inhibition of 87.62%, but in the late phase, the duration of licking was recorded as (10.33 ± 8.33 sec), with an inhibition of 76.69 % (inflammatory). Similarly, the centrally acting analgesic morphine (5 mg/kg) reduced neurogenic and inflammatory pain by 77.92% and 86.67%, respectively[17] (Figure 4).

Table 4: Formalin induced paw-licking test of EELA.The values are displayed as the Mean ± SEM (n = 3).

Treatment	Early phase (first 5 mins)	% of inhibition	Late phase (last 15 mins)	% of inhibition
Control	71.33 ± 1.20	0	44.33 ± 0.33	0
Standard	17.33 ± 0.33	75.70	16.33 ± 0.33	63.15
EELA-200	3.17 ± 0.44	95.56	3.33 ± 0.88	92.48
EELA-400	8.83 ± 5.62	87.61	10.33 ± 8.33	76.69





a= early phase licking; b=late phase licking

Figure 04: Graphical representation of Analgesic activity by paw licking test. $^{***}P<0.05$ considered as significantly different from the control (Dunnett'stest).

IV. DISCUSSION

From the beginning of civilization, people have used plants to heal a wide variety of illnesses. The field of phytopharmacology has recently emerged as a promising new avenue for the identification of plant-derived pharmaceuticals with therapeutic potential in alternative medicine. It is estimated that over 30% of medicines are made from plant compounds[18-20].

The ethanolic extract causes dose-dependent paralysis and eventual death of earthworms in an anthelminthic activity assay. The extract concentrations were shown to have a negative relationship with the time it took for earthworms to become paralyzed and die. The anthelmintic activity has been attributed to tannins, flavonoids, alkaloids, and phenolic chemicals, according to a number of studies[21][18]. A parasite's demise can be hastened when a tannin binds to a glycoprotein on its cuticle or a free protein in the host animal's digestive tract. Tannins may be able to exert their killing potential by disrupting the worms' oxidative phosphorylation pathways, thus cutting off their supply of energy. As opposed to this, alkaloids paralyze worms by disrupting their central nervous system[18]. There were phenolic substances, tannins, tannic acids, and flavonoids found in our phytochemical analysis. Therefore, *L. aequata*'s bloom has potential as a new source for anthelmintic medicines.

Most of the time, thrombin turns fibrinogen into blood clots. Drugs that are antithrombotic or that break up thrombi can stop them from forming. The main goal of thrombolytic therapy is to break up fibrin with plasmin, which can be turned on from inactive plasminogen by activators[22]. Bacterial contaminants in plants have been discovered to have plasminogen receptors that bind plasminogen. Plasminogen on the cell surface is easily converted to plasmin, which could result in fibrinolysis[23]. Bacterial plasminogen activators, such as staphylokinase and streptokinase, operate as cofactor molecules that aid in exosite synthesis and substrate presentation to the enzyme. Staphylokinase stimulates plasminogen, causing clots to disintegrate while also destroying the ECM and fibrin fibers that hold cells together[24]. A mild to moderate amount of substantial thrombolytic activity was detected when compared to the positive and negative controls. This shows that EELA has less capacity to lyse the clot. Further study on cell viability tests and in vivo investigations will have significant significance for the treatment of cardiovascular disorders, which are on the rise.

The presence of numerous inflammatory mediators, such as histamine, prostacyclin, and bradykinin, stimulates pain in the body. Inflammation in localised locations generated pain perception in this experiment due to the release of free arachidonic acid via COX and prostaglandin production[25]. In the current investigation, we established that EELA has a dose-dependent anti-nociceptive activity, with efficient suppression of both central and peripheral nociceptive pathways. This was proven by chemical pain models using acetic acid and formalin. Research on nociception often focuses on determining whether or not the opioid

system is involved in the anti-nociceptive action. This is an essential part of the research[17]. In this investigation, the analgesic effects of both morphine and EELA were dramatically inhibited by the co-administration of naltrexone in both chemical pain models. This finding confirmed that opioid receptors were responsible for mediating the pain-relieving action of the plant extract.

V. CONCLUSION

Based on the results of the current investigation, it has been established that the ethanol extract of *L. aequata* leaves exhibits a wide range of pharmacological actions. The results of the laboratory tests suggest that this plant has potential as an anthelmintic, thrombolytic, and anti-nociceptive agent, lending credence to its long-standing traditional uses. The future plan may include isolation of the plant parts responsible for its anthelmintic, thrombolytic, and pain-relieving effects.

VI. ACKNOWLEDGMENT

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VII. CONFLICT OF INTEREST

The authors explicitly stated that they do not possess any conflicts of interest.

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