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Implement of biotechnology techniques for the production of anticoagulants from *Lagestromia tomentosa* L.

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Abstract

Blood coagulation (Blood clotting) has emerged as a dangerous consequence associated with coronavirus disease, hypercholesteremia, heart failure, obesity, and smoking. Therefore, the utilization of medicinal plants containing anti-coagulant compounds and biologically safe has become imperative. *Lagerstroemia tomemtosa* characterized by having polyphenols which have highly antioxidant activity and may have a thrombolytic activity. Biotechnological techniques play a crucial role in the enhancement of the selective compounds. The main object is the production and scaling up of target compounds with anticoagulant effects using biotechnological techniques. Callus production and hairy root culture and evaluating their activity through *in-vivo* study. Seeds were used for the production of calli and cell suspension cultures. Hairy root culture was established by *Agrobacterium rhizogenes* bacteria. Ethanol extracts for cell suspension culture and hairy root culture were prepared. Moreover, crude ethanol (70%) and polar and non-polar extracts were prepared from leaves for the comparative study. Chemical analysis was performed on all prepared extracts using HPLC, along with *in-vitro* antioxidant evaluation by three assays and *in-vivo* anticoagulant activity. MS medium fortified with 1 mg/L BA+ 0.1 mg/L NAA is the best medium for calli production and cell suspension cultures. The system of hairy root biotransformation was established. The major compounds were separated and identified from crude extract. Hairy root extract showed the highest value of total phenolic and total flavonoid contents either quantitatively or qualitatively. All extracts had antioxidant activity, but the hairy root extract showed the highest activity (IC_{50}; 11.70 ug/ mL). Also, it exposed potent anti-inflammatory and anticoagulant activity more than the crude, non-polar, and polar extracts via intrinsic and/or extrinsic pathways and was safer than warfarin medication. Five major compounds were isolated and identified as ellagic acid, gallic acid, catechin, vitexin, and isovitexin. In conclusion, biotechnological studies achieved the goal of the enhancement of the phenolic constituents and, thus, their activity. *Lagestromia tomentosa is* suggested to be used as a potent anticoagulant drug.

Keywords: *Lagerstroemia tomentosa*, medicinal plant, tissue culture, anticoagulant, antioxidant stroke, thrombosis.

Introduction

Thrombosis is a prevalent underlying pathology of ischemic heart disease, stroke, vascular damage venous thromboembolism, and arterial thrombosis [1] It is well known that thrombosis is closely related to activated platelet adhesion, aggregation, secretion functions, and activation of intrinsic and extrinsic coagulation systems, which cause blood coagulation and fibrin formation. Lately, there are many pathological causes of thrombosis, such as hypercholesteremia, smoking, and viral infection [2]. It was observed that infection with SARS-CoV-2 (COVID-19) was accomplished by stroke or thrombosis in many cases [3]. The different waves of SARS-CoV-2 caused the formation of thrombosis as a side effect in different organs, not only in the lungs. The relationship between the inflammation response and the formation of thrombosis is still not identified [4]. The usage of anticoagulants, such as heparin and warfarin, may be accomplished by some restrictions and side effects. The most prominent negative side effect is bleeding[5]. Therefore, it was necessary for using medicinal plants which have dual properties of being biologically safe and containing compounds with anticoagulant effects.

Biotechnological techniques, such as plant tissue culture, bioreactors, and hairy root cultures, are the proper methods for the production and enhancement of secondary metabolites, especially from rare plants.

The infection of a plant by *Rhizobium rhizogenes* (RI) bacteria produces hairy roots that may be acquired from a broad range of plants. Hairy roots are capable of synthesizing and secreting complex active glycoproteins from a wide variety of species [6]. Utilizing biotechnological techniques, it is possible to manipulate the biosynthetic pathways of plants in order to increase the synthesis of phytocompounds of therapeutic value. Diverse researches have investigated the hairy root culture for the synthesis of an extensive array of bioactive chemicals [7].

Lagestroemia tomentosa (F. Lytharceae or the Loosestrife family) is known as Crape or Crepe myrtle. This species is one of 50 species around the world. It is a small deciduous tree that is native to India, China, South Asia, and North

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Australia. It is cultivated in a warm climate[8]. There are several reported medicinal properties of the different parts of this tree as the leaves and flowers are used traditionally as purgative while the bark acts as a febrifuge and stimulant[9]. The methanolic extract of *L. tomentosa* aerial parts was reported to contain several phytochemical constituents, such as, phenolics, flavonoids, coumarins, and tannins. It also has highly antioxidant and highly antimicrobial effects against gram-positive bacteria, *Bacillus subtilis*, fungi *Candida albicans*, and moderate effects against gram-negative bacteria *Klebsiella pneumonia*, and weak effect against different strains of *Mycobacterium tuberculosis* (MTB) [10].

Lagerstroemia tomemtosa L. is considered one of the rare plants, so the utilization of biotechnological approaches, including plant tissue culture and hairy root culture, was opted by authors for the enhancement of the plant secondary metabolites with anticoagulant activity. This effect was assessed *in vivo* by an experimental animal model, and the serum parameters were measured and validated by histopathological examinations.

The target of this study was focused on the production and enhancement of the active phenolic compounds that have the properties of anticoagulant activity through biotechnological techniques, including calli cultures, cell suspension culture, and hairy root cultures. *In-vivo* study was used to assess the anticoagulant activity, and different parameters were estimated to prove the activity.

Material and methods

Collection and identification of plant material

Lagerstroemia tomentosa leaves were collected from Al Zohriya Botanical Garden in January 2020 and stored under No. 20200120 as voucher specimens. The plant was notarized by the Consultant of Plant Taxonomy, Dr. Tereez Labib, Orman Botanical Garden, Giza, Egypt.

Mass calli production

In continuation of our work[11], freshly collected internodes of *L. tomentose* were treated according to the method by Daud et al. [12] for surface sterilization. Firstly, sterilized internode explants were cultured in aseptic conditions into full MS basal media[13] for *in-vitro* plantlets initiation. The obtained leaflets were cut and re-cultured on MS basal media supplemented with BA (1mg/l) and different concentrations of NAA (1, 3, 5 mg/l) for calli induction. All cultures were incubated for 4 weeks (28 days) under either light (fluorescent lighting lamps 3000 lux for 16/8h per day) or dark conditions, at 26 ±1ºC. After the chosen of the best media for calli production, the treatment was represented with five replicates. The following parameters were measured: calli formation percentage and fresh and dry weights (g/jar) [14]. For a detailed method of surface sterilization, *see the supplementary file.*

Cell suspension culture

The friable calli (0.5 g fresh weight) were re-cultured for cell suspension production into 125-mL Erlenmeyer flasks containing the best medium obtained for calli production according to the method of Kenneth [15]. Some parameters were assessed every 4 days from cultivation, including (a) Cell number (growth parameter) and (b) Packed cell volume (P.C.V).[16]. For a detailed method, *see the supplementary file.*

Hairy root culture

Bacteria strain preparation

Agrobacterium rhizogenes strains, A4 and 15834 were utilized (VTT Bio and Chemical processes, Plant Biotechnology Group). Bacteria strains were activated by culture on liquid Luria Bertani medium (LBA) supplemented with yeast extract (5 g/l), NaCl (10 g/l), tryptone (10 g/l), and agar (7 g/l). The bacteria strains were cultured for two days at 28°C. Using a sterile bacterial loop, the developing bacteria colonies were transferred into a liquid LBA medium and cultured overnight at 28°C in darkness on an orbital shaker at 100 rpm. A spectrophotometer was used to measure the OD to 0.5-0.6 at 600 nm.

Inoculation of several explants

For 20 minutes, sterilized leaf, stem, and root explants were submerged in *A. rhizogenes* solution supplemented with 200 M Acetosyringon. To eliminate excess germs, explants were blotted and dried with sterile filter paper (Whatman), then put on solidified free MS media at 28°C in the dark for two days. Following the incubation period, the explants were washed five times with sterile distilled water to remove excess bacteria before being sub-cultured on fresh MS medium holding Cefotaxime (400 mg/l) and kanamycin (50 mg/l) and incubated under complete darkness at 25°C to eliminate the overgrowth of bacteria in the explants. Three weeks later, the hairy roots were plucked and placed in sterile jars containing liquid MS, where they were incubated on an orbital shaker at 60 revolutions per minute, 16/8 photoperiod.

The following parameters were measured: a) fresh and dry weights (g/jar), b) the transformation effectiveness (%) was measured as the total number of explants that induced hairy roots divided by the total number of explants cultivated * 100.

Phytochemical analysis

Preparation of different extracts

The total ethanol of crude extract (T-Lt) was prepared from *L. tomentosa* dried leaves from the original plant by maceration process into boiled ethanol (70%, 3 times). Fractionation was done for a part of concentrated T-Lt suspended in water with n-hexane to form non-polar (N-Lt) and polar (P-Lt) fractions. The cell suspension culture (C-Lt) and the hairy root culture (HR-Lt) were extracted with 70% ethanol after defatting with n-hexane.

All prepared extracts (T- Lt, P1- Lt, N-Lt, C-Lt, and HR-Lt) were subjected to phytochemical and biological investigations.

Qualitative and Quantitative estimations of total phenolic &total flavonoids by spectroscopic and chromatographic techniques.

Folin–Ciocalteau [17] and aluminum chloride [18] methods were used for the estimation of total phenolic (TPC) and total flavonoid (TFC) contents of T-Lt, P1-Lt, C- Lt, and HR-Lt samples quantitatively. The measurements were recorded by microplate reader FluoStar Omega. The results were conveyed as gallic acid (GAE) and rutin (RE)

equivalents, respectively. For the sample preparation, *see the supplementary file*.
High-performance liquid chromatography (HPLC, Agilent 1260 series, Germany) was used for the quantitative analysis of the phenolic compounds on T-Lt, P1-Lt, C-Lt, and HR-Lt samples. For the separation process and their condition, *see the supplementary file*.
Biological analysis

***In-vitro* antioxidant activity**
All prepared samples of T-Lt, P1-Lt, C-Lt, and HR-Lt were evaluated for their antioxidant activity using 1, 1-diphenyl-2-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), and 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays, for the verification of the activity. Assays were adopted according to Boly *et al.* [19], Benzie *et al.* [20], Arnao *et al*. [21], respectively. IC_{50} values represented the DPPH assay result, while μM Trolox equivalent antioxidant capacity (TE) / mg sample represented the FRAP and ABTS results. For detailed methods, *see the supplementary file.*

***In-vivo* anticoagulant experiment**
Both sexes of adult albino Wistar rats (150–200 gm) were purchased from the animal house at the National Research Centre (Cairo, Egypt) and housed in an air-conditioned room (22–25°C, 12 h light/dark cycle), received human care and fed by tap water ad libitum and a standard diet of laboratory. The study protocol was carried out under the umbrella of ethical approval from the Medical Research Ethics Committee (MREC) of the National Research Centre (Egypt), number 19,478.

Experimental design of acute toxicity study
Normal saline (2 ml/kg body weight p.o.) was administered to the control group (mice). Doses of the tested extracts at 500,1000 and 2000 mg/kg were given orally to other groups. The observation was started immediately (twice/day) after the doses of all tested extracts were given and continued for 14 days to find out the rate of mortality, if any.

Experimental design of the experiment
Rats were randomly divided into 20 groups (8 rats /group). Group 1-2 All rats of both sexes were provided with distilled water (normal control). Group 3-4: All rats of both sexes were provided with *warfarin sodium* (2 mg/kg, 5 days/week) regarding Liu et al. [22].
Group 5-6: Both sexes of rats took HR-Lt extract (200 mg/kg).
Groups (7,8) and Groups (9,10): Both sexes of rats took T-Lt extract at low doses (100 mg/kg) and high doses (200 mg/kg), respectively.
Groups (11,12) and Groups (13, 14): All rats of both sexes received N-Lt extract at low doses (100 mg/kg) and high doses (200 mg/kg), respectively.
Groups (15,16) and Groups (17, 18): All rats of both sexes received P-Lt at low doses (100 mg/kg) and high doses (200 mg/kg), respectively.
All treatments (group 5 to group 18) were taken orally daily for 7 days, except warfarin sodium was taken for 5 days/ week.

All rats were anesthetized after completing the experiment with chloral hydrate injection (mild type, 350 mg/kg i.p.)[23], and three blood samples were drawn from the retro-orbital vein of each animal. The serum was obtained from the first sample after centrifugation (3000 rpm,15 min.) (Laborezentrifugen,2k15, Germany), and the following parameters were measured: Cyclooxygenase 1 (COX1) and Thromboxane B2 (TXB2) using ELIZA kits (Elabscience, USA).
After 30 minutes, the second sample was collected in tubes containing Ethylene diamine tetra acetate (EDTA). The following parameters of hematology: red blood cell (RBC) count, hemoglobin (Hb), total white blood cell (WBC), count mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), platelet count (PLC), lymphocytes count (LYM), Mid-Cell count (MID) and Granulocytes count (GRN) were analyzed by automated cell counter (Helena C-2, UK)
The third sample was collected in the test tube containing sodium citrate (3.8%), then centrifuged (1000 rpm,10 min), and the coagulation factors of plasma, such as activated partial thromboplastin time (aPTT) and Prothrombin time (PT), were measured. Clotting time (CT) and bleeding time (BT) were determined according to published methods [24]. *For the detailed method, see the supplementary file*

Histological analysis
The sections from the spleen (5 µm) were preserved in paraffin and fixed immediately in formalin (10%) at room temperature. Haematoxylin and eosin (H&E) stain was utilized to examine the histopathological changes of the slices under the light microscope[25].

Isolation and structural elucidation of major compounds from the most active extract/fraction.
After biological evaluation of the extracts/fraction. The most active extract and fraction HR-LT (0.5g) which also analysed by HPLC and determined its phenolic constituents. The preparative separation for each was performed by the PuriFlash 4100 system (Interchim Software 5.0, France). The major compounds were separated after the collection of similar fractions for each sample, purified on column Sephadex, and identified by physical, chromatographic, and spectral analyses (UV,

NMR, and MS) and/or co-chromatography with a reference sample. For the detailed method, *see the supplementary file*

Statistical analysis

Biotechnology experiments were designed in a completely randomized design, and results were statistically analyzed using standard error (SE) regarding the method designed by Snedecor and Cochran[26].

Data from the biological study were expressed as mean ± SE. Statistical analysis was done using *Graph Pad Prism 5* Software version 5 (San Diego, CA). Significance was tested at $P< 0.05$ using level one-way analysis of variance test (ANOVA) followed by Dunnett multiple comparisons test.

Fig. (1). *In vitro* plantlet initiation of *Lagerstroemia* plant (Basal MS media)

Results

Calli, cell suspension, and hairy root cultures

In vitro plantlet initiation of *L. tomentosa* was successfully achieved from the sterilized internodes on basal MS media for the preproduction of calli cultures (Fig.1). After several trials using different combinations of NAA and BA, the best medium achieved the production of the calli culture from the explant was MS culture medium supplemented with 1 mg/L BA+ 1 mg/L NAA (Fig.2).

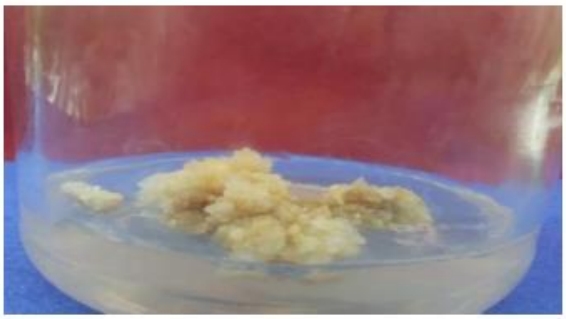

Fig. (2). Calli production of *Lagerstroemia tomentosa*

The maximum cell number recorded in the cell suspension culture experiment was 4.40 ($x10^5$) after 14 days of cultivation, while the highest packed cell volume reordered was 1.36 after 24 days of cultivation. The significant and economic period of cell culturing was 14 days of cultivation. *L. tomentosa* cell cultures showed the maximum cell production and P.C.V. accumulation (Fig. 3).

Regarding the hairy root formation, the effect of the two types of *Agrobacterium* strains on the efficiency of hairy transformation was illustrated in Fig (4). Generally, the highest percentages of hairy root transformation were recorded with the A4 strain compared with strain 15834. The maximum percentages of 65.4, 56.8, and 43.7(%) were recorded with leaf, stem, and root of *L. tomentosa* explants treated with A4 strain, respectively. The leaf explants showed the best results compared with stems and root explants, respectively (Fig. 5).

The effect of *A. rhizogenesis* strains on hairy root fresh and dried weights (mg/Jar) was investigated. The maximum fresh weights 90.1, 69.6, and 57.7(mg/jar) were recorded with leaf, stem, and root explant of *L. tomentosa* treated with 15834 strains, respectively. Regarding the dry weights (mg/jar), the highest values, 7.16, 5.19, and 4.063 were recorded with leaf, stem, and root explants, respectively. In conclusion, it could be concluded that *A. rhizogenesis* strain A4 is more efficient in hairy root transformation than the strain of 15834, and the leaf explants showed higher expiration in transformation responsibility than stem or root explants (Fig.6).

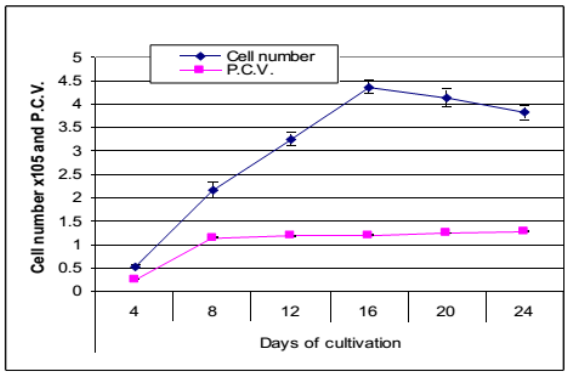

Fig. (3). Cell number x 10^5 and P.C.V. of *Lagerstroemia tomentosa* within 24 days of cultivation

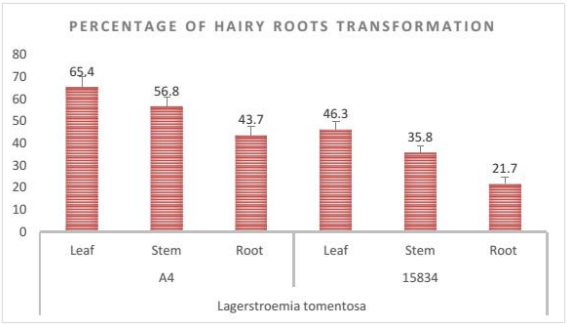

Fig. (4). Transformation efficiency of hairy roots formation in leaf, stem and root explants of *Lagerstroemia tomentosa* treated with A4 and 15834 strains of *Agrobacterium rhizogenesis.* Each value is the average of three replicates ± SE

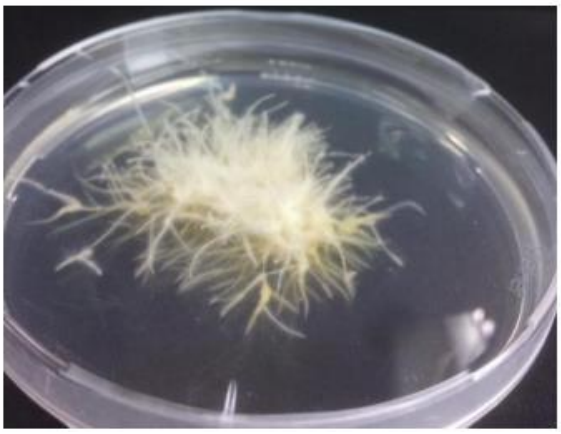

Fig. (5) Hairy root culture of *Lagerstroemia tomentosa* on A4 strain

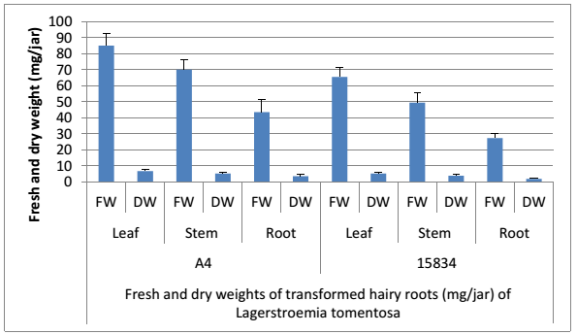

Fig. (6). Effect of two strains of *A. rhizogenesis* (A4) and (15834) on hairy roots fresh and dry weights (mg/jar) induced from leaf, stem and root explants of *Lagerstroemia tomentosa*. Each result is the mean of three replicates± SE

Phytochemical Study

Quantitative estimation of total phenolic and total flavonoid contents

TPC and TFC of *Lagerstroemia* extracts were 400.23, 323.16, 312.06, and 289.81 µg GAE/mg extract and 50.2, 38, 25.89, and 25.89 µg g RE/mg extract for HR-LT, C-LT,, T-LT, and P1-LT, respectively (**Table 1)**. It is considered the first time to evaluate the total phenolic and total flavonoid contents of *Lagerstroemia tomentosa.*

Table (1): Quantitative estimation of total phenolic (TPC) and total flavonoid (TFC) contents of *Lagerstroemia tomentosa* extracts

Samples	Total Phenolic (µg GAE/mg extract)	Total Flavonoids (µg RE/mg extract)
C-LT	323.16	38
HR-LT	400.23	50.2
T-LT	312.06	25.89
P1-LT	289.81	24.57

HPLC analysis

The analysis of *L. tomentosa* extracts by HPLC **(Table 2)**; revealed the identification of fourteen compounds on the T-Lt and P1-LT, separately with gallic acid was the major compound in each (32.369%) and (15.498%), followed by ellagic acid (13.398% and 8.5307%), respectively. Hesperetin and kaempferol were identified in T-Lt, C-Lt and HR-Lt only. There was another unknown compound detected in T-Lt and P-Lt, 28.642% and 12.298%, respectively. Fifteen compounds were identified in HR-Lt and C-Lt extracts, with gallic (33.80% and 15.0172%, respectively) and ellagic acids (18.330% and 14.1743%, respectively) being the major**s.** Besides that, there were ten compounds detected with a high percentage but cannot be identified due to the lack of authentic samples. The hairy root (HR-Lt) extract contained more compounds in folds than the cell suspension culture, and the original plant extracts, i.e., Kaempferol, was detected in 9.965 folds than T-Lt. Cinnamic acid, methyl gallate, syringic acid, vanillic acid, catechin, and quercetin were found in 7.189, 3.919, 2.4, 1.66, 1.19, and 1.14 folds, respectively, than T-Lt.

Structural elucidation of the isolated compounds from T-Lt and C-Lt

The obtained data of physical, chemical, chromatography, co-chromatography, and spectroscopic analysis for compounds were in agreement with the published data for gallic acid (**Fig. 7a)** and catechin (**Fig. 7b)** by Amer et al. [27] and for apigenin (**Fig. 7c)** by Ateya et al. [29].

Vitexin (**Fig. 7d):** Light yellow color powder with m.p. 204-205ºC. Soluble in Methanol. The UV spectrum data (λmax, nm) at MeOH: 233, 270, and 334 nm. 1H-NMR ($CDCl_3$, 400 MHz) revealed the presence of apigenin moiety as δ 7.82(2 H, d, *J* 8.4 Hz, H-2', 6', 6.94 (2H, d, *J* =8.4 Hz, H-3', H-5'), 6.58(1H, s, H-3), 6.47 (1H, s, H-8), 6.2 (1H, s, H-6) and the sugar structure δ 3.53 (1H, m, H-5"), 3.55 (1H, m, H-3"), 3.58 (1H, m, H-4"), 3.74 (1H, dd, J = 12.3, 5.5 Hz, H-6a"),3.77 (1H, dd, J = 12.3, 2.0 Hz, H-6b"), 4.14 (1H, t, J = 9.0 Hz, H-2"), 4.81 (1H, d, J = 9.9 Hz, H-1"). The data were in agreement with published data[30]. and identified as vitexin or 8-*β*-D-glucopyranosyl-apigenin.

Isovitexin (**Fig. 7e):** (6- *β*-D-glucopyranosyl-apigenin): yellow powder, m.p.220-222ºC. soluble in methanol, the 1H-NMR and 13C-NMR data, ***See the supplementary file***. The data was confirmed as compared with published data [31].

Ellagic acid (**Fig. 7f):** white powder; 348-349ºC. The obtained data from UV spectrum analysis, 1H NMR at DMSO, and co-chromatographical analysis were in agreement with published data and by Goriparti *et al.* [32]. ***See the supplementary file***

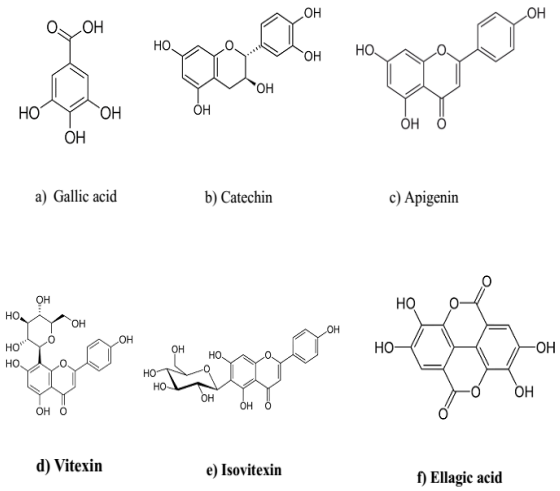

Fig. (7). The isolated and identified compounds from the hairy root extract

Table (2). HPLC analysis of *Lagerstroemia tomentosa extracts*

No	R_t	Compounds	Area%			
			T-LT	P1-LT	C-LT	HR-Lt
1	2.525	Unknown	28.6426	12.2982	ND	ND
2	**3.064**	**Gallic acid**	**32.369**	**15.498**	**15.0172**	**33.80**
3	4.022	Chlorogenic acid	ND	ND	0.4536	1.3022
4	4.239	Catechin	4.8103	1.2871	5.7652	6.5740
5	4.873	**Unknown**	ND	ND	2.3067	2.607
6	5.067	Methyl gallate	0.6796	0.9007	2.6635	3.3847
7	5.329	Caffeic acid	1.8448	2.6887	4.6635	6.770
8	5.650	**Unknown**	ND	ND	3.7710	4.8740
9	5.964	Syringic acid	2.1876	3.7129	5.2729	6.4082
10	6.572	**Unknown**	ND	ND	1.0698	1.8354
11	7.205	Rutin	0.1453	0.0712	0.4350	0.7030
12	8.003	Ellagic acid	13.3952	8.5307	14.1743	18.330
13	8.975	Vanillic acid	0.1948	0.2943	0.3236	0.7802
14	9.243	**Unknown**	ND	ND	0.5089	0.9342
15	9.578	Ferulic acid	1.2655	0.5527	1.2011	1.6254
16	10.285	Naringenin	0.8956	0.3429	1.2173	1.8403
17	10.494	**Unknown**	ND	9.4025	0.6756	2.580
18	10.779	**Unknown**	ND	1.2743	7.7619	8.7702
19	10.958	**Unknown**	ND	1.4643	11.7853	12.9706
20	11.251	**Unknown**	ND	1.9897	4.7285	5.7352
21	11.918	**Unknown**	ND	2.9454	7.0835	7.6004
22	12.799	Quercetin	3.8059	0.3264	4.3606	5.002
23	13.312	**Unknown**	ND	ND	2.1557	4.550
24	14.382	Cinnamic acid	0.8756	0.0564	6.2947	8.492
25	15.451	kaempferol	0.7028	0.2126	7.0036	9.640
26	**15.783**	**Hesperetin**	0.2972	ND	0.6403	1.708

***ND= Not detected**

***In-vitro* antioxidant activity**

The data obtained from the different methods **(Table 3);** showed that all extracts had antioxidant activity. The IC_{50} value of hairy root extract (HR-LT) was the lowest value at 11.70 (ug/ mL), followed by the polar extract (P1-LT); 23.49 (ug/ mL), total ethanol extract 70% (T-Lt), and non-polar extract (N-Lt).

Table (3). The antioxidant activity of different extracts of *Lagerstroemia tomentosa* using three assays (DPPH, FRAP, and ABTS)

Antioxidant activity	DPPH IC_{50} (ug/ mL)	FRAP	ABTS
		(µM Trolox equivalent /mg extract)	
T-LT	**28.57±0.89**	**2405.61±76.24**	**962.53±26.08**
P1-LT	**23.49±1.15**	**3903.52±228.01**	**1219.72±77.08**
N-Lt	**44.83±1.01**	**1280.53±37.03**	**860.74±22.74**
HR-LT	**11.70±0.45**	**3935.49±267.60**	**1371.91±94.22**
Trolox	**9.778±0.535**	-	-

In-vivo* anticoagulant activity of *Lagestromia tomentosa

Acute toxicity of *L. tomentosa* extracts

Our results showed that different extracts of *L. tomentosa* did not appear any toxic signs in female or male mice. All extracts represent LD_{50} values of more than 2g/Kg body weight when compared with the previously reported method [33] that they were not toxic for oral consumption[34].

Effect of *Lagerstroemia* on hematological parameters in rats

The red blood cell parameters, such as RBC, Hb, MCV, MCH, and MCHC, were evaluated to assess the effect of different extracts of *L. tomentosa* in an anemic state. The intake of HR-Lt, T-Lt, P-Lt, and N-Lt extracts did not change the parameters in comparison with the normal control groups in female and male rats, as shown in **Tables (4& 5).**

Thrombocytopenia occurred in male and female rats who were given warfarin (2mg/kg) at 45.6% and 53%, respectively, as compared to control groups in both sexes.

Therefore, administration of HR-Lt, T-Lt, N-Lt, and P-Lt (200 mg/kg) significantly resulted in thrombocytopenia at 61%, 41%, 30.6, 20.6%, and 64%, 55.6 %, 47.5 %, 45% respectively, as compared to male and female groups who administered warfarin, respectively. While rats given HR-Lt resulted in thrombocytopenia to be 69%, 63%, 70 and 68 %, 74%, and 75.5%, respectively, as compared to T-Lt, N-Lt, and P-Lt extracts groups in both sexes of rats.

Administration of warfarin led to the incidence of leucocytosis, lymphocytosis, and granulocytosis in comparison with normal control groups of both sexes. T-Lt, N-Lt, and P-Lt treatment groups showed considerable leucocytosis, lymphocytosis, and granulocytosis compared with warfarin-treatment groups in both sexes of rats (**Tables 4 & 5).**

Effect of *Lagerstroemia tomentosa* on Coagulation Factors of Plasma

Warfarin administration caused significant prolongation in the bleeding and clotting time by 50.8 % and 80.5%, and by 51.4 % and 91 %, as compared to their control groups (male and female rats), respectively. The groups treated with HR-Lt, T-Lt, and N-Lt (200 mg/kg) showed significant prolongation in the bleeding and clotting time by (35%, 20%, 10% and 29%, 15%, 10%), respectively, compared with the warfarin-treatment group (male rats). While the providing of HR-Lt, T-Lt, N-Lt, and P-Lt (200 mg/kg) caused significant prolongation in the bleeding and clotting time by 47%, 21%, 19.6%, 6% and 65%, 26%, 36.6%, 28 % respectively, compared with the warfarin-treatment group in female rats.

PT and APTT were prolonged by (34.9%, 28%) and (21%, 18%), respectively, after warfarin administration as compared to the male and female control groups. The administration of the HR-Lt, T-Lt, and N-Lt extracts significantly prolonged PT and APTT by 49%, 20.7%, 12.6 % and 39%, 16.4%, and 10%, respectively, comparing with warfarin-treatment (male rats) **(Fig. 8).** While administration of the HR-Lt, T-Lt, N-Lt, and P-Lt at high doses (200 mg/kg) caused significant prolongation on PT and APTT by 55%, 21%, 17.3 %, 9 and 12.6 %, and 35%, 22%, 19.4%, 9% respectively, as compared to warfarin-treatment (female group) **(Fig. 10).** Moreover, the intake of HR-Lt significantly prolonged the bleeding, clotting time, PT, and APTT as compared to all groups in male and female rats.

Table (4): Effect of *Lagestromia tomentosa* extracts on Hematological Parameters male rats

	Normal control	Warfarin (2mg/kg)	Hairy root extract (HR-Lt) (200mg/kg)	Crude extract (T-Lt) (100mg/kg)	Crude extract (T-Lt) (200mg/kg)	Non-polar extract (N-Lt) (100mg/kg)	Non-polar extract (N-Lt) (200mg/kg)	Polar extract (P-LT) (100mg/kg)	Polar extract (P-LT) (200mg/kg)
RBC ($\times10^{12}$ /L)	7.86± 0.17	7.04± 0.08	7.5±0.03	7.02± 0.27	7.30±0.05	7.27± 0.36	7.65± 0.27	7.32± 0.49	7.5± 0.16
Hb (g/dl)	12.48±1.9	12.6± 1.9	13.6±2.16	12.1± 1.8	13.1± 2	12.4±1.9	13.7± 2.1	12.8 ± 1.9	13 ±2
MCV%	51.74±0.58	52.12±0.52	51.24±0.10	52.4±0.4	51.5±0.73	51.3±1.4	51.2± 0.64	51.5 ±0.5	52.9± 0.69
MCH (pg)	16.46±0.24	17.4 ±0.8	17.16±0.50	17.5±0.05	17.2± 0.14	17.1± 0.06	17.0± 0.23	17 ±0.29	17.7± 0.2
MCHC (g/dl)	32.2±0.17	33.7 ±1	32.22±.19	32.7 ±0.28	31.9±0.05	32.6 ± 0.27	31.8 ±0.76	32.0±0.36	32.6±0.25
Platelets ($\times10^{9}$ /L)	995 ±54	519 ±20[a]	200.1.2±44[b]	650± 44[ac]	306 ±15[ab]	536± 79[ac]	360± 88[ab]	670± 54[ac]	412± 47[ac]
WBC ($\times10^{9}$ /L)	11.4± 1.8	16.5 ±0.14[a]	10.36±0.183[b]	12.14± 0.08[b]	11.04±1.3[b]	11± 0.87[b]	10.96±0.74[b]	11.4±0.4[b]	11.16± 0.35[b]
LYM ($\times10^{9}$ /L)	5.9± 0.56	8.12±0.38[a]	5.1±0.60	6.2 ±0.58	5.03± 0.02[b]	5.46±0.66[b]	5.18 ± 0.23[b]	7.32 ± 1	6.96± 0.25
MID ($\times10^{9}$ /L)	1.43±0.03	1.86±0.52	1.12±0.50	1.44±0.08	1.2 ± 0.05	1.36 ± 0.15	1.22 ± 0.1	1.36 ± 0.22	1.48±0.03
GRAN ($\times10^{9}$ /L)	4.24 ±0.55	6.98 ±0.69[a]	3.08±0.50[b]	4.2 ± 0.67[b]	3.1 ± 0.13[b]	3.9 ± 0.28[b]	2.78 ± 0.43[b]	3.84±0.98[b]	4.28±0.34[b]

Results are presented as the mean ± S.E. of (n=8) for each group. Statistical analysis was carried out by one-way analysis of variance followed by the Dunnett multiple comparisons test.
[a,b,c] Statistical significant from (control, warfarin, hairy root extract) groups, respectively, at P <0.05.

Table (5): Effect of *Lagestromia tomentosa* on Hematological Parameters female rats

	Normal control	Warfarin (2mg/kg)	Hairy root extract (HR-Lt) (200mg/kg)	Crude extract (T-Lt) (100mg/kg)	Crude extract (T-Lt) (200mg/kg)	Non-polar extract (N-Lt) (100mg/kg)	Non-polar extract (N-Lt) (200mg/kg)	Polar extract (P-LT) (100mg/kg)	Polar extract (P-LT) (200mg/kg)
RBC ($\times10^{12}$ /L)	7.78 ±0.26	7.02 ± 0.25	7.7±0.18	7± 0.19	7.98±0.33	7.7 ± 0.29	7.88 ± 0.36	7.06 ± 0.31	7± 0.17
Hb (g/dl)	12.84±0.39	10.28± 0.78	14.66±0.183	12.3± 0.37	14.64 ± 0.83	13.34± 0.45	13.86± 0.46	12.28± 0.47	12.36±0.29
MCV%	51.82±0.56	51.94±0.65	51.7±.184	53±0.56	51.8±0.2	52.7±0.7	51.14± 0.46	50.76±0.02	51.52± 0.95
MCH (pg)	16.42±0.17	17.34±0.024	17.34±0.08	17.24±0.51	17.48± 0.3	17.44± 0.14	17.6± 0.1	17.02 ±0.02	17.16± 0.04
MCHC (g/dl)	31.68±0.48	33.16±0.41	32.34±0.166	32.8±0.21	32.66± 0.41	32.02± 0.02	32.2±0.48	32.24±0.06	33.4±0.24
Platelets ($\times10^{9}$ /L)	855±58	400 ±55.3[a]	143.8±22[ab]	448± 60[ac]	177.6 ±10[ab]	562 ± 66[ac]	210 ± 15[ab]	544 ± 40[ac]	220 ± 20[abc]
WBC ($\times10^{9}$ /L)	11.8 ± 0.24	15 ±0.74[a]	9.2±0.182[ab]	10.5 ± 0.76[b]	10.4 ± 1.3[b]	12.1 ± 0.5[b]	11.3± 0.08[b]	11.3 ±0.53[b]	11.8 ± 0.24[b]
LYM ($\times10^{9}$ /L)	5.8± 0.56	8.22± 0.55[a]	5.22±0.09[b]	5.84±0.67	5.02± 0.95[b]	6.1± 0.69[b]	5.72± 0.03[b]	6.02± 0.86	5.92± 0.41
MID ($\times10^{9}$ /L)	1.42± 0.12	1.84± 0.32	1.22±0058	1.552± 0.038	1.12± 0.8	1.46± 0.06	1.36± 0.24	1.366± 0.15	1.48 ±0.03
GRAN ($\times10^{9}$ /L)	2.04±0.024	4.16±0.24[a]	1.64±0.163[b]	2± 0.08[b]	1.88± 0.03[b]	2.18± 0.23[b]	1.78± 0.49[b]	2.64±0.49[b]	3.16±0.71[b]

Data are presented as the mean ± S.E. of (n=8) for each group. Statistical analysis was carried out by one-way analysis of variance followed by the Dunnett multiple comparisons test.
[a,b,c] Statistical significance from (control, warfarin, hairy root extract) groups, respectively, at P <0.05.

Effect of Lagerstroemia on TXB2 and COX1.

In male rats, the administration of warfarin led to an increase in the level of serum TXB2 and COX1 formation by 38.4% and 61%, respectively, in comparison with the control group. Moreover, the administration of HR-Lt, N-Lt, and P-Lt extract (200 mg/kg) resulted in a significant inhibition in the TXB 2 and COX 1 concentrations by 46%, 25.4%, 24%, 24 %, and 46%, 19%, 18%, 14% respectively, as compared to warfarin- treatment group **(fig.9).**

In female rats, warfarin administration led to a significant increase in the TXB 2 and COX 1 levels by 52.7% and 84%, respectively, compared to the control group. Significant inhibitions at TXB 2 and COX1 were observed in groups treated with HR-Lt, T-Lt, N-Lt, and P-Lt (200 mg/kg) with 51%, 17.3%, 25%, 17% and 46%, 34.2%, 33%, 33% respectively when compared to warfarin–treated group **(fig. 11).** TXB2 and COX1 were significantly inhibited in the HR-Lt group as compared to T-Lt, N-Lt, and P-Lt extracts.

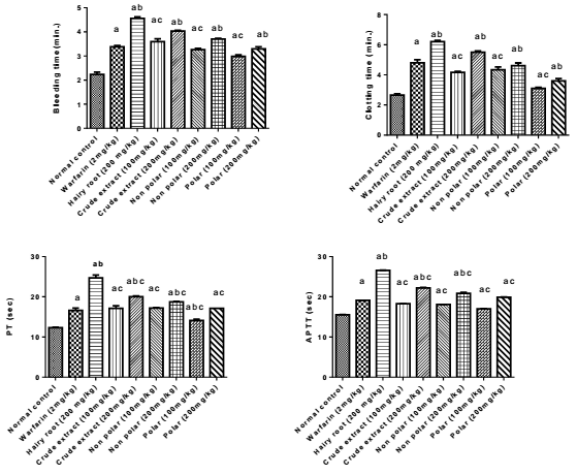

Figure (8): Effect of *Lagerstroemia* extracts on Coagulation Factors Measurement on Plasma in male rats

Results are presented as the mean ± S.E. of (n=8) for each group. Statistical analysis was carried out by one-way analysis of variance followed by the Dunnett multiple comparisons test.

[a,b,c] Statistical significance from (control, warfarin, hairy root extract) groups, respectively, at P <0.05.

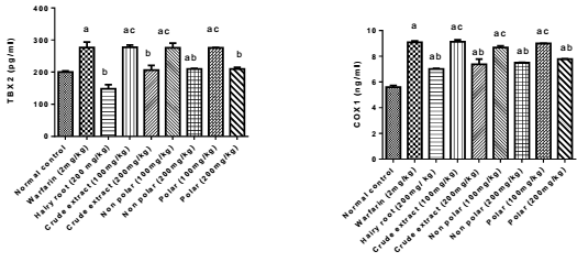

Figure (9): Effect of Lagerstroemia on TXB 2 and COX 1 in male rats

Data showed as the mean ± S.E. of (n=8) for each group. Statistical analysis was carried out by one-way analysis of variance followed by the Dunnett multiple comparisons test.

[a,b,c,] Statistical significant from (control, warfarin, hairy root extract) groups, respectively at P <0.05.

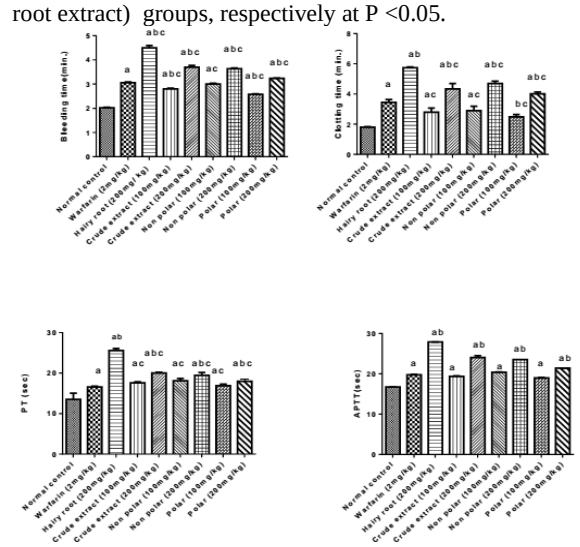

Figure (10): Effect of *Lagerstroemia extracts* on Plasma Coagulation Factors Measurement in female rats

Data are presented as the mean ± S.E. of (n=8) for each group. Statistical analysis was carried out by one-way analysis of variance followed by Dunnett multiple comparisons test.

[a,b,c,] Statistical significant from (control, warfarin, hairy root extract) groups, respectively at P <0.05.

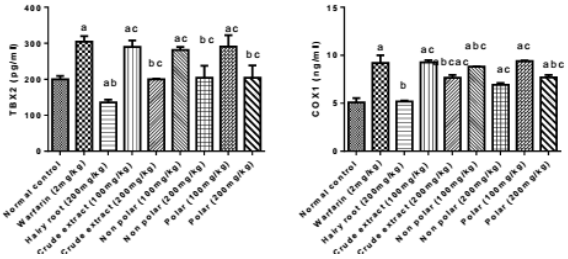

Figure (11): Effect of *Lagerstroemia* extracts on TXB 2 and COX 1 in female rats

Data are presented as the mean ± S.E. of (n=8) for each group. Statistical analysis was carried out by one-way analysis of variance followed by the Dunnett multiple comparisons test.

[a,b,c] Statistical significance from (control, warfarin, hairy root extract) groups, respectively, at P <0.05.

Histopathological examination

Sections of the spleen from all groups were examined under a light microscope after staining with H&E (X200). Sections of the control group showed white pulp (WP), with its central arteriole (CA) and the red pulp (RP) (**Fig.12a**). The reduction in the white pulp size appeared in the warfarin group, besides the loss in the distinction between red and white pulps with dilated in the CA. The RP of the spleen showed a loss of architecture. Major lymphocytes had dark, irregular nuclei with heterogeneous cytoplasm and splenic hemorrhages. The splenic parenchyma showed deposition in the hemosiderin pigment (star) with marked dilatation and congestion in the blood vessels (arrowhead) (**Fig.12b**). Splenic sections from the hairy root extract group (**Fig.12c**) showed nearly normal splenic structure (WP with CA), with slight degeneration and hemosiderin pigment deposition in the RP. Splenic sections from the group administered with *L. tomentosa* total extract (**Fig.12d**) showed more or less normal splenic structure (WP with CA), and the RP exhibited slight degeneration and deposition of hemosiderin pigment in the splenic parenchyma. Histopathological examination of sections from the non-polar *L. tomentosa* extract group (N-Lt) (**Fig.12e**) showed significant improvement, almost normal WP with CA, while the RP exhibited slight degeneration with hemosiderin pigment deposition and mild blood sinusoids dilation. Splenic sections from the polar extract of *L. tomentosa* (p-Lt) (**Fig.12f**) showed moderate improvement and nearly normal splenic structure (WP with CA), although the RP showed slight degeneration, hemosiderin pigment deposition, and mild dilated and congested blood vessels.

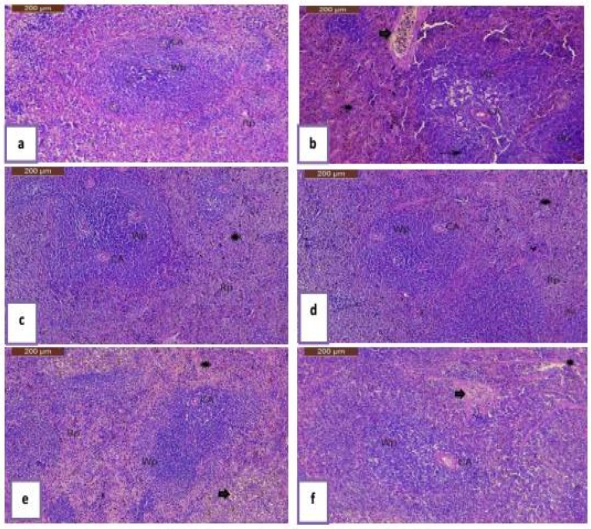

Figure (12): a photomicrograph of **(a)** the spleen of the control group, (b) the spleen of the warfarin group, (c) the spleen of the hairy root Lagerstroemia group, (d) the spleen of the *Lagerstroemia* extract group **(T-Lt)**. **(e)** spleen of the non-polar *Lagerstroemia* extract (N-Lt) group. **(f)** spleen of the Polar *Lagerstroemia* **(P.Lt)** extract group **(H&E X 200).**

Discussion

The aromatic plant produces a significant quantity of secondary metabolites, such as phenolic compounds, which have the capacity to provide advantageous impacts on human health. Considering the role of free radicals in inflammation and the association between inflammation and coagulation mechanisms [35]. Plant tissue culture and cell suspension culture techniques helped in the enhancement of phenolic constituents. MS culture medium supplemented with 1 mg/L BA+ 1 mg/L NAA was the best medium for callus production from leaf explants of the plants**.** It **might be the first report regarding the *in-vitro* cultivation of the Egyptian *L. tomentosa* either through plant tissue culture or by using hairy root culture.**

Mano [36] revealed that a culture of hairy roots caused by *A. rhizogenes* infection is an ideal starting material for the formation of secondary metabolites by plant cells due to the stability of critical properties such as secondary metabolite development and productivity. Many research on the generation of secondary metabolites, such as alkaloids, flavonoids, and steroids, by hairy root cultivation have been published in recent decades [37]

The obtained results of total phenolic and total flavonoid contents of *L. tomentosa* total ethanol extract (T-Lt) were compatible with that previously reported [10]for *L. tomentosa* aerial part ethanol extract (677.5±8.63 mg GAE/g DW) and the reported by Junaid *et al.* [38] for *Lagerstroemia speciosa* L. (325±0.01 µg GAE/mg extract). It is considered the first time to evaluate the total phenolic and total flavonoid contents of *Lagerstroemia tomentosa* for cell suspension culture and hairy root culture. Phenolics are characterized by having a wide range of biological activities, such as radical scavenging properties, antimicrobial, and anti-allergic [39].

A previous study *on Lagerstroemia indica* demonstrated that eight compounds of flavonoids, which were identified for the first time from this genus and their structures, were elucidated as isovitexin, vitexin, iso-orientin, orientin, astralagin, rutin, apigenin-7-O-$^4C_1$-β-D-glucoside, luteolin-7-*O*-$^4C_1$-β-D-glucoside, and their aglycones: apigenin, quercetin, kaempferol and luteolin were isolated from *Lagerstroemia* extract [38]. Six ellagitannins were also isolated and identified as Tellimagrandin, nilocitin, 1,3-di-O-galloyl-4,6-hexahydroxydiphenoyl-β-$^4C_1$-glucopyranose, and Decarboxy ellagic acid for the first time in the genus while 2,3 hexahydroxydiphenic acid-α, β-glucoside and brevifolin for the first time in the species[40]. These results were compatible with our results in spite of the difference of species. As in our work, apigenin, vitexin, isovitexin, gallic acid, and ellagic acid were isolated and identified from the ethanol extract of the hairy root.

Lagerstroemia tomentosa extracts were assessed for their antioxidant effect through *in vitro* study by three different methods: DPPH, FRAP, and ABTS. All methods revealed the potency of plant extracts, especially the hairy root extract by applying the role of "The low value of IC_{50} indicates a high antioxidant activity, hairy root extract showed the highest activity". The result of DPPH was confirmed by FRAP and ABTs. It was reported that *Lagestroemia speciosa* L. seed extract had an IC50 value (9.63±0.20 µg/mL) in the DPPH assay. The extract exhibited reducing activity that was dependent on its concentration, demonstrated by an increase in the reaction mixture's absorbance [38]. Major phenolic acids and flavonoids such as catechin and quercetin displayed remarkable radical scavenging activity, contributing to the high radical scavenging activity of the callus [41].

There is a shred of indicator that suggests a close connection between coagulation and the inflammation process, highlighting their significant influence on each other [42]. Systemic inflammation typically triggers the activation of the coagulation system; conversely, components of the coagulation system can substantially impact the inflammatory response[43]. Nitric oxide and prostaglandins, characterized as contribution mediators, define the Inflammation process. Cyclooxygenase enzymes are involved in the arachidonic acid metabolism, motivating thromboxane and prostaglandin synthesis. The inflammatory processes are availed by prostanoids produced by COX-1 [44]. On the basis of our study, the suppressive regulation of coagulation by the tested extracts could give potential anti-inflammatory mediators. This hypothesis was proven by Labib et al. [45] that anticoagulant and anti-inflammatory effects have been mostly assigned by the high content of polyphenol and flavonoid constituents in this plant.

The formation of a blood clot initiates with platelet clustering, leading to blockage of blood vessels and ultimately resulting in thromboembolic conditions such as heart attacks or strokes [46]. When adhered to the inner lining of blood vessels and activated, platelets release chemokines and proinflammatory substances [47]. The COX-1 enzyme plays a crucial role in producing prostaglandin, which aids in platelet clustering through the formation of thromboxane B2 (TXB2). By inhibiting COX-1, the production of thromboxane B2 is hindered, thereby impeding platelet aggregation [48]. Our study on *Lagestromia tomentosa* extracts showed significant anti-inflammatory activity where COX1 and TXB2 were inhibited in treated groups, especially in the group of hairy root extract than non-treated group extract, as well as PT and aPTT assays were prolonged in all tested extracts. Labib et

al. (2013) agreed with our results when they studied the aqueous methanol leaf extracts of *Lagerstroemia indica* [45]. They also act on clotting and oxidative stress *in vitro* studies. Thus, the current study has scientifically confirmed the traditional use of the *Lagerstroemia indica* aqueous extract in treating inflammatory diseases.

Histopathological examination also provided crucial evidence for the biochemical analysis and substantiated the current findings. Warfarin exhibited a noticeable decrease in the size of the white pulp, a blurred boundary between white and red pulps, along with an enlarged central arteriole under microscopic observation. The red pulp of the spleen displayed structural loss, along with a majority of lymphocytes showing dark irregular nuclei with varied cytoplasm and evident splenic hemorrhages. Furthermore, there was a presence of hemosiderin pigment deposition in the splenic tissue, distinctly dilated and congested blood vessels. Conversely, *Lagerstroemia tomentosa* extracts, especially hairy root extract, showcased a spleen structure that was almost normal, with white pulp featuring a central arteriole and red pulp displaying slight degeneration.

Conclusion

Lagerstroemia tomentosa hairy root extract exhibited potent anti-inflammatory and anticoagulant activities more than the crude, polar, and non-polar extracts via intrinsic and /or extrinsic pathways and was safer than warfarin medication. The authors suggested applying this idea at an industrial/commercial level to serve humanity in light of the pandemic.

Declarations:

Conflict of Interest

The authors declare no conflict of interest.

Contribution of Authors

The authors confirm that this work was completed by the authors listed in this article. The authors declare no competing financial interest**.**

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