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Research Article

ANTI-INFLAMMATORY STUDY (*IN-VITRO* AND *IN-VIVO*) AND HPTLC DENSITOMERIC DETERMINATION OF QUERCETIN FROM METHANOLIC LEAF EXTRACT OF *HOTTUYNIA CORDATA* THUNB. FROM MIZORAM, INDIA

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ABSTRACT

Objective: This study aimed to investigate the anti-inflammatory activity of the methanolic leaf extract of *Houttuynia cordata* Thunb. from Mizoram, India, using *in-vitro* and *in-vivo* models. In addition, the study also aimed to determine the quercetin content of the methanolic leaf extract using high-performance thin-layer chromatography (HPTLC) densitometry.

Methods: The methanolic leaf extract of *H. cordata* was prepared using successive Soxhlet extraction, and its anti-inflammatory activity was evaluated using *in-vitro* models such as inhibition of protein denaturation and inhibition of proteinase activity. The *in-vivo* anti-inflammatory activity of the extract was evaluated using carrageenan-induced paw edema in rats. The quercetin content of the extract was determined using HPTLC densitometry.

Results: The methanolic leaf extract of *H. cordata* showed significant anti-inflammatory activity in both *in-vitro* and *in-vivo* models. The extract exhibited a dose-dependent inhibition of protein denaturation and proteinase activity and also significantly reduced paw edema in the rat model. The IC50 values of egg albumin and bovine serum albumin denaturation studies were 317.365 µg/mL and 198.918 µg/mL, respectively. The results showed that the methanolic leaf extract of the plant was 200 mg/kg b.w. and 400 mg/kg b.w., which considerably reduced carrageenan-induced paw edema, and their effects were comparable to those of the standard diclofenac sodium. The HPTLC densitometry analysis revealed the presence of quercetin in the extract, with a quercetin content of 21.8 ng/µL.

Conclusion: The methanolic leaf extract of *H. cordata* Thunb. from Mizoram, India demonstrated significant anti-inflammatory activity in both *in-vitro* and *in-vivo* models. The presence of quercetin in the extract may contribute to its anti-inflammatory properties. These findings provide scientific evidence for the traditional use of *H. cordata* as an anti-inflammatory agent.

Keywords: *Houttuynia cordata* Thunb, Anti-inflammatory, High-performance thin layer chromatography, Quercetin.

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INTRODUCTION

Natural products possessing anti-inflammatory properties have been used for a long time as a traditional treatment for inflammatory conditions such as fevers, pain, and migraines [1]. Inflammation is a normal response of our body against harmful microorganisms, physical stress, cancer, etc. In most cases, the development of inflammation involves both the innate immune response and the adaptive immunological response. The innate immune system, which involves the action of numerous cells, including macrophages, mast cells, and dendritic cells, is the primary defense mechanism against invading microbes and cancer cells. More specialized cells, such as B- and T-cells are active in the adaptive immune system and are in charge of eliminating foreign infections and cancer cells by creating specialized receptors and antibodies [2].

High-performance thin-layer chromatography (HPTLC) is an advanced and sophisticated form of chromatography, mostly used for the identification and quantification of phytochemical compounds in the case of natural products. This advanced chromatography depends on the principle of adsorption, where the components having a higher affinity toward the stationary phase (adsorbent) travel slower and the components with a lesser affinity toward the stationary phase travel faster. The phytochemical identification is done by comparing the retention factor of the unknown sample with that of the known standard. This form of chromatography is widely used for its proven accuracy and cost-effective reasons [3].

Houttuynia cordata Thunb, also known as Uithinthang in the Mizo language, is a member of the Saururaceae family, found in countries such as China, Japan, Korea, Thailand, Vietnam, and India. It often grows between 300 and 2600 meters above sea level in hilly, wet, and shaded regions. The tribal populace of India has been using it for a very long time to treat a variety of illnesses and disorders. It contains biologically active substances such as flavones, phenolic components (quercetin and chlorogenic acid), essential oils, and alkaloids, according to studies. Although there is currently no high-quality clinical evidence to support the safety or efficacy of such usage, this ethnomedicinal plant was widely utilized in traditional Chinese medicine, notably by Chinese scientists in an effort to cure SARS and a number of other illnesses.

METHODS

Collection and authentication of plant

Fresh leaves of *H. cordata* Thunb. were collected from Tanhril, Aizawl, Mizoram, India. Authentication was done in Shillong, Eastern Circle, and the Botanical Survey of India. The specimen has been identified as *H. cordata* Thunb. (Saururaceae) with the reference BSI/ERC/Tech/2021/287.

Experimental animals

In this investigation, Wistar albino rats (150–200 g, any sex) were employed. The animals were kept at the Regional Institute of Paramedical and Nursing Sciences' Department of Pharmacy's animal house. The animals were kept in sizable propylene cages

Table 1: Percentage inhibition of egg albumin of methanol extract of *Houttuynia cordata* thunb leaves and diclofenac sodium

Concentration (µg/mL)	Percentage inhibition (%)	
	Extract Mean±SEM	Standard Mean±SEM
100	17.789±0.099**	26.943±0.049**
200	32.037±0.086**	41.882±0.049**
300	42.918±0.049**	56.303±0.179**
400	63.903±0.049**	75.906±0.217**
500	79.879±0.049**	85.837±0.149**

SEM: Standard error of the mean. All value is Mean±SEM, n=3. One-way Analysis of Variance (ANOVA) followed by Dunnett's test was performed as the significance. The minimum value of *p<0.05 considered significant, **p<0.01,***p<0.001 as compared with control group

Table 2: Percentage inhibition of bovine serum albumin denaturation of methanol extract of *Houttuynia cordata* thunb leaves and diclofenac sodium

Concentration (µg/mL)	Percentage inhibition (%)	
	Extract Mean±SEM	Standard Mean±SEM
100	44.696±0.126**	45.328±0.145**
200	47.474±0.145**	53.156±0.218**
300	57.07±0.193**	63.383±0.126**
400	66.792±0.145**	71.843±0.072**
500	72.348±0.126**	82.33±0.192**

SEM: Standard error of the mean. All value is Mean±SEM, n=3. One-way Analysis of Variance (ANOVA), followed by Dunnett's test was performed as the significance. The minimum value of *p<0.05 is considered significant, **p<0.01,***p<0.001 as compared with the control group

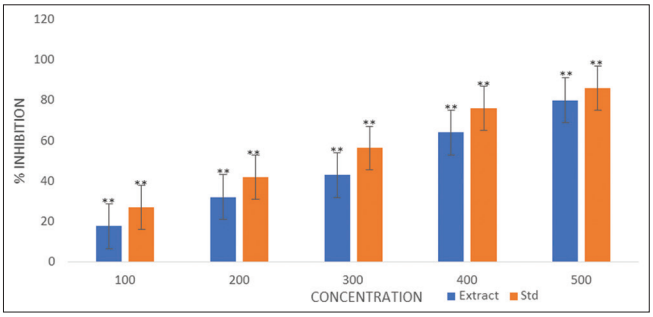


Fig. 1: Percentage inhibition of protein denaturation of methanol extract of *Houttuynia cordata* thunband standard diclofenac sodium

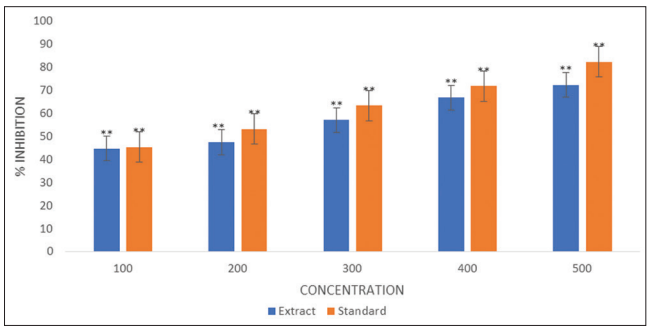


Fig. 2: % inhibition of BSA denaturation of methanol extract of *Houttuynia cordata* thunband standard diclofenac sodium

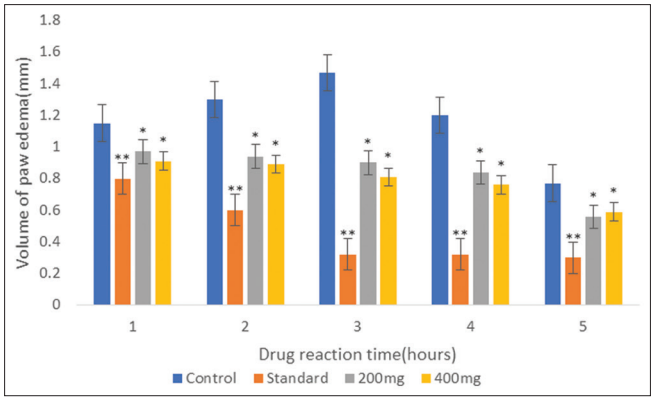


Fig. 3: Comparison of the anti-inflammatory effects of *Houttuynia cordata* thunb with control and standard group

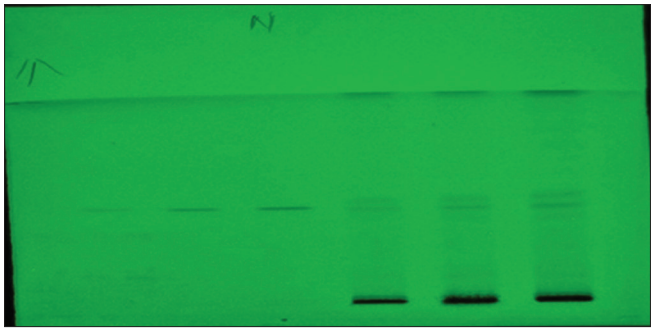


Fig. 4: TLC plates seen at 254 nm for methanolic extract of *Houttuynia cordata* thunb

Track	Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	1	0.45 Rf	1.5 AU	0.49 Rf	54.8 AU	100.00 %	0.50 Rf	3.2 AU	581.3 AU	100.00 %	Quercetin
2	1	0.45 Rf	3.4 AU	0.48 Rf	123.8 AU	100.00 %	0.50 Rf	0.4 AU	1279.0 AU	100.00 %	Quercetin
3	1	0.44 Rf	0.9 AU	0.48 Rf	216.8 AU	100.00 %	0.50 Rf	0.4 AU	2127.5 AU	100.00 %	Quercetin
4	1	0.44 Rf	0.4 AU	0.47 Rf	71.2 AU	100.00 %	0.49 Rf	0.6 AU	918.4 AU	100.00 %	Quercetin
5	1	0.44 Rf	0.1 AU	0.47 Rf	87.5 AU	100.00 %	0.49 Rf	0.1 AU	1217.3 AU	100.00 %	Quercetin
6	1	0.44 Rf	0.3 AU	0.47 Rf	99.7 AU	100.00 %	0.49 Rf	0.5 AU	1748.0 AU	100.00 %	Quercetin

Fig. 5: Peak table of methanolic extract of *Houttuynia cordata* thunb

at a temperature of 22+2°C with a 12-h cycle of darkness and light and were given access to water and rat pellet meal at will. Before the experimental session, all animals underwent at least an 1-week acclimatization period. Following approval (approved number: Institutional Animal Ethics Committee's [IAEC]/RIPANS/78), all experimental procedures were carried out in accordance with the IAEC recommendations.

Preparation of extract

The *H. cordata* Thunb. fresh leaves were properly washed under running water and allowed to dry in the shade for 3 weeks. Following that, dried leaves were mechanically ground into a coarse, homogenous powder and stored in airtight containers for extraction. After drying, the plant material was ground into a powder. Different solvents, including petroleum ether, chloroform, and methanol based on polarity, were used to macerate the plant powder for 72 h. The solvents were then recovered using steam distillation, and the extracts were stored for later use at 4°C in the refrigerator [4].

Table 3: Results of methanolic extract in carrageenan induced paw edema

S. No.	Groups	Dose (mg/kg)	Initial paw thickness 0 h	Change in paw thickness in mm (Mean±SEM) value+ % inhibition of paw edema				
				1hr	2hr	3hr	4hr	5hr
1	Control	0.5 mL/kg	3.5±0.15	4.6±0.06	4.75±0.09	4.92±0.10	4.65±0.18	4.27±0.10
2	Standard	10 mg/kg	3.54±0.02	4.4±0.09** 30.43%	4.2±0.09** 53.84%	3.92±0.09** 78.23%	3.92±0.09** 73.33%	3.9±0.07** 61.03%
3	Extract (Low dose)	200	3.57±0.11	4.54±0.01* 15.65%	4.52±0.006* 27.11%	4.48±0.01* 38.26%	4.42±0.02* 29.37%	4.14±0.03* 26.29%
4	Extract (High dose)	400	4.075±0.1	4.98±0.008* 20.86%	4.97±0.006* 31.15%	4.88±0.008* 44.72%	4.83±0.02* 36.66%	4.66±0.03* 22.72%

SEM: Standard error of mean

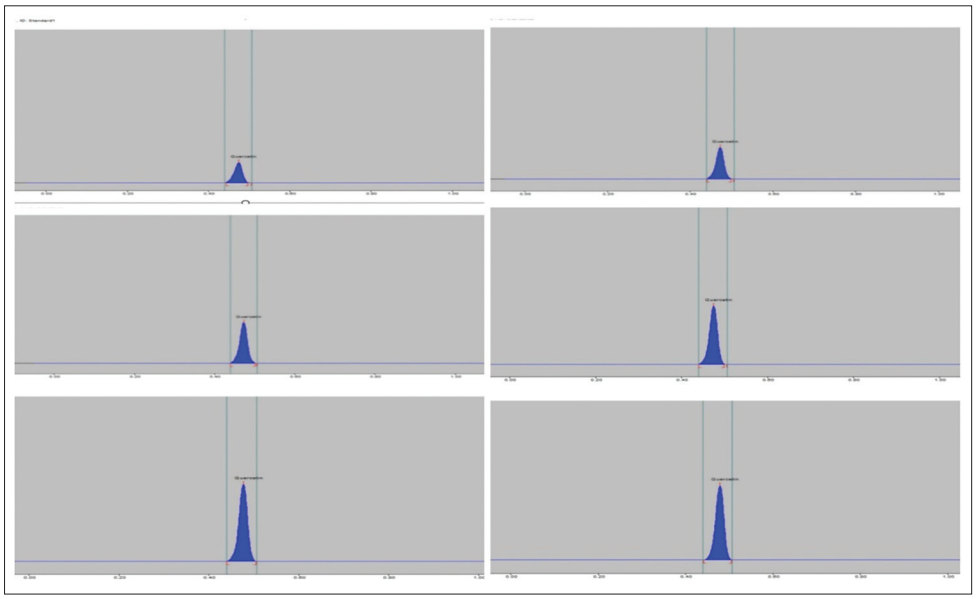


Fig. 6: Peak diagram of six standard levels (2, 3, 4, 5, 6 and 7 µL) respectively

Phytochemical screening

The preliminary phytochemical screening of leaves of *H. cordata* Thunb. extracts using different solvents was already reported in prior published articles [5,6].

Acute toxicity test

H. cordata Thunb. underwent an acute toxicity test, which was already completed and published [7]. There was no mortality and no appreciable weight loss in the *H. cordata* extract. In addition, all mice were alert and well-groomed before being put to death.

***In vitro* anti-inflammatory activity**

Egg albumin denaturation method

The reaction mixture (5 mL) consists of 0.2 mL of egg albumin (from a fresh hen's egg), 2.8 mL of phosphate-buffered saline (PBS) (pH 6.4), and 2 mL of varying concentrations of plant extracts. A similar volume of double-distilled water served as a control. Then the mixtures were incubated at 37±2°C in an incubator for 15 min and then heated at 70°C for 5 min. After cooling, their absorbance was measured at 660 nm by using the vehicle as a blank. Diclofenac was used as a reference drug and treated similarly for the determination of absorbance [8]. The percentage inhibition of protein denaturation was calculated as follows:

$$\text{Percentage inhibition } (\%) = 100 - \frac{\text{Absorbanceof control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Bovine serum albumin (BSA) denaturation method

The test solution (0.5 mL) consists of 0.45 mL of BSA (5% w/v aqueous solution) and 0.05 mL of the test solution (100, 200, 300, 400, and 500 µg/mL). The test control solution (0.5 mL) consists of 0.45 mL of BSA (5% w/v aqueous solution) and 0.05 mL of distilled water. The standard solution (0.5 mL) consists of 0.45 mL of BSA (5% w/v aqueous solution) and 0.05 mL of diclofenac sodium (100, 200, 300, 400, and 500 µg/mL). All the above solutions were adjusted to pH 6.3 using 1N hydrochloric acid. The samples were incubated at 37°C for 20 min, and the temperature was increased to keep the samples at 57°C for 3 min. After cooling, 2.5 mL of PBS was added to the above solutions. The absorbance was measured using ultraviolet (UV), a visible spectrophotometer at 416 nm [9]. The percentage inhibition of protein denaturation was calculated by the formula:

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

***In vivo* anti-inflammatory activity**

The carrageenan-induced rat paw edema experiment was used to assess the extract's anti-inflammatory efficacy. Four equal groups of 16 Wistar albino rats (150–200 g) of either sex were formed. Distilled water was administered as a control to Group I. Groups II and III received methanolic leaf extract of *H. cordata* Thunb. at doses ranging from 200 mg/kg body weight to 400 mg/kg body weight, whereas Group IV received the standard dose of diclofenac (10 mg/kg) [10]. All

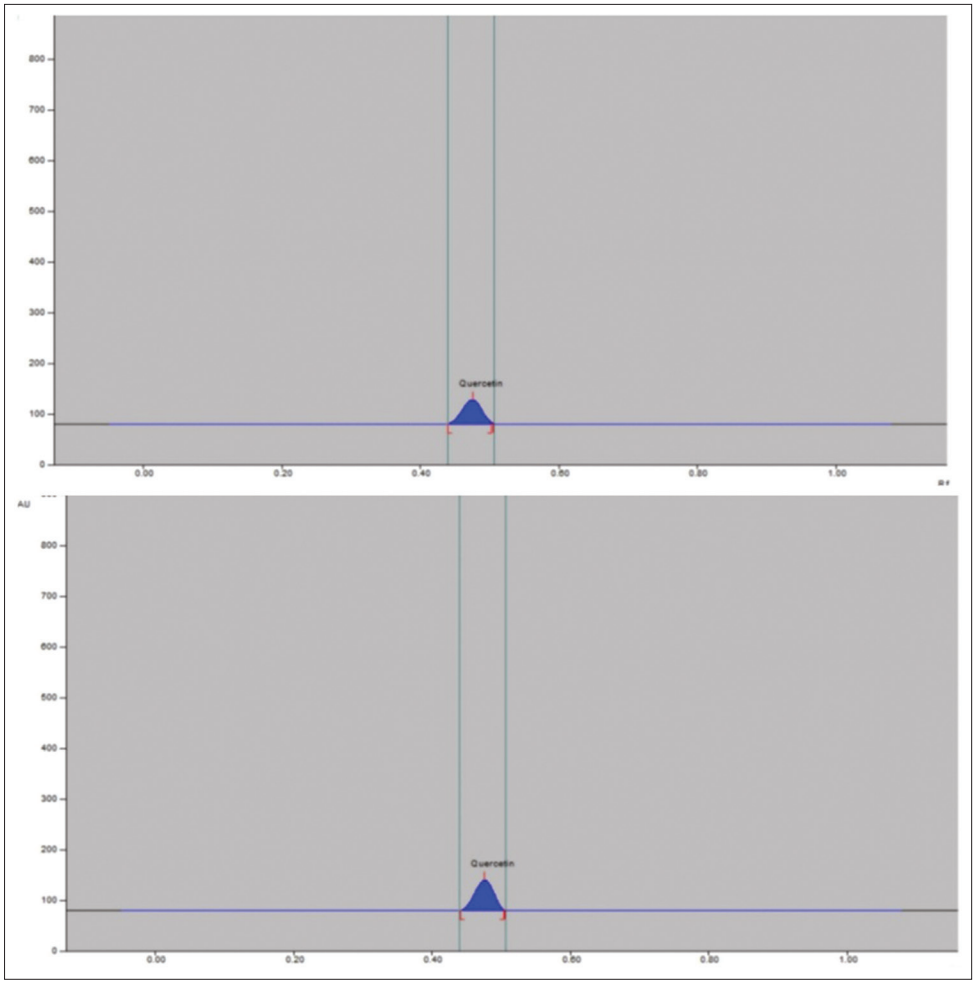


Fig. 7: Peak diagram of sample concentrations (6 and 8 µL) respectively

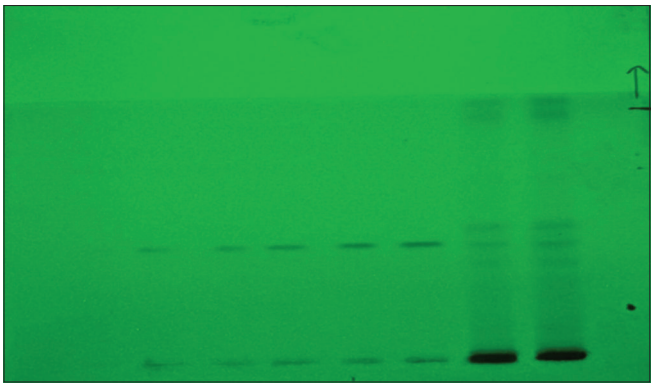


Fig. 8: TLC plate under UV showing five standard levels and two sample of different concentration

medications were administered orally. 0.1 mL of a 1% w/v carrageenan solution in normal saline was injected into the subplantar tissue of the left hind paw an hour after the medications had been administered. The paw volume of the rats was measured in the digital Vernier caliper, at the end of 0 min, 1 h, 2 h, 3 h, 4 h, and 5 h. The percentage increase in paw edema of the treated groups was compared with that of the control group and the inhibitory effect of drugs was studied. The relative potency of the drugs under investigation was calculated based on the percentage inhibition of inflammation [11]. The percent inhibition was calculated using the formula:

$$Percentage\,inhibition\,(\%) = \frac{V_c - V_t}{V_c} \times 100$$

where Vc and Vt represent the average paw volume of control and treated animals, respectively.

High-performance thin-layer chromatography fingerprinting

By dissolving 30 mg of the plant's extract in 1.5 mL of methanol, a sample working solution of *H. cordata* Thunb. with a concentration of 20 mg/mL was created. A stock solution of the standard quercetin of 1000 μg/mL concentration was prepared. A standard working solution of 50 μg/mL concentration was prepared by taking 50 μL of the stock solution and adding 950 μL of methanol. The mobile phase was prepared by mixing toluene, ethyl acetate, and formic acid (5:4:0.2) [12]. During the development of each plate, a fresh mobile phase was prepared. Bands of the sample (2 µL, 4 µL, and 6 µL) and standard (4 µL, 6 µL, and 8 µL) were applied in duplicate on pre-coated TLC silica gel 60 F254 luminum sheets (10×10 cm) with the help of a Linomat 5 applicator connected to the CAMAGHP TLC system programmed through WINCATS software. After the sample application, the chromatogram was developed in a twin-trough glass chamber (10×10 cm) saturated with the developed solvent for 20 min. The air-dried plate was viewed in UV radiation as shown in Fig. 8 and mid-day light. The developed plate was documented using a digital documentation system under UV light at 366 nm and 254 nm. The chromatogram was scanned by the CAMAG TLC Scanner for densitometric evaluation of HPTLC chromatograms. The fingerprint data were recorded by WINCATS software.

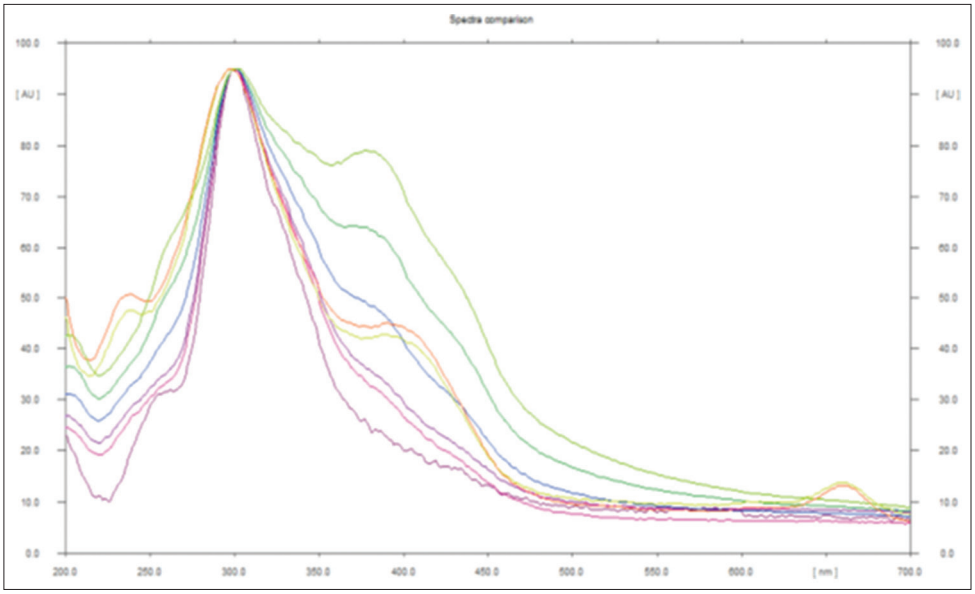
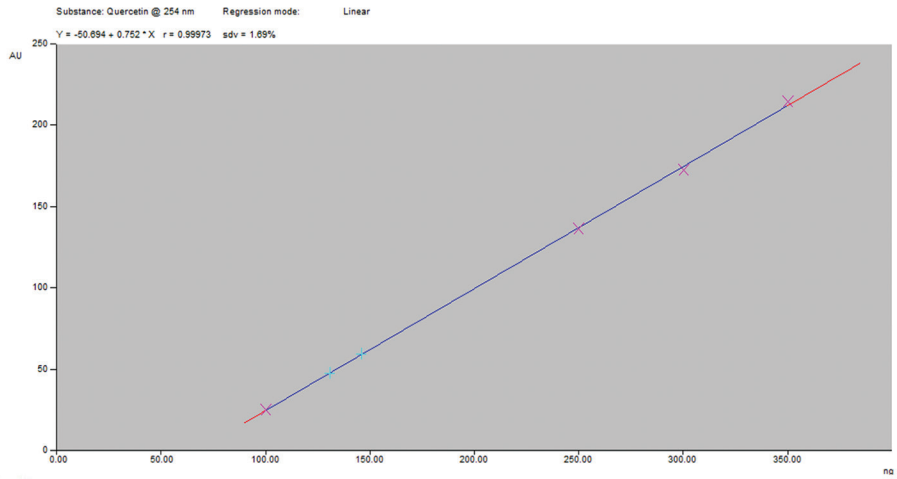


Fig. 9: Spectral comparison of quercetin and sample

Track	Vial	Rf	Amount	Height	X(Calc)
1	1	0.47	100.00 ng	25.23	
2	1				
3	1				
4	1	0.47	250.00 ng	136.78	
5	1	0.48	300.00 ng	172.72	
6	1	0.48	350.00 ng	214.78	
7	2	0.48		47.72	130.82 ng
8	2	0.48		59.27	146.17 ng



winCATS summary report

Calibration results per Analysis

Sample from vial 2: H. cordata

Result via height

Substance	Rf	X(average)	CV [%]	n	Regression	Remark
Quercetin	0.48	138.50 ng	7.835	2	Linear	

Fig. 10: Standard curve with calibration result

HPTLC quantification of quercetin

HPTLC was utilized for the quantification of quercetin on the sample, which was equipped with a Linomat-5 applicator (Camag), a TLC scanner smart Digi (Camag), and Wincats software. 6 µL and 8 µL of the sample extract were applied on the HPTLC plate with silica gel 60 F254 (10×10 cm), and for the standard calibration curve, pure quercetin at the concentration range of 100 ng, 150 ng, 200 ng, 250 ng, 300 ng, and 350 ng was applied on the same plate using the Linomat 5 applicator. The plate was developed in a twin chamber, and the mobile phase consisted of toluene, ethyl acetate, and formic acid (5:4:0.2) v/v.

Densitometric analysis of quercetin was carried out in the absorbance mode at a wavelength of 254 nm using a TLC scanner [13].

RESULTS

***In-vitro* anti-inflammatory activity**

Egg albumin denaturation activity

In-vitro anti-inflammatory activity was performed by the egg albumin denaturation method [14] at different concentrations and showed various degrees of inhibition as compared to standard Diclofenac sodium at similar concentrations. The percentage inhibition of methanolic extract and standard is shown in Table 1 and Fig. 1. The IC_{50} of *H. cordata* Thunb and standard diclofenac sodium were found to be 317.365 µg/mL and 2 52.781 µg/mL, respectively. From this experimental result, the methanolic plant extract showed significant inhibition of denaturation of egg albumin in a concentration-dependent manner when compared to the diclofenac sodium standard drug. The results of anti-inflammatory activity studies indicated that the methanol extract of the leaf of *H. cordata* Thunb possesses promising anti-inflammatory activity. It also revealed that the plant possesses a certain percentage of inhibition of inflammation as there is the presence of lavonoids.

BSA denaturation activity

The denaturation inhibition capability of the methanolic extract of *H. cordata* Thunb was found to be dose-dependent as with the increase in there was a decrease in absorbance as well. The percentage inhibition of methanolic extract and diclofenac standard are shown in Table 2 and Fig. 2. IC50 values were calculated for standards as well as for extracts [15]. The IC_{50} values of both the diclofenac sodium standard and the methanol extract of *H. cordata* Thunb were 158.24 µg/mL and 198.918 µg/mL, respectively.

***In-vivo* anti-inflammatory activity**

Carrageenan-induced paw edema

Carrageenan was injected subcutaneously into the paws of rats, and this resulted in edema, indicating acute inflammation. Table 3 displays the paw edema that the various rat groups had after receiving treatments with regular diclofenac sodium and a methanolic extract of:

H. cordata thunb

According to the findings, methanolic extract at doses of 200 and 400 mg/kg of body weight significantly decreased the paw edema caused by carrageenan, and their effects were on par with those of diclofenac sodium. It was discovered that the extract's anti-inflammatory effect was dose-dependent. Fig. 3 displays a comparison of medication responses.

All the results presented here are mean±standard error of mean of n=4, compared with the control by one-way ANOVA, Dunnet's multiple comparison tests, and the statistical significance taken at the level of $p<0.05$, $p<0.01$ as significant, and $p<0.001$ is very significant.

HPTLC fingerprint profiling

HPTLC fingerprinting of *H. cordata* Thunb was done using quercetin as a standard. The development of the chromatogram was done in a twin-thorough chamber with the mobile phase: toluene, ethyl acetate, and formic acid (5:4:0.2) [16]. Visualization of the dried TLC plates under UV at 254 nm showed that the bands of the methanolic extract of *H. cordata* Thunb aligned with those of the bands of the standard quercetin (Fig. 4)

The TLC plates were scanned at 386 nm, which showed peaks of quercetin in the methanolic extract of *H. crodata* Thunb. The peak table showed that the sample and the standard quercetin peaks have similar R_f (Fig. 5). A spectral comparison of the peaks was done, which further confirmed the presence of quercetin.

HPTLC quantification

HPTLC quantification was done by taking six standard levels (2, 3, 4, 5, 6, and 7 µL) and two different sample concentrations (6 and 8 µL). The plates were developed in a twin-thorough chamber using the mobile phase:-Toluene, ethyl acetate, and formic acid (5:4:0.2) [17]. The detection of the peaks was done by scanning the plates at 254 nm. The peak diagram of the chromatogram (Figs. 6 and 7) shows that the extracted sample contains quercetin.

The spectra of the peaks of standard quercetin and the sample overlapped, which confirmed the presence of quercetin as given in Fig. 9. The standard calibration curve was plotted as shown in Fig. 10. The amount of quercetin present in the methanolic extract of *H. cordata* was found to be 21.8 ng/mL.

CONCLUSION

The current study investigated the anti-inflammatory activity of methanolic leaf extracts of *H. cordata* Thunb. using both *in-vitro* and *in-vivo* methods. The results showed that the extract exhibited anti-inflammatory activity in a concentration-dependent manner, which may be due to the presence of phytochemicals. The exact mechanism of action and specific compounds responsible for the anti-inflammatory effects require further study.

The study also identified the presence of quercetin in the extract through HPTLC examination and quantified it at 21.8 ng/µL. Quercetin is a known antioxidant and anti-inflammatory compound with potential health benefits, including anti-viral, anti-allergic, and anti-cancer properties, as well as improved exercise performance and reduced muscular fatigue.

Based on the results, it can be inferred that the presence of quercetin in *H. cordata* Thunb. The extract may contribute to its anti-inflammatory activity. However, further research is needed to determine the specific role of quercetin and other compounds in the extract's overall health benefits.

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AUTHOR CONTRIBUTION

Equal contributions were made by all the respected authors.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS FUNDING

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