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Phytochemical screening, antioxidant, anthelmintic and protein denaturation activities of *Barleria lupulina* leaf extract

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Abstract
In South Asian countries, *Barleria lupulina* Lindl is used ethno-medically to treat rheumatoid arthritis, diabetes, fever, pain, mental illness, and snake bites. This work aims to examine the pharmacological activities of ethanolic leaf extract of *B. lupulina*, including anthelminthic, antioxidant, and protein denaturation activity, with phytochemical screening. Secondary metabolites such as carbohydrates, tannins, flavonoids, saponins, steroids, glycosides, and quinones were identified by preliminary phytochemical tests. The ethanol extract of *B. lupulina* leaves showed considerable free radical scavenging activity (IC50 = 8.65 µg/ml) in the quantitative assay, compared to ascorbic acid (IC50 = 4.72 µg/ml) as a standard. The total phenolic content (TPC) of *B. lupulina* was measured at 153±0.11 µg GAE/g, whereas the total flavonoid content (TFC) was 32.26 ± 0.09 µg QE/g dry weight extract. Additionally, the anthelmintic properties of *B. lupulina* leaves against *Paramphistomum cervi* revealed encouraging outcomes at 200 µg/ml, compared to the reference standard (Albendazole). Moreover, Egg albumin denaturation was used to measure the protein denaturation activity. *B. lupulina* leaves extract and Diclofenac Na (standard) demonstrated percentage inhibition in the protein denaturation test of 53.78%, 55.25%, 60.6%, and 63.3%, 75%, 79%, respectively. Consequently, additional research is required to isolate and characterize the pure chemicals found in the extract, as they may provide a natural remedy for oxidative stress.

Keywords: Antioxidant, anthelmintic, inflammation, DPPH, *Barleria lupulina*

Introduction
Natural products are extremely useful in a variety of areas, including organic synthesis, bioorganic chemistry, medicine, and natural product chemistry. Natural products are an important source of leads for drug development. Figures about the sources of novel pharmaceuticals between 1981 and 2007 show that over 50% of medications licensed since 1994 are derived from natural sources as active metabolites [1]. Pharmacological models of medicinal plants and natural products have been systematically considered as alternative therapies for the treatment of a range of illnesses [2]. Natural plants have unique effects on situations in terms of efficacy, safety, and economy [3]. Plants or their synthetic or semisynthetic derivatives provide a large number of compounds that are currently utilized as antimalarial, antioxidant, anthelminthic, anti-inflammatory, anti-diabetic, anti-aging, anti-tumor, and anticancer medicines [4, 5]. Although their exact dosage concentration, molecular mechanism of action, and structural characterization are not always fully defined and documented, several natural substances have been utilized for hundreds of years as natural medicines to cure human ailments.

In Bangladesh, *Barleria lupulina* Lindl., a member of the *Acanthaceae* family, grows wild and on fallow land. It is referred to as "Hophead Philippine violet" in English and "Lal-tarokh, Kali-chondal, or Rahu-chondal" in Bengali. It is a perennial shrub with branches that is evergreen and reaches a height of 150 cm. In Bangladeshi and other traditional medical systems, the herb is regarded as therapeutic. It was interesting to evaluate the plant as a potential source of lead compounds and innovative drugs because it has also been discovered to possess bioactive components with potential therapeutic properties and exhibit significant pharmacological actions [6].

In traditional Thai medicine, the herb is very well-known. Among the many uses are as an anti-inflammatory for amebic illnesses and as a defense against insect as well as snake bites [7, 8.] The paste from the leaves is used to cure acne and relieve pain; leaf juice is used to stop bleeding [8]. The plant is used as a diuretic to treat various illnesses in India, including diabetes, rheumatoid arthritis, fever, pain, mental disease, and snake bites. The plant is used as a diuretic to treat various illnesses in India, including diabetes, rheumatoid arthritis, fever, pain, mental disease, and snake bites [6]. Several carcinogenic models have investigated the antitumor efficacy of some *B. lupulina* components. Furthermore, reports have indicated that *B. lupulina* possesses strong antimicrobial, anti-inflammatory, analgesic, antiulcerogenic, antidiabetic, neuropharmacological, antibacterial, anticancer, anti-arthritis, acute and sub-chronic diuretic and antiviral properties [9].

These findings support our desire to find out more about this plant. We are now investigating in our lab the pharmacological properties of this plant extract. To provide pharmacological proof for the folklore claim of significant medicinal properties of *B. lupulina*, we assessed the antioxidant, anthelmintic, and protein denaturation efficiency of an ethanol extract of the plant's leaves in this work.

Materials and Methods

Plant collection and extraction

B. lupulina leaves were collected from Khulna, Bangladesh. After removing unwanted materials, the collected leaves were washed with water and allowed to dry in the shade for a week. The leaves were then ground into a coarse powder using a suitable grinder (Capacitor start motor, Wuhu motor manufacture, China), and ethanol was used as a solvent in the Soxhlet extraction process. It was stored for five days at room temperature. At last, the supernatant was obtained and put to use for several *in vitro* examinations.

Phytochemical analysis

The following screening experiments were performed on *B. lupulina* leaves extract to identify phytochemicals according to the previously published method [10, 11] after slight modifications.

Carbohydrate detection

Molish test (General test for carbohydrates)

One milliliter of molish reagent and two milliliters of aqueous extract were carefully mixed. A layer was then formed under the aqueous solution by adding a few drops of concentrated sulfuric acid to the test tube. A purple or reddish violet ring appeared at the confluence of the two layers, indicating the presence of carbohydrates.

Fehling's Test (Standard Test for reducing sugar)

Two milliliters of the sample were mixed with 1ml of Fehling's solutions A and B in equal amounts. Boil for a couple of minutes. There was no precipitate observed, indicating the lack of reducing sugar.

Tannins detection

Ferric Chloride Test

About 5 ml solution of the extract and 1 ml of 5%Ferric chloride solution were mixed in a test tube. The formation of black color signifies the existence of tannins.

Potassium Dichromate test

5 ml extract solution was added with 1 ml of 10% Potassium Dichromate solution in a test tube. A yellow precipitate indicates the presence of Tannins. Here, Gallic acid was used as standard.

Flavonoids detection

Test 1: Added a few drops of concentrated hydrochloric acid to 1 ml of extract. A red color was not found which indicates the presence of Flavonoids.

Test 2: 2 ml of each extract was added with a few drops of 20% sodium hydroxide, formation of an intense yellow color was observed. To this, a few drops of 70% dilute hydrochloric acid were added and the yellow color disappeared. The presence of yellow color indicates the presence of flavonoids in the sample extract.

Saponins detection

After diluting 1 ml of the extract solution with 20 ml of distilled water, it was agitated for 15 minutes in a graduated cylinder. There was no layer of foam, indicating the absence of saponin.

Gums Detection

5 ml solution of the extract was taken in a tube. Then, Molish reagent and sulphuric acid were added consequently. At the intersection of the two layers, a reddish-violet ring appeared, signifying the existence of gum.

Steroids Detection

Libermann-Burchard test

1 ml solution of chloroform extract was taken and then 2 ml Libermann-Burchard reagent was added. A greenish color was found which indicates the presence of steroids.

Glycosides detection

2 ml extract was taken in 1 ml of water. Then, 3ml chloroform and 10% ammonia solution were added. Pink color was found which is considered as an indication for the presence of glycosides.

Proteins detection

A small amount of 1% copper sulfate and 1 milliliter of 40% sodium hydroxide were added to 2 milliliters of each extract; the formation of a violet color suggests the existence of peptide-linking molecules in the sample.

Terpenoids detection

When two milliliters of chloroform and a few drops of concentrated sulfuric acid were added to 0.5 milliliters of extract, a reddish precipitate formed, suggesting that terpenoids were present in the extract.

Quinone detection

1 ml of extract was taken in a tube. 1 ml of conc. $H_2SO_4$was added. The formation of red color indicates the presence of quinone.

DPPH free radical scavenging assay

Using the DPPH model, the antioxidant capacity of the *B. lupulina* leaf extract was assessed using the prior procedure [12] with a few minor modifications. One milliliter of various sample concentrations (1, 1.95, 3.90, 7.81, 15.62, 31.25, 62.2, 125, 250, and 500 µg/ml) was mixed with three milliliters of DPPH 0.004% ethanol solution. The samples were placed in the dark for thirty minutes, and then a UV spectrophotometer

was used to quantify the OD (optical density) at 517 nm. The following formula was used to determine the radical scavenging activity:

% inhibition = (Blank OD-Sample OD/ Blank OD) × 100

Total Phenolic Content (TPC) assay
The TPC of the ethanol extract of *B. lupulina* leaves was determined by Folin–Ciocalteu (FC) reagent [13]. In a nutshell, 20ml of 80% ethanol was added to 200mg leaves powder and sonicated for 15 minutes using ultrasonic bath. After sonication, 2 ml of sample mixture was taken and centrifuged at 20000 × g for 5 minutes. Then the supernatant was transferred into a volumetric flask. On the other hand, Gallic acid (standard) was prepared in 80% ethanol at the concentrations of 15.62, 31.25, 62.5, 125, 250, 500 µg/ml. A separate volumetric flask was used to take each concentrations of gallic acid and samples. Each volumetric flask was then filled with 9 mL of distilled water. Each volumetric flask received 1 mL of diluted Folin-Ciocalteu's reagent, which was shaken continuously. After 5 minutes, 10 mL of 7% Na_2CO_3 solution was added to the mixture. The distilled water was incorporated to get a final amount of 25 mL. After 60 minutes of incubation at room temperature, absorbance was determined at 750 nm compared to the blank. Blanks were made similarly to samples, without extracts or standards. The extract's phenolic content was calculated using a calibration curve for gallic acid. The extract's total phenolic content was quantified in mg the equivalent of gallic acid (GAE) per gram dried leaves extract.

Total Flavonoid Content (TFC) assay
A 10 mL volumetric flask was filled with 1 mL of the extract or a standard solution containing quercetin (15.62, 31.25, 62.5, 125, 250, and 500 µg/mL). Four milliliters of distilled water were then added. Subsequently, 0.3 mL of 5% $NaNO_2$ had been added to the mixture. 10% $AlCl_3$ (0.3 mL) was inserted into the mixture after 5 minutes. After adding 1 mol/L NaOH (2 mL) to the mixture, the final amount of liquid was adjusted to 10 mL using distilled water, and the mixture was given a vigorous shake. After that, absorbance was calculated against the blank at 510 nm. Plotting absorbance versus concentration allowed for developing the quercetin standard calibration curve. The extract's total flavonoid content was reported as mg of quercetin equivalent (QE)/g of dried tuber extract.

Anthelmintic activity test
Parasite collection: *Paramphistomum cervi* was obtained from freshly slaughtered cattle given by the slaughterhouse and verified by experts. Parasites were cleaned and kept in 0.9% PBS (pH 7.4) at 37 ± 1 °C.

Standard drug: The standard anthelmintic medicine was albendazole suspension (15 µg/ml), obtained from Square Pharmaceuticals Ltd., Bangladesh.

***In vitro* tests:** The methodology for the anthelmintic effect was described by Ahmed *et al.* [14]. Cattle *P. cervi* live parasites were selected at random for the current investigation. The parasites were placed in a lab setting before research. The parasites were separated into four groups, each containing six parasites. The standard medicine, albendazole suspension, was put into a petri dish at 15 µg/ml. Different concentrations of the sample extracts (25, 50, 100, and 200 µg/ml) emerged. Water containing 0.2% tween-80 was administered to the control group. In each Petri dish, six similar parasites were set up and monitored at room temperature. In comparison to the control group, the time required for total paralysis and death was noted. Every sample was used to investigate the paralysis and death times. The worms have been reported to have become paralyzed throughout that period. Each worm was continuously exposed to external stimuli to ensure its death, suggesting that if the worm survived, its mobility would be promoted.

Protein denaturation activity test: Protein denaturation causes inflammatory and arthritic disorders, and it could be the reason for auto-antigen yield in several arthritic conditions. Therefore, substances with the ability to denaturize proteins can be employed in the creation of anti-inflammatory medications. The anti-inflammatory properties of *B. lupulina* were assessed in the % inhibition against the denatured state of egg albumin. The reaction mixture consisted of 3 ml of egg albumin with 2.8 ml phosphate buffer (PBS, pH 6.4), 3 ml of various concentrations of *B. lupulina* extract (250, 500, 750 µg/ml), and. As a control, the same volume of deionized water was used. After 15 minutes of incubation at 37 °C, it was then heated for 5 minutes at 70 °C to denaturize. Following that, absorbance was determined at 660 nm [15].
The following formula is used for calculating inhibition of protein denaturation:

$$\% \text{ inhibition of protein denaturation} = 100 \times [1 - \{(A_2 - A_1) \div (B_2 - B_1)\}]$$

Where A_1= Test sample unheated, A_2= Test sample heated, B_1= Control sample unheated, and B_2= Control sample heated.

Results and Discussions
This study was conducted to assess the antioxidant capability of a crude ethanol extract of *B. lupulina* leaves in terms of its total phenol content, total flavonoid contents, free radical scavenging activity, membrane stabilizing activity, and anthelmintic activity.

Phytochemical analysis
The significance of medicinal plants for both individual and community health is evident. Phytochemicals that are found in medicinal plants naturally have defensive mechanisms and offer protection against a range of illnesses. There are two types of phytochemicals: primary and secondary. Primary ingredients include proteins, common carbohydrates, and chlorophyll; secondary substances have alkaloids, phenolic compounds, and terpenoids. In herbal medicine research, the initial step toward identifying novel and bioactive lead chemicals is called phytochemical screening. Plant material is made up of a wide variety of naturally occurring compounds, each with a unique polarity that determines how it is soluble [16]. Many phytochemicals found in *B. lupulina* leaves can have medicinal properties. *B. lupulina* leaves were performed in qualitative chemical screening. Table 1 summarizes the findings of numerous phytochemical tests used to find and identify chemical ingredients. These phytochemical evaluations confirmed the presence of carbohydrates, tannins, flavonoids, saponins, steroids, glycosides, and quinones. These plant-based constituents have a role as antioxidant, anthelmintic, and membrane stabilizing agents.

Table 1: Chemical groups present in the leaves extract of *B. lupulina*

Name of the group	Indications
Carbohydrate	+
Tannins	+
Flavonoids	+
Saponins	+
Proteins	-
Terpenoids	-
Gums	-
Steroids	+
Glycoside	+
Quinone	+

(+) = Presence of constituents; (-) = Absence of constituents

Antioxidant activity

Antioxidants are well known for their ability to scavenge reactive species like ROS and RNS, among others, or to stop cell oxidation, which offers disease prevention. Antioxidants control the overproduction of oxidants by preventing or delaying oxidative processes. Phenolic chemicals are frequently utilized to treat a variety of conditions, including cancer, hypoglycemia, oxidative stress, wounds, infections on the skin, and immune system failure. In the same way, phenols and flavonoids offer numerous health advantages such as anti-inflammatory, anti-cancer, antiviral, anthelmintic, and antioxidant properties. Additionally, they have neuroprotective and cardio-protective qualities [17]. The potential for antioxidants and electron donation capacity of an extract, molecule, or other biological source-both of which are necessary for phenolic antioxidant activity-can be assessed using DPPH.

The DPPH free radical scavenging activity of *B. lupulina* leaves at different concentrations is shown in Table 2 and Fig. 1. The DPPH radical activity of ethanol extract of *B. lupulina* leaves increased with concentration. Maximum inhibitions of sample 64.69±0.019 was observed at 500 µg/ml concentration.

Table 2: DPPH Radical Scavenging Activity of Different Concentrations of ethanol extract of *B. lupulina*.

Conc. (extract) (µg/ml)	Average	% Inhibition	IC_{50} (µg/ml)
1	0.364	10.12	8.65
1.95	0.351	13.33	
3.90	0.323	20.25	
7.81	0.300	26.25	
15.62	0.282	30.37	
31.25	0.240	40.74	
62.5	0.208	48.64	
125	0.192	52.59	
250	0.172	57.53	
500	0.143	64.69	

In the quantitative assay, ethanol extract of *B. lupulina* leaves displayed a strong free radical scavenging activity in the DPPH assay (IC_{50} =8.65 µg/ml) which is comparable to that of ascorbic acid (IC_{50} =4.72 µg/ml), a well-known standard antioxidant.

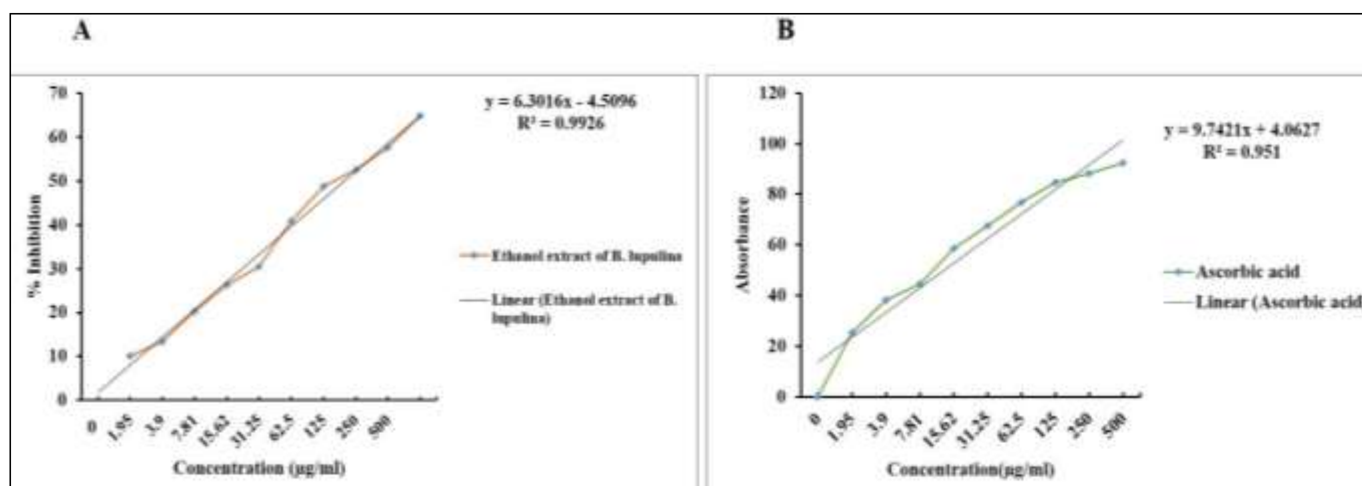

Fig 1: DPPH radical scavenging activity of different concentrations of ethanol extract of the leaves of *B. lupulina* (A), and standard ascorbic acid (B)

Additionally, FC and aluminum chloride colorimetric methods were used to estimate TPC and TFC in each sample. Table 2 shows the TPC and TFC of *B. lupulina* extracts, using gallic acid and quercetin as standards, respectively. *B. lupulina* had TPC values of 153±0.11 and µg GAE/g, with a total flavonoid content of 32.26 ± 0.09 µg QE/g dry weight extract.

Table 2: Total phenolic and flavonoid contents of *B. lupulina* leaves extract

Sample	Total Phenolic Contents(µg GAE/g Extract)	Total Flavonoid Contents(µg QE/g Extract)
Ethanol extract of *B. lupulina* leaves	153.3±0.11	32.26± 0.09

Anthelmintic activity

A growing number of countries have reported cases of helminths developing resistance to synthetic medications due to anthelmintic drug development, which indicates that control efforts that rely only on their use are unsustainable [18]. Some anthelmintic drugs such as praziquantel and albendazole, are contraindicated for certain groups of patients like pregnant and lactating women [19]. This has increased interest in using medicinal herbs to treat helminthic disorders through ethnomedical traditions worldwide [20].

Examining the anthelmintic activity of *B. lupulina* leaf extract and justifying its use in folk medicine for helminthiasis are the goals of the current investigation.

The data in Table 3 and Fig. 2 show that gradually increasing

the sample concentration of *B. lupulina* extract causes worm paralysis and death fewer times. At 25, 50, 100, and 200 mg/ml concentrations, the extract demonstrated paralysis time of 31±0.2, 19.83±0.7, 13.5±0.9 min, 9.5±0.7 min, and death time of 35.66±0.5, 35.66±0.5, 17.83±0.7 min, and 12.66±0.1 min, respectively. The results were compared to those of normal albendazole, which had a paralysis time of 8.5±0.9 min and a death time of 13.16±0.6 min at a dosage of 15 mg/mL.

Table 3: Anthelmintic activity of ethanol extract *of B. lupulina* leaves against *P. cervi*

Test Group	Time taken for Paralysis (min)	Time taken for Paralysis (min) ±SD	Time taken for Death (min) ±SD
Control (0.2% Tween-80 in water)	0	0	0
Albendazole (Standard)	15	8.5±0.9	13.16±0.6
B. lupulina leaves extract (µg/ml)	25	31±0.2	35.66±0.5
	50	19.83±0.7	25.16±0.3
	100	13.5±0.9	17.83±0.7
	200	9.5±0.7	12.66±0.1

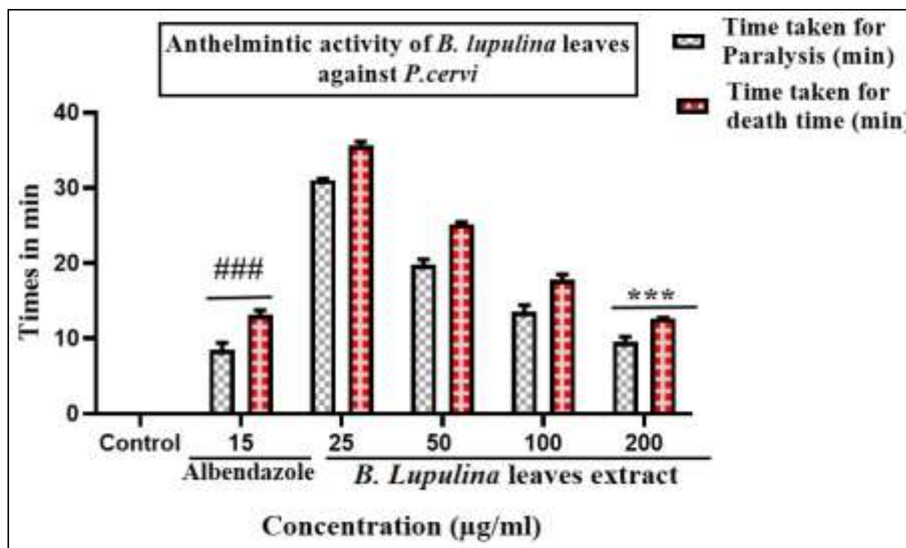

Fig 2: Anthelmintic activity of different concentrations of *B. lupulina* against *P. cervi* compared with standard drug Albendazole. Statistical analysis revealed significant differences, denoted as***p<0.001 when compared with the indicated *B. lupulina* extract treated group and ###p< 0.001 when compared with the Albendazole treated group

The anthelmintic activity of plant materials was measured depending on the loss of movement or paralysis and destruction or death of live parasites in the *in-vitro* experiments. Phytochemical investigation of the extracts exhibited the presence of flavonoids, steroids, tannins, terpenoids, glycosides, carbohydrates, quinone, etc. Tannins are responsible for producing anthelmintic activities [21]; the exact mechanism of the anthelminthic activity *of B. lupulina* cannot be explained based on our present results.

Protein denaturation activity test

Arthritis and inflammatory disorders are mostly caused by denaturation of tissue proteins. We develop auto antigens in our bodies that lead to arthritic disorders as a result of the denaturation of proteins. Treatments that reduce inflammation are developed using extracts that can stop protein denaturation [22]. As a result, the *in-vitro* anti-inflammatory efficacy of *B. lupulina* leaves crude ethanol extract was assessed in this study in comparison to egg albumin denaturation.

In the protein denaturation test, the leaves extract of *B. lupulina* and Diclofenac Na (standard) showed % inhibition of 53.78%, 55.25%, 60.6%, and 63.3%, 75%, 79% respectively (Table 4 and Fig. 3). The present study revealed that the leaf extract of *B. lupulina* may be a potent anti-inflammatory agent.

Table 4: Protein denaturation activity of the ethanol extract of *B. lupulina* leaves

Concentration (µg/ml)	% inhibition of *B. lupulina* leaves extract	% inhibition of Diclofenac Na (Standard)
250	53.78	63.3
500	55.25	75
750	60.6	79

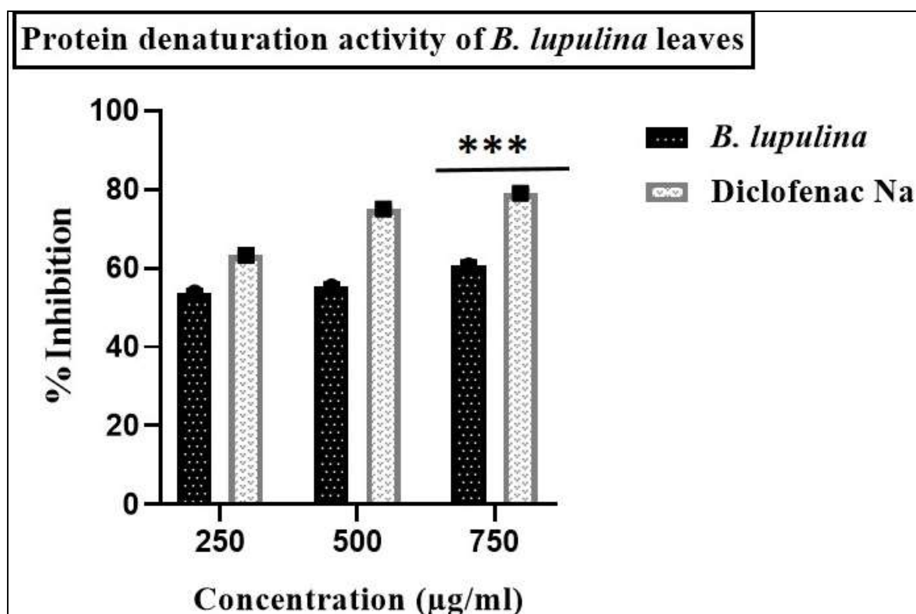

Fig 3: % inhibition of protein denaturation at different concentrations by ethanol extract of *B. lupulina* leaves compared to Diclofenac Na. Statistical analysis revealed significant differences, denoted as,***p<0.001 when compared with the indicated *B. lupulina* extract-treated group

Conclusion

The ethanolic extract of *B. lupulina* used in this investigation included a variety of compounds of major phytochemical significance, including quinone, alkaloids, tannins, flavonoids, saponins, steroids, and glycosides. The study also showed that the crude ethanolic extract exhibited possible anthelmintic, antioxidant, and protein denaturation capabilities, suggesting that *B. lupulina* may be useful in the search for novel, highly effective medications. The plant's traditional usage for treating a variety of ailments is supported by the biological activities displayed by the extracts. The current work was a preliminary effort that needs more in-depth analysis. Nevertheless, additional laboratory research is needed to validate these reported pharmacological effects.

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Data Availability Statement

The data are contained within the article.

Conflicts of Interest

The authors declare no conflict of interest.

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