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In vitro evaluation of anti-diabetic and anti-inflammatory activities of ethanolic extract of *Lawsonia inermis* L. leaves (Family: Lythraceae)

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Abstract
The study was designated to perform antidiabetic and anti-inflammatory activities of the leaves of *Lawsonia inermis* L. Phytochemical screening of the plant was also evaluated. The crude ethanolic leaf extract (CEF 5.00 gm) was fractioned with pet-ether (PEF 2.65 gm) and ethyl acetate (EAF, 1.63gm). Anti-diabetic activity was assessed using alpha-amylase inhibitory assay with Acarbose as standard and found IC_{50} (µg/ml) values of acarbose, PEF and EAF were 57.2, 206.11 and 323.1, respectively, indicating PEF as potent antidiabetic candidate compared to EAF. Anti-inflammatory activity was performed using hypotonic solution induced hemolysis and heat induced hemolysis. Ethanolic leave extract of PEF, EAF and standard aspirin at 800 µg/ml showed 80.0±0.79, 82.9±1.04 and 91.4±0.22% inhibition for heat induced haemolysis and 79.5±0.24, 84.8±0.05 and 96.3±0.16% inhibition for hypotonic solution induced haemolysis, respectively. The results showed EAF possessed strong anti-inflammatory activity compared to aspirin. The phytochemical screening showed the presence of alkaloids, flavonoids, glycosides, resins, tannins, terpenoids and steroids and absence of anthocyanin, phlobatannins, proteins and saponin. From the observation it was concluded that *Lawsonia inermis* L might be a good source for anti-diabetes and anti-inflammatory agent.

Keywords: *Lawsonia inermis* L, lythraceae, haemolysis, anti-inflammatory, anti-diabetic

1. Introduction
From the earliest recorded human civilization, people are habituated to use plants for curing a variety of diseases. The demands to use medicinal plants for primary healthcare in various parts of the world are increasing day by day due to broad spectrum of biological and medicinal values as well as higher safety margins [1-2]. The pharmacological activities of plants are due to presence of a wide variety of phytochemicals, such as alkaloids, flavonoids, tannins, terpenoids, etc. [1-3]

Lawsonia inermis, also known as henna tree, is a flowering evergreen medium sized shrub of the family Lythraceae and is native to Asia, northern Africa and Australia[3]. There is a plethora of uses of *Lawsonia inermis*. The leaf paste of this plant is considered to possess antimicrobial and fungicidal properties and has been traditionally used for cosmetic and medicinal purposes to dye hair, skin and nails for over 9000 years. The plant also possesses a well-documented folklore history to cure diabetes, oxidative stress, wound, inflammation, fever, liver diseases, rheumatoid arthritis, jaundice and gastrointestinal tracts disorders including malignant ulcers [4-6]. Phytochemical investigation reported the presence of several bioactive compounds like sterols, glycosides, terpenoids, gallic acid, glucose, mannitol, fats, resin and flavonoids [4-7].

Based on the diversified usages of *Lawsonia inermis* extracts, phytochemical reports and availability in Bangladesh, the plant was chosen for further research and the present study was designed to evaluate In vitro anti-diabetic and anti-inflammatory activities of the ethanolic leaf extract of *L. inermis* L (Family: Lythraceae, Bangladeshi name Mehndi).

2. Materials and Methods
2.1 Collection and Processing of Plant Material
The leaf of *L. inermis* was collected from Tanore Upazila, Rajshahi (northern part of Bangladesh) during the month of August 2016 and was identified by taxonomist Dr. AHM Mahbubur Rahman, Professor, Department of Botany, University of Rajshahi, Bangladesh. The leaves were washed by tap water and sun dried for several days followed by oven drying (at 45 °C) for 24 hours. The dried leaves powder plants were stored for further purpose.

2.2 Preparation and fractionation of extract
Approximately 50 gm of dried leaf powder was taken in an amber colored extraction bottle and the powder was soaked with 70% ethanol (250 ml × 3 times). The sealed bottle was kept for 7 days with regular shaking and stirring. The extracts were filtered through cotton and were concentrated by using rotary evaporator to get gummy mass with 10.1% yield (w/w). After dilution with adequate amount of water, the CEF (5 gm) was partitioned with pet-ether and chloroform to get petroleum ether (PEF 2.65 gm), chloroform (EAF, 1.63gm), and aqueous (AQF, weight was not measured) fraction.

2.3 *In vitro* Alpha-amylase inhibitory assay (Anti-diabetic assay)
In vitro anti-diabetic activity was evaluated by the method called "Alpha-amylase inhibitory assay" described by Rege and Choudhary, 2014 [8]. In brief, the mixture of phosphate buffer (100 µl of PH 6.8), alpha-amylase (20µl) and various concentrations of plant extracts (40 µl each) were incubated at room temperature for 10 min and then 40µl soluble starch was added and incubated for the next 15 min at 37 ˚C. The reaction was stopped by adding 40 µl 1N HCl followed by the addition of 200 µl iodine reagent (which was prepared by mixing 5 mM KI and 5 mM I_2).
Absorbance of each mixture was recorded at 620 nm by spectrophotometer and the experiment was repeated thrice. The negative control (blanks) samples were prepared without plant extract and Acarbose at various concentrations was used as the reference alpha amylase inhibitor. The inhibition percentage was calculated from given formula and from plots of percent inhibition versus log inhibitor concentration, the IC_{50} values were calculated.

Inhibition (%) = [(Absorbance of Negative control – Absorbance of Sample)/Absorbance of Negative control] ×100.

2.4 *In vitro* Anti-inflammatory assay
In vitro anti-inflammatory activity of the fractions were assessed by evaluating their ability to inhibit hypotonic solution and heat-induced haemolysis of human erythrocytes [9-10]. In brief, red blood cells (RBCs) suspension was prepared by collecting fresh human blood (10 ml) from healthy human volunteers who have not a record of taking oral contraceptive or anticoagulant therapy and was transferred to the centrifuge tubes. The tubes were centrifuged for 10 min at 3000 rpm and were washed three times with equal volume of normal saline. The volume of blood was measured and reconstituted as 10% v/v suspension with normal saline.

2.4.1 Heat induced haemolysis
5 ml isotonic buffer containing five concentrations (50, 100, 200, 400 and 800 µg/ml) of ethanol solution of plants extracts were put into centrifuge tubes. 50µl erythrocyte suspension was added to each tube, mixed gently and was incubated for 20 min at 54 ^{0}C in a water bath. The reaction mixture was centrifuged at 5000 rpm for 5 min and the absorbance of the supernatant was measured at 540 nm using spectrophotometer. Same concentration of aspirin was used as a reference standard and the vehicle, in the same amount, was added to another tube as control. The percentage inhibition [11] of haemolysis was calculated according to the equation:

Percentage (%) inhibition of haemolysis = 100 × [1 – (OD_2-OD_1)/(OD_3-OD_1)]

Where OD_1 and OD_2 are test sample (unheated and heated, respectively), and OD_3 is heated control sample.

2.4.2 Hypotonic solution induced haemolysis
Five concentrations (50, 100, 200, 400 and 800 µg/ml) of ethanol solution of plants extracts were taken and 2 ml hyposaline (0.25% NaCl) was added to each. 1 ml (0.15M) $Na_3(PO_4)$ buffer and 0.5 ml 10% RBC solution was added in each test tube. Mixtures were incubated for 10 min at RT. Then the mixtures were centrifuged at 5000 rpm for 5 min. Absorbance was taken at 540 nm. Aspirin with same concentrations were used as standard [12-13]. The percentage inhibition of haemolysis was calculated according to the equation:

Percentage (%) inhibition of haemolysis = 100 - [(Absorbance of test - Absorbance of control)/Absorbance of Blood control] × 100

2.5 Phytochemical screening of CEF
The crude ethanolic extracts, CEF was tested for the presence of variety of phytochemicals including steroids, saponin, anthocyanin, betacyanin, starch, glycosides and terpinoids and the qualitative results are expressed as (+) for the presence and (-) for the absence of phytochemicals. Detail methods are explained in previously published paper [10, 14].

2.6 Statistical analysis
Statistical comparisons were performed using Microsoft Excel, 2020. Mean values ± SD. were calculated for the parameters where applicable.

3. Results and Discussion
***In vitro* Anti-diabetic assay:** The result of *In vitro* Anti-diabetic assay of petroleum ether (PEF) and ethyl acetate (EAF) fractions obtaining from crude ethanolic extract of leaf of *Lawsonia inermis* L. through fractionation is shown in Table 1 and the concentration vs % of inhibition is represented in Figure 1.

Table 1: *In vitro* Anti-diabetic assay

Conc (µg/ml)	% of inhibition		
	Acarbose	PEF	EAF
500	95.81	83.56	64.65
250	90.69	65.73	46.51
125	68.99	56.58	38.29
62.5	53.48	19.37	24.03
31.25	27.13	16.27	10.07
IC_{50}	57.2	206.11	323.1

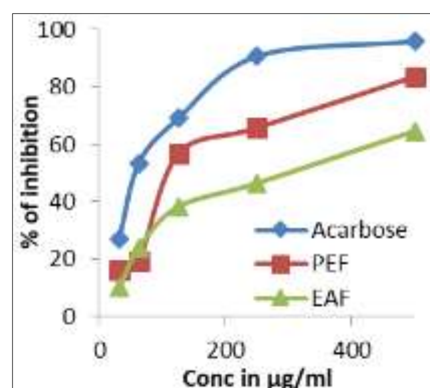


Fig 1: *In vitro* anti-diabetic assay of two fractions from *Lawsonia inermis*

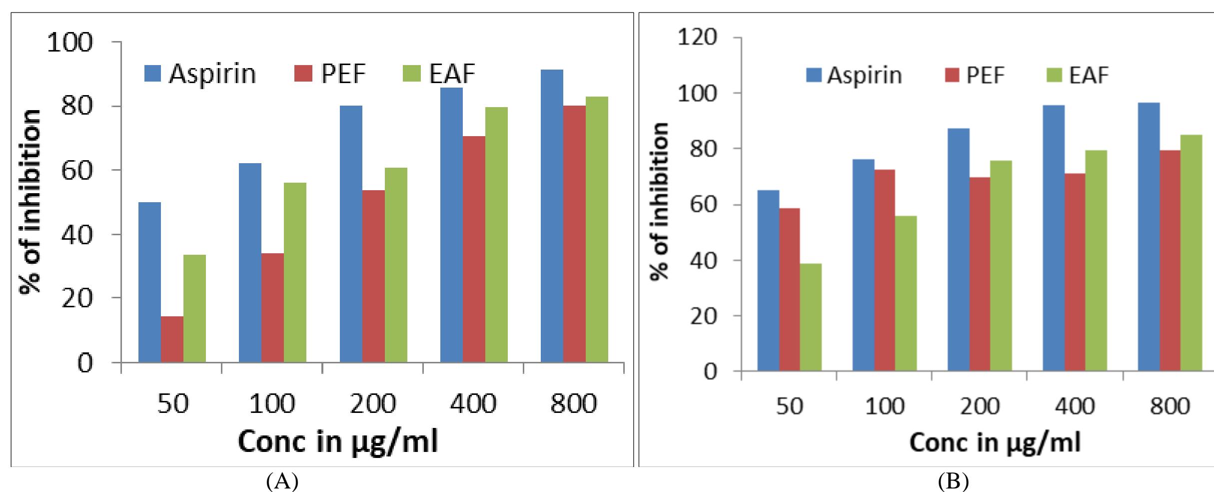
From the results of *In vitro* anti-diabetic assay, it was found that pet-ether fraction of leaves of *L. inermis* L. has significant ability to inhibit alpha amylase activity when compared to standard. The IC_{50} values of PEF, EAF and acarbose are 206.11, 323.1 and 57.2 µg/ml, respectively (Table 1 and Figure 1).

***In vitro* anti-inflammatory activity:** The observed anti-inflammatory activity of PEF, EAF and standard was concentration dependent, and the percent of protection increased with increase in the concentration of the samples. PEF, EAF and standard showed 14.2±0.47, 33.8±1.23 and 50.1±0.81, and 80.0±0.79, 82.9±1.04 and 91.4±0.22 inhibition of heat induced RBC haemolysis at a concentration of 50 and 800 µg/ml, respectively. Among the two fractions, EAF showed higher anti-inflammatory activity than EAF and is closed to that of the standard (Table 2, Figure 2A).

Table 2: Effect of PEF and EAF on heat and hypotonicity-induced haemolysis

Sample	Conc. (µg/ml)	% of inhibition of Haemolysis	
		1	2
Aspirin	50	50.1±0.81	65.0±1.84
	100	62.3±0.15	76.0±5.48
	200	80.0±0.09	87.0±0.12
	400	85.6±0.66	95.5±0.32
	800	91.4±0.22	96.3±0.16
PEF	50	14.2±0.47	58.8±3.85
	100	33.9±0.20	72.5±3.30
	200	53.9±4.03	69.7±0.56
	400	70.8±1.01	71.1±0.06
	800	80.0±0.79	79.5±0.24
EAF	50	33.8±1.23	38.6±3.88
	100	55.9±0.70	55.8±0.22
	200	60.6±1.27	75.7±0.25
	400	79.7±1.29	79.6±0.11
	800	82.9±1.04	84.8±0.05

NB: 1: Heat Induced and 2: Hypotonic solution Induced RBC haemolysis



(A) (B)

Fig 2: Anti-inflammatory activity of PEF and EAF using (A) Heat induced haemolysis and (B) Hypotonicity induced haemolysis.

Hypotonicity solution induced RBC membrane stabilization results are shown in Table 2 and Figure 2B. At a concentration of 50 and 800 µg/ml, PEF, EAF and standard showed 58.8±3.85, 38.6±3.88 and 65±1.84 and 79.5±0.24, 84.8±1.04, 96.3±0.45% of inhibition, respectively. Comparing with standard Aspirin, EAF showed remarkable activity and the activity is higher than PEF.

Phytochemical screening: The result of phytochemical screening is shown in Table 3. From the table it is shown that the leave extract is rich of alkaloid, flavonoids and other polyphenols as well as sterols.

Table 3: Phytochemical screening of *L. inermis*

Test	Result
Alkaloids	+
Flavonoids	+
phenols	+
Phlobatannins	-
Proteins	-
Resins	+
Tannins	+
Steroids	+
Saponin	-
Anthocyanin and Betacynin	-
Starch	+
Glycosides	+
Terpinoids	+

NB: +: presence, -: absence

4. Conclusions

Modern world is going to turn into plant medicine due to minimum side effects, availability and cost effectiveness. According to the WHO, medicinal plants would be the best source for obtaining variety of drugs. Reported multi-pharmacological activities of *L. inermis* made the plant interesting for pharmacologist as well as natural product chemist. Results of our studies showed that two different fractions of leaf ethanolic extract have significant anti-diabetic and anti-inflammatory activities, hence the extract as well as phytochemicals present in the extracts like alkaloid, flavonoids and polyphenols might be a potential alternative[15]. Since our study was carried out using crude ethanolic extract and its fractions, it is necessary to determine the toxicity of the extracts and active constituents, their side effects and pharmaco-kinetic properties.

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