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Evaluation of Physicochemical Standards, Phytochemical Screening and in-vitro Anti-inflammatory Evaluation of Echinops niveus Leaves from the Western Himalayas

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ABSTRACT:

Background: Medicinal plants from the Himalayan region represents an important source of bioactive phytochemicals with potential therapeutic potential. Echinops niveus (EN), traditionally used as folk medicine, has gained attention due to its diverse phytoconstituents and pharmacological activities. However, its physicochemical standardization as well as scientific validation of its anti-inflammatory potential is limited.

Objectives: The present study aimed to investigate the pharmacognostical standards, physicochemical standardization, preliminary phytochemical screening of EN leaves which may be helpful in terms of its validity, purity, and quality. As well as to evaluate its in-vitro anti-inflammatory activity.

Methods: Macroscopic and microscopic parameters of EN leaves were observed. Physicochemical parameters, including moisture content, ash values, and extractive values, were determined according to standard pharmacopeial procedures. Preliminary phytochemical screening was carried out to identify the presence of various plant metabolites. The in vitro anti-inflammatory activity was evaluated using protein denaturation inhibition and membrane stabilization assays.

Result: Phytochemical screening of EN leaves confirmed the presence of alkaloids, glycosides, phenolics, tannins, flavonoids, saponins, triterpenoids and steroids. Quantitative analysis showed total phenolic content of 58.78 ± 0.60 mg GAE g^{-1} and total flavonoid content of 320.30 ± 1.33 mg RE g^{-1}. Ethanol extract of EN leaves demonstrated significant dose dependent inhibition of protein denaturation and stabilization of erythrocyte membranes.

Conclusion: These findings highlight EN as a promising source of bioactive compounds to develop novel anti-inflammatory therapeutics. Further studies focussing on isolation of active phytoconstituents and in vivo pharmacological evaluation are still warranted.

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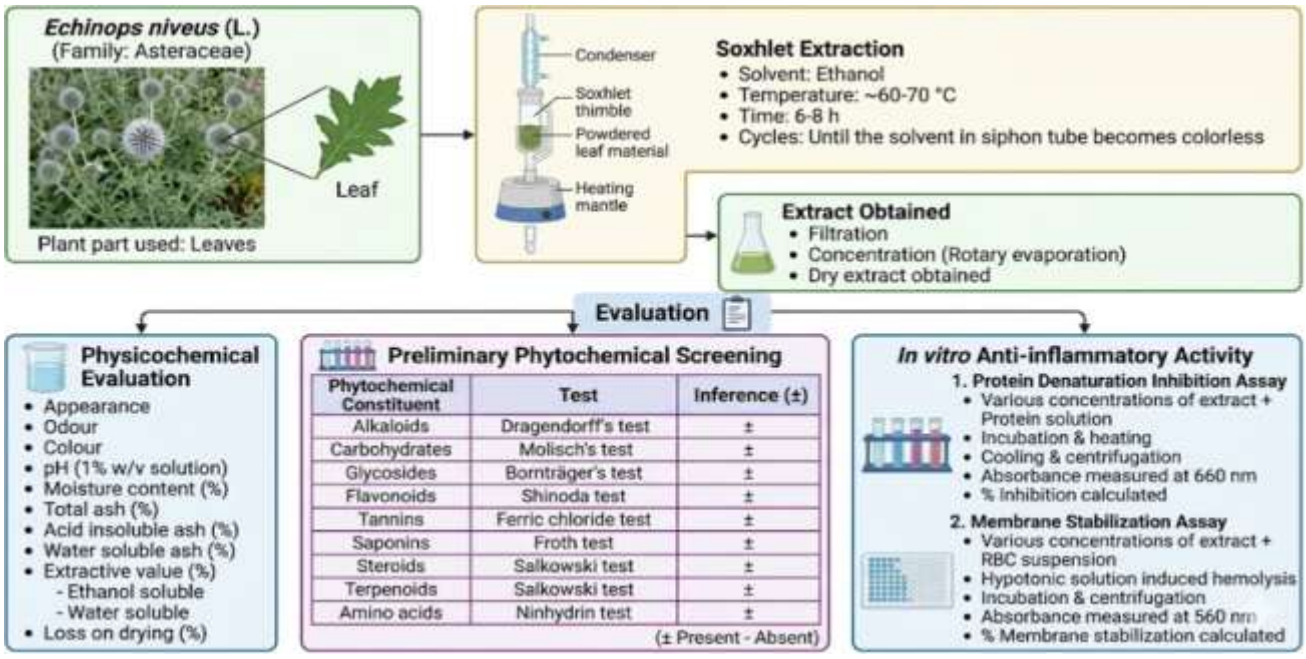
Graphical Abstract



1. INTRODUCTION

Medicinal plants that can be used in medicine have been important components of healthcare systems in the world over the centuries. The Siddha, Unani and Ayurveda systems of traditional medicine which made extensive use of compounds of plants derivatives in treatment for many diseases. As, India is considered to be among richest nations with regard to biodiversity and medicinal plant resources with almost 45,000 plant species being reported to exist in the country with about 15,000 of them having medicinal use. Researchers have been interested in the potential healing effects of phytochemicals in medicinal plants by spurring their research on these natural products over the last several decades [1]. The medication derived by plants usually has fewer side effects and offers a variety of biologically active substances like as flavonoids, phenolics, alkaloids and terpenoids. Such phytoconstituents are characterized by acts of antioxidant, anticancer, antimicrobial and anti-inflammatory. Himalayan area is an exclusive ecosystem of medicinal plants. Himachal Pradesh is specially located in the Western Himalayas and it is especially renowned in the various medicinal plant resources. Climatic factors and differences in altitude in the region promote the proliferation of many medicinal species, which have been used in the practice of local healthcare since the time immemorial [2].

There is a notable ethical medical plant among these plants *Echinops niveus* (*EN*) is an important ethnomedicinal plant that is widely spread in the Western Himalayan area and the plant belongs to the Asteraceae family and is commonly called White Globe Thistle and locally referred to as "Oontktara." It is typically found at altitudes ranging from 1500 to 3000 meters and is characterized by an erect branched stem, spiny leaves and spherical flower heads composed of tubular florets. *Echinops* species have been investigated to show a presence of numerous bioactive substances such flavonoids, alkaloids, terpenoids and lipid compounds by using phytochemical methods [3,4]. The key phytochemicals obtained in *EN* are identified as nivetin, neo flavonoids, echinopsine and echinopsidine among others as lipid derivatives. These are the compounds which are believed to be crucial in the plant's pharmacological activities. *EN* used traditionally has been a diuretic, nerve tonic and cough remedy, indigestion, ophthalmic and many more. Although this is a traditional matter, Scientific research on this is limited phytochemical characterization and the biological activity of this plant. Other researchers have reported antioxidant and wound-healing properties of the genus *Echinops* in previous studies but no proper pharmacological analysis on this respect has been described on *EN* [5]. Inflammation is a biological process connected to infection or tissue damage and is related to a number of chronic illnesses such as arthritis and cardiovascular disorders [6]. Given the paucity of scientific evidence on *EN*, the current research was aimed to evaluate its physicochemical standards, preliminary phytochemical screening and determine its *in vitro* anti-inflammatory effects.

2. MATERIALS AND METHODS

2.1. Instruments, Chemicals and solvents

Gallic Acid, Rutin and all chemicals used were sourced from Sigma Aldrich, Germany. The chemical reagents and distilled water were prepared fresh in the laboratory.

2.2. Collection of Plant Material

EN leaves were gathered from Salano (Sub-district of Solan) from Himachal Pradesh (31.68171° N, 77.286026° E). The collected plant parts were identified by Mr. Kumar Ambrish, Scientist "F" Botanical survey of India, Dehradun (Accession No: 742_Date: 29-07-2024). After authentication, the plant leaves were cleaned, dried and pulverized into a fine powder for further analysis.

2.3. Organoleptic Evaluation

Colour, odour, taste, shape, size, texture of *EN* leaves were determined as per pharmacopoeia to determine quality and purity contributing to deeper understanding of its pharmacognostical profile.

2.4. Microscopical Analysis

The surface morphology and powder microscopy of *EN* leaves were studies by using Field Emission Scanning Electron Microscopy (FESEM) to examine microscopical features of the leaf. The prepared sample was analysed under varying magnifications to investigate the microstructural features of the leaf surface [12] thereby providing valuable pharmacognostical information for identification and authentication of the sample.

2.5. Physicochemical Analysis

Various physicochemical parameters, including Moisture level, extraneous organic material, overall ash content, water-soluble ash and extractive values for the powdered *EN* leaf sample were assessed according to WHO guidelines to assess the quality and purity of the plant material [14].

2.6. Plant Extraction

Air-dried *EN* leaves were pulverized into coarse powder by using mechanical grinder and subsequently subjected to phytochemical extraction employing maceration and Soxhlet extraction [7]. For maceration, the powdered *EN* leaves were immersed in ethanol and aqueous solvent for 24 hours with intermittent agitation to enhance solvent penetration and extraction efficiency. Following maceration, the mixture. The marc was separated from the filtrate through filtration. Subsequently, the filtrate was concentrated with a rotary evaporator under reduced pressure. get rid of the solvent. The yield of obtained crude extract was noted and stored at airtight conditions at 4°C until further phytochemical analysis was done [9].

For Soxhlet extraction, the powdered plant sample was packed in thimble and extracted continuously with ethanol solvent under reflux condition inserted into Soxhlet equipment. Ethanol served as the extraction solvent until the siphon solvent turned clear, indicating exhaustive extraction of soluble phytoconstituents. The resulting Crude ethanolic extract was obtained by concentrating the extract processed under reduced pressure with a rotary vacuum evaporator [8].

2.7. Phytochemical Screening

Preliminary phytochemical screening of *EN* leaves was performed in accordance with protocols described in Trease and Evans.

2.8. Total Phenolic Content

The Folin-Ciocalteu colorimetric method was used to measure the TPC. that exists in ethanolic extracts of *EN*. Then 0.2 mL of plant extracts was collected & Folin-Ciocalteu reagent -2.5 mL was added to it & stirred to start the reaction. The mixture was then left to stand about 5 mins and 2 mL of 7.5% $NaCO_3$ was added in order to induce the production of colour. Then the reaction The solution was brought to a final volume of 7 mL with distilled water and then prepared for use and stored under dark temperature during approximately 90 minutes at controlled temperature of 25°C to enable maximum reaction of the phenolic substances and the reagent. After incubation, the solution's A UV-Vis spectrophotometer was used to measure absorbance at 760 nm. A calculation of the conc. of phenolic compounds in the extract was done using a calibration curve

established with gallic acid standard solutions ranging from 20 to 100 µg/ml. The results are shown as milligrams of GAE per gram of dried plant material. [31,32].

2.9. Total Flavonoid Content

The flavonoid content in the ethanolic extract of EN was quantified utilizing aluminium chloride colorimetry. To analyse it, the plant extract (0.5 mL) was loaded into a test tube and stirred with 0.15 mL of a 5% $NaNO_2$ solution and 0.15 mL of a 10% $AlCl_3$. The mixture was left to rest of about 5 mins so that the mixture could react appropriately. Then, 2 mL of 4 percent NaOH solution was incorporated into the mixture, which was then diluted with distilled water to reach a final volume of 5 mL. The solution was well blended so that there would be no localisation of reagents, and a coloured complex would be formed. Measurement of the absorbance of the developed solution was then done in a UV spectrophotometer at a wavelength of 510 nm relative to a reagent blank. A rutin A calibration curve The flavonoid concentration in the extract, ranging from 20 to 100 µg/mL, was determined using this method. Results were expressed as milligrams of rutin equivalents divided by the weight of dry plant sample in grams [33-35].

2.10. *In vitro* Anti-inflammatory Activity

2.10.1 Protein denaturation assay

Protein denaturation has employed to evaluate the plant extract's anti-inflammatory effect.. The protein substrate used in this assay was bovine serum albumin (BSA) solution (0.4%) made in Tris-buffered saline. The plant extract with different concentrations of 50-250 µg/ml has added to reaction mixture & aspirin was used as reference standard drug. The mixtures were ready and placed in a water bath for incubation 37±2°C for 10-15 minutes, then heated accordingly. 72°C for about 10 minutes to carry out the denaturation of proteins. The absorbance of all the solutions at 660 nm was recorded at room temperature in a spectrophotometer after cooldown. The degree of inhibition of the denaturation of the proteins generated by the plant extract was determined and quantified as percentage inhibition as compared to control [36].

$$\% \text{ Inhibition} = \frac{Absorbance\ of\ control - Absorbance\ of\ text}{Absorbance\ of\ control} 100$$

2.10.2 Membrane stabilization assay

The plant extract method was used to measure the membrane stabilizing activity using the human red blood cell (RBC) haemolysis method. A healthy volunteer was taken to get fresh blood that was centrifuged then washed with isotonic saline solution in an attempt to get erythrocyte suspensions. The experiment involved the preparation of the final suspension consisting of about 40 percent of erythrocytes. Hypotonic solution at different levels of the plant extract a range from 50 to 250 µg/ml was subsequently applied to the RBC suspension as a suspension containing the required levels of the same whereas aspirin served as standard drug in comparison. Incubated and later centrifuged samples were then measured at 540 nm through the spectrophotometer. To analyse the extract's anti-inflammatory potential, represented by the percentage inhibition of haemolysis was computed to assess the membrane stabilizing property of the extract [37].

2.10.3 Statistics:

All measurements were done in triplicate, with data reported as mean ± standard error. Statistical analysis was carried out using SPSS version 20 (IBM, Somers, NY, USA). The inhibition rates across different groups were compared using an independent samples t-test. Results were deemed statistically significant when $P < 0.05$.

2.11. Thin Layer Chromatography

Preliminary TLC analysis was conducted to optimize the solvent systems for the phytochemical profiling of *EN* leaves extract. Different solvent combinations with varying polarities were evaluated to achieve efficient resolution, maximum separation, and distinct visualization of phytoconstituent spots. TLC analysis was carried out using pre-coated silica gel plates with a 60. F_{254} designation and 0.2 mm thickness these served as the stationary phase. The extract samples were carefully applied manually as discrete spots using capillary tubes and allowed to air-dry prior to chromatographic development. Subsequently, the prepared plates were prepared in the TLC chamber is pre-saturated with the selected mobile phase at room temperature. The solvent moved until it nearly reached the edge of the plate, usually about 1 cm from the end. All plates were then examined under ultraviolet light, and the Rf values were determined. [38].

3. RESULTS AND DISCUSSIONS

3.1. Macroscopical evaluation

Various macroscopical features of *EN* Leaves were noted (Figure 1) & the results are shown in Table 1 below:

Table 1: Macroscopical Parameters of *EN* Leaves

Macroscopic Characters	Observation
Colour	Green
Odor	Characteristics
Taste	Characteristics
Texture	Rough and spiny
Size	15-20 cm long
Type	Unipennate compound leaf
Margin	Recurved spiny
Apex	Acuminate
Base	Regular, Petiolate
Shape	Oblong Lanceolate
Texture	Rough (Hairy surface)

Figure 1: *Echinops niveus* Leaves

3.2. Microscopical evaluation

3.2.1. Leaf Microscopy: Microscopical evaluation using Field Emission Scanning Electron microscopy showed leaf surface with numerous diacytic stomata, epidermal cells, uniseriate trichomes with pollen grains, glandular trichomes. Detailed photographs at different magnifications revealed epidermal cells, stomata and trichomes on the leaf surface. The diacytic stomata had subsidiary cells arranged perpendicular to guard cells (Figure 2).

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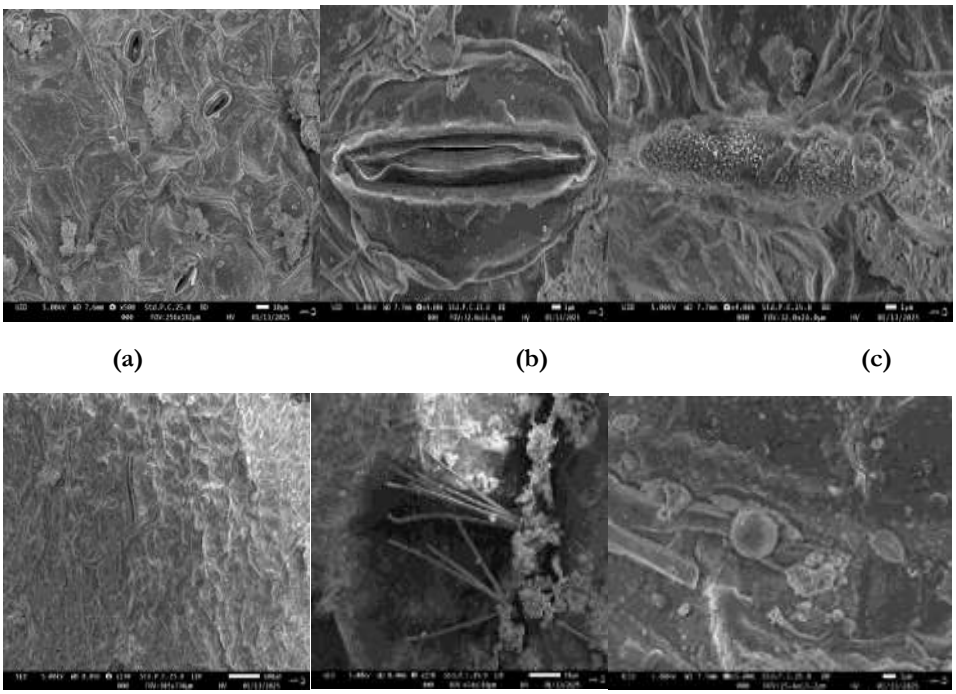

Figure 2: Samples of leaf surfaces showing (a) leaf surface with stomata (b) Single diacytic stomata (c) Single cell eukaryote (d) Leaf surface epidermal cells (e) uniseriate trichomes with Pollen grains (f) glandular trichomes

3.2.2. Powder: Powder microscopy of *EN* leaves showed epicuticular wax, Spherical Pollen Grain, anomocytic stomata, acicular shaped calcium oxalate crystals, lignified Fibres, parenchymatous structures. The powdered drug exhibited different cellular structures and components which are useful for identification and evaluation of the plant material (Figure 3).

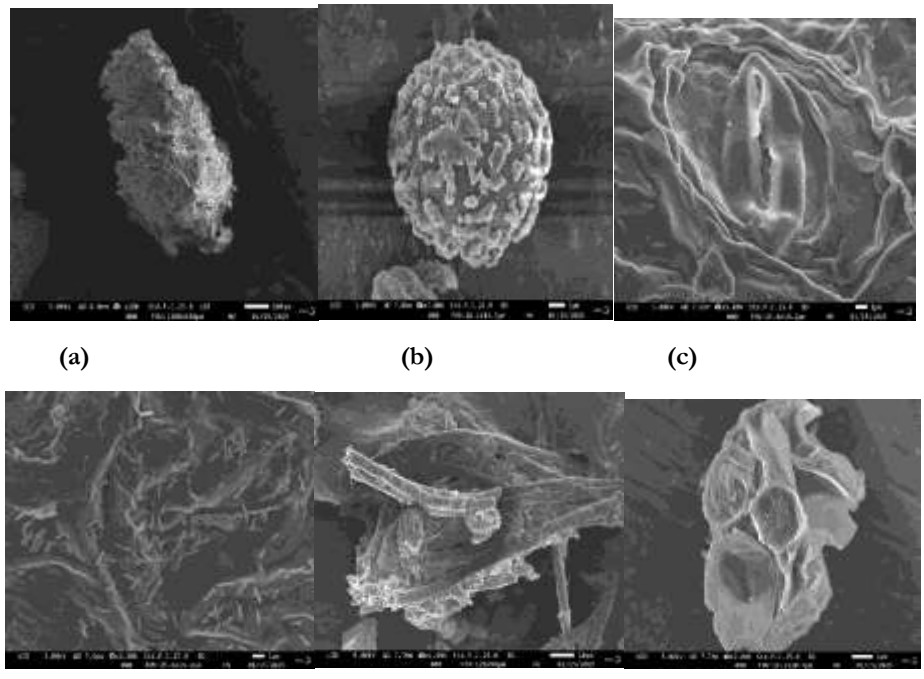

Figure 3: Powdered drug showing (a) *epicuticular wax* (b) *Spherical Pollen Grain* (c) *Anomocytic stomata* (d) *Acicular Crystals* (e) *Lignified Fibres* (f) *Parenchymatous structures*

3.3 Physicochemical Evaluation

The results of physicochemical evaluation of EN leaf powder are shown in Table 2.

Table 2: Physicochemical Evaluation of *EN* leaves

Sr. No.	Parameters	Observations
	Ash Values	
1.	Total ash Value	9 ± 0.2 %
	Water soluble ash value	2 ± 0.08%
	Acid insoluble ash value	1 ± 0.04%
2.	**Extractive Values**	
	Water soluble	1 ± 0.05% w/w
	Chloroform soluble	4 ± 0.12%w/w
	Pet-Ether soluble	6 ± 0.18% w/w
	Ethanol Soluble	3 ± 0.10% w/w
3.	Foaming Index	0.42 ± 0.01%
4.	pH	8.4 ± 0.03
5.	Moisture Content	3 ± 0.08%
6.	**Heavy Metal analysis (mg/kg)**	
	Zinc	5.38
	Iron	1817.44
	Manganese	49.02
	Copper	9.69
	Nickel	2.52
	Cadmium	0.81
	Lead	1.43
	Arsenic	2.38
7.	Foreign Organic Matters	0.5 ± 0.02%

3.3 Qualitative phytochemical screening

Preliminary qualitative phytochemical evaluation the ethanol extract of EN leaves showed evidence of presence. of various plant metabolites including carbohydrates, glycosides, tannin, phenolic compounds, flavonoids, saponins, alkaloids, triterpenoids & steroids as shown in the table below (Table 3) suggests that this plant could be utilized to treat various ailments and potentially produce valuable drugs for humans.

Table 3: Preliminary Phytochemical screening of *Echinops niveus* leaves

Chemical Class	Chemical Test	Result
Carbohydrates	Molisch's	+
	Fehling Solution	
Proteins	Ninhydrin	
	Biuret's	
Lipids	Sudan III	
Alkaloids	Mayer's	
	Wagner's	
	Hager's	
	Dragendroff's	
Glycosides	Borntrager's	
	Killer Killiani	
	Baljet	
	Legal	-
Flavonoids	Shinoda	+
	Bismuth Antimony Trichloride	
Tannins & Phenolic Compounds	Ferric Test	
	Lead Acetate	
Triterpenoids	Gelatine	
	Salkowski	
	Libermann-Burchard's	

3.4 Total Phenolic Content

The standard (gallic acid) of different concentrations was used to prepare calibration curve and linear regression equation $y = 0.005x + 0.0049$ was obtained with regression coefficient $R^2 = 0.9995$ as shown in Figure 4. The TPC in EN ethanolic leaves extract was found using that calibration curve and it has express as mg GAE / gram of extract. The TPC value ethanol extract of the *EN* was determined to be 58.78 ± 0.60 mg GAE g^{-1} of ai-dried ethanol extract.

3.5 Total Flavonoid Content

Standard calibration curve has developed by different conc. of rutin as the equation for linear regression $y = 0.0039x + 0.0052$ & regression coefficient $R^2 = 0.9988$ (shown in Figure 5). The A calibration curve of the TFC of the ethanolic extract of EN was measured and measured in milligrams of rutin equivalents for each gram of extract. The TFC of the ethanolic extract of EN was of significant value, estimated to be 320.30 ± 1.33 mg RE g^{-1} of extract.

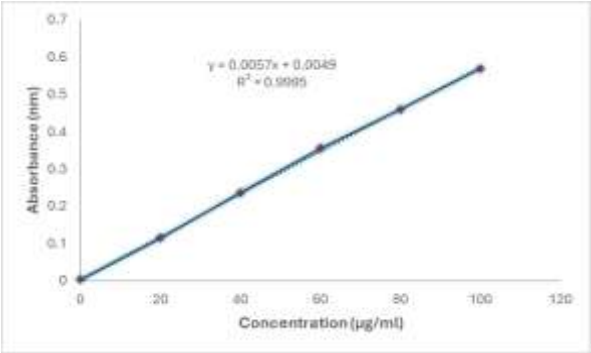

Figure 4: Calibration curve of gallic acid

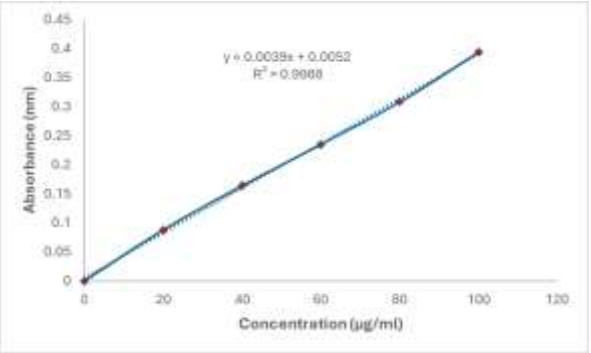

Figure 5: Calibration curve of rutin

Table 4: Results of TPC and TFC of *Echinops niveus*

Echinops niveus	**TPC (mg GAE/g of dried extract) Mean±SD**	**TFC (mg RE/g of dried extract) Mean±SD**
Ethanol extract	58.78 ± 0.60 mg GAE g^{-1}	320.30 ± 1.33 mg RE g^{-1}

3.6 *In vitro* Anti-inflammatory Activity

3.6.1 Protein Denaturation Assay

Ethanolic extract of *EN* inhibited protein denaturation in a concentration dependable way (50-250 µg/ml). The percentage inhibition was 39.89% - 68.03% and with an IC_{50} of 120.583 µg/ml. Aspirin used as the standard drug showed stronger activity with an IC_{50} of 20.326 µg/ml, indicating effective of the plant extract as anti-inflammatory potential which result in the determination of successful anti-inflammatory activity of plant extract.

3.6.2 RBC membrane stabilization Assay

It was tested by the human RBC method where the stabilization of membranes by the *EN* extract was calculated. The extract had concentration dependent inhibition of haemolysis at 50-250 µg/ml where percentage inhibition was varying between 41.27% to 68.38%. The resultant IC_{50} was 112.015 which is a moderate membrane stabilizing activity. Comparatively, of the usual drug aspirin increased activity was indicated by an IC_{50} value of 11.845 (shown in Figure 6). According to these findings, the extract has a good anti-inflammatory effect via erythrocyte membrane stabilization.

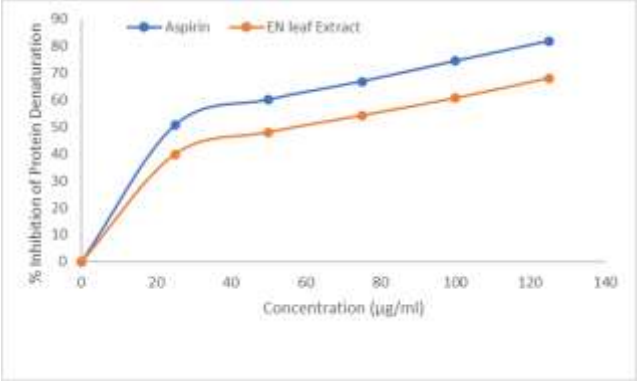

Figure 6A

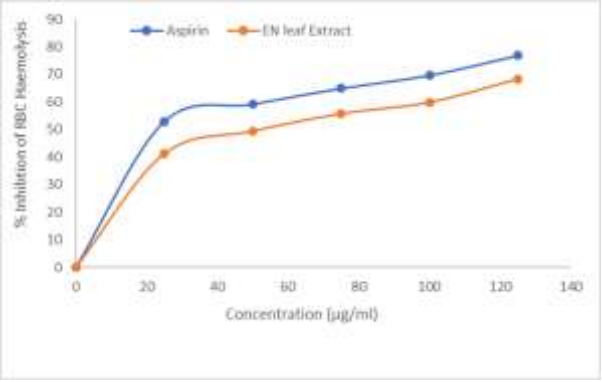

Figure 6B

Figure 6: In vitro Anti-inflammatory activity of Echinops niveus

3.7 Thin Layer Chromatography

TLC analysis the ethanolic extract of EN has been tested using different solvent systems, including Toluene, Ethyl acetate, and Acetic acid. (6:4.2:0.5) provided the best separation and was selected as the ideal mobile phase. Under UV light (254 and 365 nm) about 14 spots with R_f values ranging from 0.12-0.98 were observed that indicating multiple phytoconstituents with different polarities suitable for further isolation studies (shown in Table 5).

Table 5: TLC Analysis of Ethanolic Extract of *EN*

Solvent System	Spot No.	Colour at 365 nm	Colour at 254 nm	Rf Value
Toluene: Ethyl acetate: Acetic acid (6:4:2:0.5)	1	Dark blue (EN)	Green (EN)	0.12
	2	Fluorescence	Light green	0.28
	3	Fluorescence		0.38
	4	Fluorescence	No spot	0.46
	5	Light pink	Light green	0.52
	6	Dark pink	Green	0.56
	7	Blue	Dark green	0.60
	8	Light pink	No spot	0.64
	9	Dark blue	Blue	0.72
	10	Pink	Light green	0.76
	11	Yellow	Dark green	0.80
	12	Fluorescence		0.86
	13	Purple	Green	0.92
	14	Pink	Light green	0.98

4. DISCUSSION

The increasing emphasis on natural products and traditional medicines offers exciting opportunities for drug discovery. Medicinal plants, rich in unique chemical and molecular compounds, are central to ongoing pharmaceutical research. India's extensive biodiversity, which includes many medicinal plants, it plays a crucial role in promoting drug development and healthcare within traditional systems. Nonetheless, rising demand has caused problems such as counterfeit products and quality issues in the market. Adulteration often results from insufficient knowledge about local growing conditions, vernacular names, and plant morphology and microscopy. Therefore, developing standardized methods and procedures is crucial to guarantee the quality of both raw materials and finished products. This study assessed important physicochemical parameters such as ash value and acid-insoluble ash include water-soluble ash to analyze the plant's inorganic content. Extractive values in various solvents indicate the existence of active chemical compounds within the plant material. These findings aid in precise identification and verify the authenticity of Echnipos niveus, ensuring its quality and purity. Furthermore, this approach helps prevent adulteration with drugs from similar or different genera that may be less effective. The initial phytochemical analysis offers insights into the chemical makeup of the extract's active ingredients. The screening revealed several secondary metabolites, such as glycosides, tannins, alkaloids, phenolic compounds, saponins, triterpenoids, and steroids, likely contributing to the plant's varied pharmacological activities. The quantification of TPC (58.78 ± 0.60 mg GAE g^{-1}) and TFC (320.30 ± 1.33 mg RE g^{-1}) in EN leaves primarily indicates their antioxidant effects, including free radical scavenging, inhibition of peroxidation, and transition metal chelation—all connected to inflammation. Phenolic compounds are known to effectively neutralize oxygen-derived free radicals by donating hydrogen atoms or electrons [40]. Both laboratory (in vitro), animal (in vivo), and clinical studies confirm the significance of flavonoids in managing oxidative stress, inflammation, metabolic

syndrome, adipogenesis, cell growth, programmed cell death (apoptosis), autophagy, and the process of forming new blood vessels (angiogenesis) [41].

The EN extract demonstrated dose dependent anti-inflammatory property by preventing protein denaturation and membrane stabilization assay. EN leaves extract showed significant and dose dependent membrane stabilizing property compared with the standard drug aspirin that may be due to the inhibition of the release of phospholipases [42].

5. CONCLUSION

EN leaves have been found to be rich in significant phytochemicals, especially phenolics and flavonoids may contribute to the significant in-vitro anti-inflammatory effects. Thus, the plant has potential to be used as a natural therapeutic agent in the prevention of inflammation diseases but medicinal validation has to be done by using *in-vivo* studies, toxicity assessment and clinical trials.

AVAILABILITY OF DATA AND MATERIALS

All data generated in the study are provided in the manuscript. No extra data is available.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not Applicable.

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CONSENT FOR PUBLICATION

All authors have read and approved the final manuscript.

DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

Authors' contributions

Panshul Sharma: Did experimental work and wrote manuscript; Nitin Verma: Ph.D supervisor, guidance throughout the completion of work and manuscript. Parul Sood: Manuscript checking and finalization

ABBREVIATIONS

EN: *Echinops niveus;* ***FESEM:*** *Field Emission Scanning Electron Microscopy;* ***FT-IR:*** *Fourier Transform Infrared Spectroscopy;* ***GAE:*** *Gallic Acid Equivalent;* ***HPTLC:*** *High-Performance Thin Layer Chromatography;* ***IC_{50}:*** *Half Maximal Inhibitory Concentration;* ***MS:*** *Mass Spectrometry;* ***NaCl:*** *Sodium Chloride;* ***NaOH:*** *Sodium Hydroxide;* ***NMR:*** *Nuclear Magnetic Resonance;* ***RBC:*** *Red Blood Cells;* ***RE:*** *Rutin Equivalent;* ***TFC:*** *Total Flavonoid Content;* ***TLC:*** *Thin Layer Chromatography;* ***TPC:*** *Total Phenolic Content;* ***UV:*** *Ultraviolet;* ***WHO:*** *World Health Organization.*

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