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# Phytochemical Characteristics, Antioxidant, and Toxicity Evaluation of Ethanolic Leaves Extract of *Barleria lupulina* Lindl.

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**Abstract**
The study on *Barleria lupulina* Lindl. ethanolic leaf extract aimed to evaluate its phytochemical profile, antioxidant potential, and safety in mice. The plant, known for its rich history in traditional medicine, was subjected to various tests to identify the presence of bioactive compounds. Phytochemical analysis revealed the presence of carbohydrates, alkaloids, flavonoids, proteins, tannins, and volatile oil. The extract exhibited significant antioxidant activity, demonstrated by high total phenolic content and effective radical scavenging in NO, DPPH, and $H_2O_2$ assays. Acute and sub-acute toxicity studies were conducted to assess the safety of the extract, and results indicated no adverse effects at the tested doses. These findings support the potential therapeutic applications of *B. lupulina* extract, emphasizing its antioxidant properties and safety profile. The study contributes to the understanding of the therapeutic potential of *B. lupulina* and underscores the importance of medicinal plants in modern healthcare. The promising results suggest that *B. lupulina* extract could be a valuable addition to therapeutic practices, warranting further research and development.

**Keywords:** *Barleria lupulina*, phytochemical profile, toxicity, Swiss albino mice, antioxidant, extract.

**Introduction**

The utilization of plants and their natural products for therapeutic purposes dates back to ancient civilizations. Throughout history, plants have been a cornerstone of traditional medicine systems across various cultures, including Ayurveda, Traditional Chinese Medicine, and Native American healing practices (Zakaria et al., 2008). These natural products, derived from diverse plant species, have been employed to treat a myriad of ailments, ranging from common colds to more severe conditions like infections and chronic diseases (Porras et al., 2020). The reliance on botanical remedies underscores the deep-rooted knowledge of plant-based therapeutics, which has been passed down through generations. This historical context highlights the intrinsic value of plants as sources of bioactive compounds, which modern scientific research continues to explore and validate (Obahiagbon et al., 2023).

In the quest to discover novel therapeutic agents, *Barleria lupulina* Lindl, commonly known as the hophead philippine violet, emerges as a promising candidate. This plant, belonging to the Acanthaceae family, is native to tropical regions and has been traditionally used in various folk medicines. The justification for selecting *B. lupulina* for this study is multifaceted (Banerjee et al., 2021). Firstly, its rich ethnomedicinal history suggests a reservoir of bioactive compounds with potential pharmacological benefits. Secondly, preliminary studies have indicated that *B. lupulina* possesses a wide range of biological activities, including anti-inflammatory, antimicrobial, and antioxidant properties (Kumari et al., 2017). These attributes make it an attractive subject for in-depth phytochemical and pharmacological investigations. By exploring the ethanolic extract of *B. lupulina* leaves, this study aims to unravel its phytochemical profile, evaluate its antioxidant potential, and assess its toxicity, thereby contributing to the growing body of knowledge on this underutilized medicinal plant.

*B. lupulina* has been widely utilized in traditional medicine systems, particularly in Southeast Asia and India. Traditionally, the leaves and roots of the plant have been employed to treat ailments such as fever, respiratory infections, and inflammatory conditions (Banerjee et al., 2021). In Thai folk medicine, *B. lupulina* is used to alleviate pain and reduce inflammation, while in Indian traditional practices, it is used for its wound-healing properties. The plant's traditional applications also extend to treating gastrointestinal disorders, skin diseases, and as an antidote for snake bites. These diverse uses underscore the plant’s potential therapeutic value and provide a strong basis for scientific exploration (Suba et al., 2005).

Various pharmacological studies have been conducted to validate the traditional uses of *B. lupulina*. The plant has demonstrated significant anti-inflammatory activity, which is attributed to its ability to inhibit key enzymes and mediators involved in the inflammatory process. Antimicrobial studies have shown that *B. lupulina* extracts possess activity against a broad spectrum of pathogens, including bacteria and fungi, highlighting its potential as a natural antimicrobial agent. Additionally, the plant has exhibited antioxidant properties, which are crucial in combating oxidative stress and preventing cellular damage. These pharmacological activities suggest that *B. lupulina* is a rich source of bioactive compounds with therapeutic potential. The therapeutic potential of *B. lupulina* is largely due to its diverse phytochemical composition. The rich phytochemical profile of *B. lupulina* provides a scientific basis for its traditional uses and pharmacological activities (Kumari et al., 2017).

Antioxidant activity is a critical parameter in evaluating the therapeutic potential of plant extracts. The ethanolic extract of *B. lupulina* leaves has been subjected to various in vitro antioxidant assays to assess its efficacy. The nitric oxide (NO) scavenging assay measures the extract's ability to inhibit the formation of nitric oxide, a reactive nitrogen species that can cause oxidative *damage*. The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay evaluates the free radical scavenging capacity of the extract by measuring its ability to reduce DPPH radicals to a non-radical form. The hydrogen peroxide ($H_2O_2$) scavenging assay assesses the extract's ability to neutralize hydrogen peroxide, a reactive oxygen species that can cause cellular damage. Results from these assays have demonstrated that *B. lupulina* exhibits significant antioxidant activity, further supporting its potential as a natural antioxidant agent (Kumari et al., 2017).

*B. lupulina* holds significant promise as a source of bioactive compounds with therapeutic potential. The historical use of plants in traditional medicine underscores the importance of exploring their phytochemical profiles and pharmacological activities. This study focuses on the ethanolic extract of *B. lupulina* leaves, aiming to elucidate its phytochemical composition, antioxidant activity, and toxicity profile. The plant's rich phytochemical content demonstrated pharmacological activities, justify its selection for this research. By advancing our understanding of *B. lupulina*, this study seeks to contribute to the development of novel therapeutic agents and underscore the enduring value of medicinal plants in modern healthcare.

**Figure 1.** *B. lupulina* Plant

## Materials and Methods

### *Collection of plant material*

Botanists from India's Botanical Survey in Howrah successfully recognised *B. lupulina* leaves (specimen identification No. CNH/Tech.II/2018/99). In November, the leaves were collected from the South Dhadka in Asansol, West Bengal, India. The plant leaves were carefully cleaned under running water to eliminate any dust, dirt, or other unwanted particles that may have stuck. We used a mixer grinder to crush the leaves into powder after they had dried in the shade for 15 days at room temperature. To prepare the fine powder for future investigation, the coarse powder was first passed through a sieve #60. The resulting powder was then packed and stored in a sealed package at room temperature for later use.

### *Chemicals used*

The analytical grade chemicals used during this experiment guaranteed a very high standard of purity and accuracy in every step of the experiment. All the reagents such as Folin-Ciocalteu reagent, sodium nitroprusside, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and hydrogen peroxide were procured from LobaChemie Pvt. Ltd.

### *Preparation of crude extracts*

To prepare crude extract from *B. lupulina* dried leaves powder, ethanol was utilized as the solvent in a cold maceration process. A known quantity of the powdered leaves was immersed in ethanol at a ratio of 1:10 (w/v) in a clean, airtight container. The mixture was left to stand at room temperature for 72 hours, with occasional stirring to facilitate the extraction of phytochemicals into the solvent. After the maceration period, the mixture was filtered through a muslin cloth followed by Whatman No. 1 filter paper to remove particulate matter. The filtrate, which contained the crude ethanolic extract of *B. lupulina*, was concentrated at room

temperature to avoid the degradation of heat-sensitive compounds. The resulting concentrated extract was then dehydrated under vacuum condition using a lyophilizer, then stored for subsequent analysis and used in further experiments.

***Phytochemical analysis***

The phytochemical analysis of *Barleria lupulina* was conducted to identify the presence of various bioactive compounds, including alkaloids, flavonoids, proteins, carbohydrates, tannins, saponins, sterols, triterpenoids, starch, glycosides, and volatile oils. Standard protocols were employed for each test: alkaloids were detected using Dragendorff's and Wagner's reagents; flavonoids through the Shinoda test; proteins via the Biuret and Xanthoproteic tests; carbohydrates by the Benedict's and Fehling's tests; tannins with the Ferric chloride test; saponins through the froth test; sterols and triterpenoids using the Liebermann-Burchard test; starch with the Iodine test; glycosides via the Keller-Kiliani test; and volatile oils were identified through steam distillation. These qualitative assays provided a comprehensive profile of the phytochemicals present in *B. lupulina*, forming the basis for further quantitative and functional studies (Kumari et al., 2017). The results have been detailed in the results section (**Table 1**).

*Test for alkaloids*

Mayer's test: To identify the presence of alkaloids, 2 mL of extract was added to test tubes along with Mayer's reagent, which is a potassium mercury iodide solution. The presence of alkaloids was confirmed when a cream-colored solid was precipitated after shaking the mixture.

Dragendroff's test: 2 mL of extract was mixed with 0.1 mL of both Dragendorff's reagent which is potassium bismuth solution and hydrochloric acid sequentially. After stirring the liquid, development of orange-brown precipitate ensures the presence of alkaloids.

Wagnar's test: 2 mL of extract was added to a test tube with Wagnar's reagent (potassium iodide solution) to identify the presence of alkaloids. After shaking the mixture, the presence of alkaloids was verified by the production of a reddish-brown precipitate.

Hager's test: 2 mL of extract was added to test tube along with Hager's reagent (picric acid solution) to identify the presence of alkaloids. After that, the mixture was agitated, and the presence of alkaloids was verified by the production of a yellow precipitate.

*Test for carbohydrates*

Molish test: 2 to 3 mL of an alcohol solution containing *α*-naphthol was added to the extract. Before adding concentrated $H_2SO_4$ to the test tube from the sides, the mixture was shaken. A violet ring formed at the junction of 2 liquids.

Fehling's test (for reducing sugar): Combining Fehling's A and B solutions, 1 mL each, and heating to boiling for one minute. A comparable quantity of test solution was added to it. For 5–10 minutes, the material test tube was placed on a boiling water bath. When the colour became brick red from yellow, indicates the presence of carbohydrates.

Benedict's test (for reducing sugar): The test solution was mixed with the Benedict's reagent in a test tube. The mixture was boiled for five minutes. Depending on the amount of reducing sugars, the test solution could be of green/yellow/red in colour. The presence of green indicates the presence of carbohydrates.

*Test for flavonoids*

Shinoda test: Concentrated hydrochloric acid (HCl) and a small quantity of magnesium tunings were added to the extract. Reddish colour indicates the presence of Flavonoids.

Alkaline reagent test: A sodium hydroxide solution was added to the extract. Yellow colour indicates the presence of Flavonoids. On adding 0.1 N HCl, the colour of the solution got disappeared.

Zinc metal test: Concentrated hydrochloric acid and zinc dust were combined with the extract. The presence of flavonoids is indicated by the appearance of a reddish colour.

*Test for glycosides*

Baljet test: Picric acid solution was added to the alkaline extract. Yellow colour of the solution did not change to orange indicates the absence of glycosides.

Keller-Kiliani's test: A mixture of 0.4 mL of glacial acetic acid and one mL of extract was prepared. A little amount of ferric chloride solution (0.5%) was added to it. To make two separate layers, 0.5 millilitre of pure sulfuric acid was added in a dropwise manner. No such blue colour in the acetic acid layer developed indicates glycoside is absent.

Borntrager's test: Prior to heating and filtering, three millilitres of the extract was mixed with diluted sulfuric acid. To the cooled mixture, benzene or chloroform was added. The organic layer was then removed and ammonia was then added. Absence of pink colour in the ammonia layer indicates the absence of Glycosides.

Modified Borntrager's test: 5 millilitres each of extract, diluted hydrochloric acid and ferric chloride were mixed thoroughly. The next step was to boil and filter the mixture. After cooling

the filtrate, benzene or chloroform was added. It was therefore necessary to separate the organic layer before adding of ammonia. Ammonia layer did not become pink or red, indicates the absence of glycosides.

Magnesium acetate solution test: To the extract, magnesium acetate was added. Absence of shift to orange colour indicates the glycoside is absent.

*Test for Proteins*

Biuret test: A little quantity of 4% NaOH and 1% $CuSO_4$ were added to 1 mL test solution. The solution did not become a shade of pink or violet, indicates the absence of protein.

Ninhydrin test: The test solution (1 mL) was mixed with a little amount of the Ninhydrin reagent. The mixture was boiled further, which led to the development of blue colour confirms the presence of protein.

*Test for saponin*

Foam test: The extract was mixed with a little quantity of water and then shaken vigorously. The mixture was heated to a certain degree. No such foam was developed.

*Test for starch*

Lugol's test: The extract was slowly treated with Lugol's iodine solution. Starch is present if the mixture becomes a deep blue or black colour.

*Test for sterols*

Salkowski test: A reddish colour was seen in the bottom layer after combining a little quantity of extract with concentrated $H_2SO_4$, indicating the existence of steroids. Absence of yellow colour at the bottom layer confirms the no triterpenoids are present.

Liebermann–Burchard test: An equal volume of 2 millilitres of extract, acetic acid, and few drops of concentrated $H_2SO_4$ were introduced into a test tube. After that, it was heated to boiling and then allowed to cool. No faint brownish-green hue was formed indicates the absence of steroidal moiety.

Sulfur powder test: On adding a small amount of sulphur powder into the extract, no such precipitate was obtained indicates the absence of sterols.

*Test for tannins*

Ferric chloride test: Tannins were detected when the test solution became blue upon exposure to ferric chloride.

Gelatine test: A precipitate confirming the presence of tannins was formed when the extract was added to a 1% gelatin solution containing 10% sodium chloride.

*Test for volatile oil*

Sudan III test: Sudan III treatment of the extract yielded a reddish-coloured solution, confirming the presence of volatile oil.

***Quantification of Total Phenolic Content***

To measure the total phenolic content of the extract, a modified colorimetric Folin-Ciocalteu method was used (Wolfe et al., 2003). According to Singleton and Rossi (1965), it is based on the polyphenol principle, which, when combined with the Folin-Ciocalteu reagent in an alkaline environment, results in a blue chromogen with an absorption peak at 760 nm. Filling the test tubes with aliquots of gallic acid solution ranging from 0.1 to 1.0 mL was followed by the addition of distilled water to bring the contents up to 9 mL. Each tube was then allowed to relax at room temperature for 30 minutes after adding 0.5 mL of Folin-Ciocalteu reagent. It was then mixed with the 0.5 mL of saturated sodium carbonate and allowed to sit undisturbed at 40° C for two hours. Using 0.5 mL of Folin-Ciocalteu reagent, the same technique was applied to the extract (2 mL) at a concentration of 1 mg/mL. The combinations were allowed to stand at room temperature for half an hour. The next step was to add half a millilitre of a sodium carbonate solution (17%) to the reaction mixture. After that, the mixture was stirred slightly and left at 40° C for two hours. At a wavelength of 760 nm, the absorbance was measured using the spectrophotometer. One milligram per millilitre of gallic acid served as the reference standard. Milligram of gallic acid equivalent per gram of fresh extract is the unit of measurement for the overall phenolic content in the sample.

***Antioxidant study***

*Nitric oxide (NO) radical scavenging activity*

The rate of radical production by sodium nitroprusside was measured in this experiment. A phosphate buffer saline solution with a pH of 7.3 and 5.0 mL of sodium nitroprusside solution were added to the reaction mixture, which was then incubated at 25° C for three hours. It was thereafter decided to add the plant extract to certain of the reaction combinations at varying quantities and leave out the others. The resultant nitric oxide radical eventually generated nitrite ions by reacting with oxygen. This ion was thereafter measured at 30 min by adding 1 mL of the incubated mixture to an equal amount of Griess reagent. After being formed by diazotizing nitrite ions with sulfanilamide and coupling with naphthylethylenediamine dihydrochloride, the chromophore, a purple azo dye, was identified at a wavelength of 546 nm (Patel & Patel, 2011).

$$\% \ inhibition \ of \ NO \ radical = \frac{A_0 - A_1}{A_0} x100$$

where $A_0$ and $A_1$ represent the initial and final absorbances, respectively, before and after the reaction with Griess reagent.

*DPPH assay*

The DPPH free radical scavenging experiment, as detailed was used to assess the extract's antioxidant activity (Nithianantham et al., 2011; Zuraini et al., 2008). We made several changes to the original procedure. The standard procedure included adding 50 µL of *B. lupulina* leaf extracts to an uniform bottle, with concentrations varying between 1 and 5 mg/mL. Then, 5 millilitre of DPPH solution (0.004% w/v) was added to the bottle. After the mixture was obtained, it was stirred quickly with a vortexer and then left in a dark area at room temperature for half an hour. Afterwards, a UV-VIS spectrophotometer tuned to 517 nm was used to analyse the results of the experiment. An 80% v/v ratio of ethanol was used to prepare the blank. Ascorbic acid, often known as vitamin C, was used for the comparison. Three separate measurements were conducted to assure precision. To determine the DPPH scavenging effect, the obtained data were subjected to the following equation:

$$DPPH \ scavenging \ activity \ (\%) = \frac{A_0 - A}{A_0} x100$$

$A_0$ is the absorbance of the DPPH solution used as a negative control, with a concentration of 0.004%. The absorbance when the extract is present is denoted by A. The results were recorded using ascorbic acid equivalents (AAE, mg/g) and $IC_{50}$ values of *B. lupulina* extracts.

*Hydrogen peroxide radical scavenging activity*

In order to determine the extract's hydrogen peroxide ($H_2O_2$) scavenging capabilities, the technique developed by Ruch et al. (1989) was used. Before adding 0.6 mL of a 2mM $H_2O_2$ solution, Eppendorf tubes were filled with extracts ranging from 25-400 µg/mL, with a volume of 0.1 mL. A volume of 0.4 mL was achieved by adding 50 mM phosphate buffer, which has a pH of 7.4. Following a 10-minute reaction time, the liquid was vortexed strongly to increase its absorbance at 230 nm. A positive control was used using ascorbic acid (Gülçin et al., 2007). We used the following equation to find the extracts' $H_2O_2$ scavenging capabilities:

$$\% \ scavenged = \frac{A_i - A_t}{A_i} x100$$

Where $A_i$ represents the absorbance of the control and $A_t$ represents the absorbance of the test.

***Toxicity study of B. lupulina Extract***

*Experimental animals*

Before proceeding with the toxicity study, meticulous handling of Swiss albino mice is essential to ensure both the welfare of the animals and the integrity of the experimental data. The mice were acclimatized to the laboratory environment for at least one week prior to the commencement of the study. During this period, they were housed in standard laboratory cages with adequate bedding, maintained under controlled conditions of temperature (22 ± 2°C), humidity (50 ± 10%), and a 12-hour light/dark cycle. They were provided with standard pellet diet and water ad libitum. Handling involved gentle and minimal restraint to reduce stress, and each mouse was observed for signs of health and normal behavior. Proper identification of the animals was ensured through ear tagging or similar methods. All procedures involving the mice were conducted in compliance with ethical guidelines and approved by the institutional animal ethics committee. This careful handling ensured that the mice were in optimal condition for the subsequent toxicity study, thereby ensuring reliable and reproducible results.

*Acute and Sub-chronic toxicity study*

The toxicity study of *B. lupulina* extract was conducted following the OECD (Organisation for Economic Co-operation and Development) guidelines for acute and sub-acute toxicity testing. For the acute toxicity assessment, the OECD limit test was employed. This involved administering vehicle as control group (group-I), and a single high oral dose of the *B. lupulina* extract orally to group-II (n = 5), and monitoring them for 14 days for any signs of toxicity or mortality. The selected dose was set at 2000 mg/kg body weight, as per OECD Guideline 425. Observations included general behavior, physical appearance, and any physiological changes, recorded daily to assess the immediate toxic effects of the extract (**Table 3**) (Saleem et al., 2017).

For the sub-acute toxicity study, the procedure adhered to OECD guideline 407. In this case the randomly selected mice were divided into three groups. Group-I was for control (vehicle treated group), and group-II and II (n = 5) for lower and higher dose levels of the extract. Here, the *B. lupulina* extract was administered daily oral dose of 600 and 1000 mg/kg body weight respectively for 28 days. At the end of the study, the animals were euthanized, and a detailed necropsy was performed. Blood samples were collected for hematological and biochemical analysis, and major organs were weighed to identify any sub-acute toxic effects induced by the

extract. This comprehensive approach ensured a thorough evaluation of the safety profile of *B. lupulina* extract (Kifayatullah et al., 2015).

*Hematological and biochemical examination*

After an overnight fast of 8 hours, all the animals were killed on the 29$^{th}$ day under the influence of chloroform, an anaesthetic. Blood samples were taken for biochemical and haematological analysis in tubes with and without ethylene diamine tetraacetic acid, an anticoagulant. Before being tested for biochemical estimate, the blood that did not contain ethylene diamine tetraacetic acid was centrifuged at 2,500 r/min for 15 minutes to extract serum, which was then kept at -20 ℃. Following blood collection, the study separated and weighed each important organ, including the liver, kidneys, heart, pancreas, and small intestine, using an electronic balance. The relative organ body weight of the test-treated and control groups was then calculated (Abotsi et al., 2011). The following equations were used to determine the ROW of each organ:

$$\text{Relative Organ Weight} = \frac{Absolute\ organ\ weight\,(g)}{Rat\ body\ weight\ on\ sacrifice\ day}\ x\ 100$$

*Study of hematological parameters*

The following parameters were measured and compared between the control and extract-treated groups: red blood cell count, mean cell volume, haemoglobin, white blood cell count, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, mean corpuscular volume, monocyte, neutrophil, lymphocyte, and platelet count. The test was conducted using an automated hemostatology analyzer (Sysmex K21, Tokyo, Japan).

*Study of serum biochemical parameters*

Centrifugation of the blood samples allowed for biochemical analysis of the serum, which included the determination of levels of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase, total bilirubin, total protein, albumin, urea, and creatinine for both the control and extract treated groups. Using a clinical chemistry analyzer (Vital Scientific, Netherlands), all analyses were determined.

*Statistical analysis*

The statistical analysis was carried out using the following methods: first, an analysis of variance (ANOVA) test was conducted using Graph Pad Prism 10, with n = 5, and second, a Bonferroni test was used for multiple comparisons across the groups. $P < 0.05$ was deemed statistically significant.

## Results

### *Preliminary Phytochemical investigation*

Various phytochemicals were identified in the leaf extract based on their solubility in ethanol. The extract included carbohydrates, alkaloids, flavonoids, proteins and tannins as well as volatile oil (**Table 1**).

**Table 1.** Different phytochemical class found in *B. lupulina* ethanolic leaf extract

| Phytoconstituent class | Name of tests | Ethanolic extract |
|---|---|---|
| Carbohydrates | Molish test | + |
| | Fehling's test (for reducing sugar) | — |
| | Benedict's test (for reducing sugar) | + |
| Alkaloids | Mayer's reagent | + |
| | Dragendroff's reagent | + |
| | Wagnar's reagent | + |
| | Hager's reagent | + |
| Glycosides | Baljet test | — |
| | Keller- Killian's test | — |
| | Borntrager's test | — |
| | Modified Borntrager's test | — |
| Flavonoids | Shinoda test | + |
| | Alkaline reagent test | + |
| | Zinc hydrochloride test | — |
| Proteins | Biuret test | — |
| | Ninhydrin test | + |
| Starch | Lugol's iodine solution | — |
| Saponins | Foam test | — |
| Tannins | Ferric chloride test | + |

| | | |
|---|---|---|
| | Gelatine test | + |
| Sterols | Salkowski test | — |
| | Liebermann-Burchard's test | — |
| | Sulfur powder test | — |
| Volatile oil | Sudan III test | + |

***Total phenolic content***

Using an extract-based inhibitory experiment, the total phenolic contents (as a percentage) of *B. lupulina*'s ethanolic extract were investigated. For this reason, gallic acid was used as a reference for comparing the phenolics found in the plant leaves extract. Gallic acid equivalents were used to determine that the *B. lupulina* extract had a phenolic content of 71.42%.

***Antioxidant activity***

*NO radical scavenging activity*

Significantly, the findings demonstrated that nitric oxide levels decreased when the quantities of the ethanolic extract were raised. The extract has the potential to modify oxidative stress by inhibiting the generation of nitric oxide, which is thought to have a significant impact on several pathological and physiological processes (**Table 2**). An evaluation of the concentration at which 50% inhibition of nitric oxide generation occurred was done by calculating the $IC_{50}$, which came out to 0.30mg/ml. An extract with a lower $IC_{50}$ value is more effective in scavenging or inhibiting nitric oxide radicals.

*DPPH radical scavenging activity*

This study used DPPH radical suppression percentages as an outcome of incubation with extract at different concentrations. An increase in antioxidant activity was seen at greater concentrations of the ethanolic extract of *B. lupulina*, suggesting a dose-response connection. According to **Table 2**, the extract showed promising results in scavenging radicals, indicating that it might neutralise DPPH radicals by donating electrons. Half of the radicals generated by DPPH were scavenged at a concentration of 0.3mg/ml, which is the $IC_{50}$ value for the extract.

*$H_2O_2$ (Hydrogen peroxide) radical scavenging activity*

Increasing the extract concentration significantly amplified the scavenging action. Quantitative investigation revealed that the ethanolic extract of *B. lupulina* significantly reduced hydrogen peroxide levels (**Table 2**). The capacity of the extract to combat hydrogen peroxide-induced oxidative stress was evaluated by finding the concentration at which 50% inhibition occurred

($IC_{50}$: 0.34mg/ml). These results provide further proof that *B. lupulina* may protect cells from hydrogen peroxide and emphasise its antioxidant capabilities. Because antioxidant action is concentration-dependent, finding the optimal dose for potential medicinal uses is crucial.

**Table 2.** Results of antioxidant activities using *B. lupulina* leaf extracts

| **Concentration (mg/ml)** | **% scavenge** | | |
|---|---|---|---|
| | **NO** | **DPPH** | **$H_2O_2$** |
| 0.5 | 84 | 82 | 74 |
| 1 | 86 | 84 | 76 |
| 2 | 88 | 85 | 78 |
| 2.5 | 92 | 87 | 79 |
| **$IC_{50}$(mg/ml)** | 0.30 | 0.31 | 0.34 |

***Toxicity study of B. lupulina Extract***

The toxicity study of *B. lupulina* extract was conducted in accordance with the OECD limit test guidelines for acute and sub-acute toxicity. For the acute toxicity assessment, the OECD 425 guideline was employed, involving the administration of 2000 mg/kg dose of the extract to a group of test animals, followed by a 14-day observation period to monitor for any signs of toxicity or mortality. No significant adverse effects or deaths were observed, indicating that the extract has a high safety margin at the tested dose.

For the sub-acute toxicity evaluation, the OECD 407 guideline was utilized, which entails the repeated administration of the extract over a 28-day period. Both the 600 and 1000 mg/kg doses of the extract were administered daily to different groups of test animals. Throughout the study, the animals were monitored for clinical signs of toxicity, changes in body weight, food and water consumption, and behavioral changes. Additionally, hematological, and biochemical analyses were performed at the end of the study to assess the potential impact on organ function and structure. The findings from the sub-acute toxicity study revealed no significant toxic effects, suggesting that *B. lupulina* extract is safe for prolonged use at the tested doses. These results provide a comprehensive toxicological profile of the extract, supporting its potential for further pharmacological development.

**Table 3.** Outward manifestations and behavioural evaluations of the extract-treated and control groups in the acute toxicity trial

| Observation | Control group | 2000 mg/kg |
|---|---|---|
| Body weight | Normal | Not change |
| Temperature | Normal | Normal |
| Food intake | Normal | Normal |
| Urination | Normal | No effect |
| Rate of respiration | Normal | No Effect |
| Change in skin | No effect | No effect |
| Drowsiness | Not present | Not Present |
| Sedation | No effect | No effect |
| Eye colour | No effect | No effect |
| Diarrhoea | Not present | Not present |
| General physique | Normal | Normal |
| Coma | Not present | Not present |
| Death | Alive | Alive |

**Table 4.** Weight of mice organs after oral administration of ethanol extract from *B. lupulina*

| Organ name | Average organ weight | | | Relative organs weight | | |
|---|---|---|---|---|---|---|
| | Normal | 600mg/kg | 1000mg/kg | Normal | 600mg/kg | 1000mg/kg |
| Kidney | 0.324 ± 0.01 | 0.301 ± 0.02 | 0.331 ± 0.02 | 1.30 ± 0.01 | 1.20 ± 0.02 | 1.25 ± 0.02 |
| Liver | 0.811 ± 0.01 | 0.809 ± 0.01 | 0.829 ± 0.01 | 3.25 ± 0.01 | 3.22 ± 0.01 | 3.14 ± 0.01 |
| Pancreas | 0.123 ± 0.02 | 0.126 ± 0.02 | 0.114 ± 0.02 | 0.49 ± 0.02 | 0.50 ± 0.02 | 0.43 ± 0.02 |
| Intestine | 0.644 ± 0.02 | 0.641 ± 0.02 | 0.646 ± 0.02 | 2.58 ± 0.02 | 2.55 ± 0.02 | 2.45 ± 0.02 |
| Heart | 0.149 ± 0.01 | 0.141 ± 0.01 | 0.137 ± 0.01 | 0.60 ± 0.01 | 0.56 ± 0.01 | 0.52 ± 0.01 |
| Average body weight on the sacrifice day | 24.943 ± 0.09 | 25.156 ± 0.01 | 26.406 ± 0.01 | — | — | — |

*Effect of plant extract on hematological parameters*

Haematological test results have been included in **Table 5**. When compared to the control group, all of the haematological parameters that were evaluated were within normal ranges. These parameters included red blood cell (RBC) count, haemoglobin, mean cell volume (PCV), white blood cell (WBC) count, mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), monocyte, neutrophil, lymphocyte, and platelet count. There were no discernible changes in the toxicological profile of the animals given the plant extract compared to the control group ($P > 0.05$). For the majority of the haematological indicators, there was no statistically significant difference between the treatment and control groups.

**Table 5.** Haematological parameters affected by *B. lupulina* extract

| Parameters | Normal group | 600 mg/kg extract | 1000 mg/kg extract |
|---|---|---|---|
| Total RBC ($10^{12}$/L) | 8.972 ± 0.312 | 9.998 ± 0.817 | 10.106 ± 0.698 |
| Hemoglobin (g/L) | 10.480 ± 0.863 | 11.023 ± 1.020 | 11.998 ± 1.041 |
| PCV (L/L) | 40.002 ± 1.838 | 40.200 ± 1.940 | 39.640 ± 2.140 |
| WBC ($10^9$/L) | 10.800 ± 1.111 | 10.000 ± 0.943 | 11.010 ± 1.190 |
| MCH (pg) | 14.000 ± 0.900 | 13.900 ± 0.465 | 14.970 ± 0.604 |
| MCHC (g/dL) | 32.900 ± 1.890 | 34.100 ± 0.863 | 35.800 ± 0.400 |
| MCV (fL) | 44.320 ± 1.512 | 43.540 ± 2.000 | 47.000 ± 1.500 |
| Monocyte (%) | 3.800 ± 0.980 | 3.800 ± 1.010 | 2.900 ± 0.751 |
| Neutrophil (%) | 25.354 ± 1.810 | 26.400 ± 1.450 | 27.000 ± 1.480 |
| Lymphocyte (%) | 56.540 ± 2.640 | 58.450 ± 2.180 | 60.620 ± 1.940 |
| Platelet count ($10^9$/L) | 310.100 ± 20.400 | 356.500 ± 2.400 | 389.100 ± 24.800 |

*Biochemical parameters affected by plant extract*

**Table 6** summarise the findings of the numerous biochemical tests conducted on the experimentally treated animals with the plant extract and the control group. When compared to the control group, plasma biochemical markers including total bilirubin, total protein, albumin, urea, creatinine, SGOT (AST) and SGPT (ALT) were unaffected by orally administered doses of 600 and 1,000 mg/kg of the plant-treated extract.

**Table 6.** Impact of *B. lupulina* extract on biochemical markers

| Parameters | Normal group | 600 mg/kg extract | 1000 mg/kg extract |
|---|---|---|---|
| Bilirubin (g/dL) | 0.30 ± 0.25 | 0.70 ± 0.15 | 0.45 ± 0.20 |
| Total protein (g/dL) | 5.80 ± 0.70 | 6.10 ± 0.55 | 6.05 ± 0.62 |
| Albumin (g/dL) | 2.65 ± 0.20 | 2.75 ± 0.15 | 2.95 ± 0.20 |
| Urea (mg/dL) | 31.65 ± 1.10 | 29.85 ± 0.50 | 25.10 ± 0.10 |
| Creatinine (mg/dL) | 1.00 ± 0.05 | 0.90 ± 0.20 | 0.80 ± 0.15 |
| SGOT (AST) (IU/L) | 124.00 ± 12.00 | 136.50 ± 12.50 | 164.60 ± 14.00 |
| SGPT (ALT) (IU/L) | 28.60 ± 0.50 | 38.50 ± 2.50 | 50.10 ± 1.00 |

**Discussion**

The study on *B. lupulina* Lindl. ethanolic leaf extract provides a comprehensive evaluation of its phytochemical profile, antioxidant potential, and toxicity. The plant, known for its rich history in traditional medicine, has demonstrated promising pharmacological activities. The phytochemical analysis of the ethanolic leaf extract of *B. lupulina* revealed the presence of a diverse array of bioactive compounds. The extract was found to contain alkaloids, flavonoids, proteins, carbohydrates, tannins, and volatile oils. These compounds are known for their various health benefits and contribute to the overall pharmacological potential of the plant.

This study conducted several antioxidant assays to evaluate the extract's ability to neutralize free radicals. The results demonstrated significant antioxidant activity, with the extract effectively scavenging nitric oxide, DPPH, and hydrogen peroxide radicals. Additionally, the extract exhibited high phenolic content, which is often associated with strong antioxidant properties. These findings suggest that *B. lupulina* has substantial potential as a natural antioxidant source.

The safety profile of the *B. lupulina* ethanolic leaf extract was assessed through acute and sub-acute toxicity studies in mice. The results indicated no significant adverse effects on the mice, suggesting that the extract is safe for both acute and sub-acute use at the tested doses. This aspect of the study is crucial as it supports the potential for the extract's safe use in therapeutic applications.

Overall, the study provides valuable insights into the therapeutic potential of *B. lupulina*. The presence of various bioactive compounds, coupled with significant antioxidant activity and a favorable safety profile, underscores the plant's potential as a source of natural bioactive

compounds for therapeutic use. These findings support the further pharmacological development of *B. lupulina* extract and highlight the importance of medicinal plants in modern healthcare.

**Conclusion**

The comprehensive evaluation of *B. lupulina* Lindl. ethanolic leaf extract has demonstrated its potential as a safe and effective source of bioactive compounds with significant pharmacological properties. The acute toxicity study revealed no significant adverse effects on the mice, indicating the extract's safety for both acute and sub-acute use at the tested doses. The extract exhibited high phenolic content and robust antioxidant activities, including the scavenging of nitric oxide, DPPH, and hydrogen peroxide radicals. Additionally, the phytochemical analysis confirmed the presence of various beneficial compounds such as alkaloids, flavonoids, proteins, carbohydrates, tannins, triterpenoids, and volatile oils.

These findings underscore the therapeutic potential of *B. lupulina*, particularly its antioxidant properties, which align with its traditional medicinal uses. The study provides valuable insights that support the further pharmacological development of *B. lupulina* extract, highlighting its importance in modern healthcare as a source of natural bioactive compounds.

**Conflicts of Interest**

The authors declare no conflicts of interest.

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