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PHYTOCHEMICAL SCREENING, ANTI-INFLAMMATORY ACTIVITY AND IDENTIFICATION OF HYPERICIN FROM HYPERICUM CORDIFOLIUM AN ENDEMIC SPECIES FROM NEPAL

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ABSTRACT

Medicinal plants have long been a reliable source of bioactive compounds, contributing significantly to modern drug discovery. The *Hypericum* genus, known for its diverse pharmacological properties, contains over 490 species, including *Hypericum perforatum*, which has been extensively studied for its antidepressant and anti-inflammatory activities. However, other species, such as *Hypericum cordifolium*, remain underexplored despite their traditional use in inflammatory disorders, burns, and infections. This study investigates the phytochemical composition and anti-inflammatory potential of *H. cordifolium*, focusing on the extraction and characterization of hypericin, a key bioactive compound. The methanolic extract of *Hypericum* was first subjected for phytochemical screening and then for biological activity i.e. anti-inflammatory property in vitro using egg albumin denaturation assay. The phytochemical screening revealed presence of phenols alkaloids flavonoids in the extract. The anti-inflammatory property was comparable with diclofenac 83%% and the extract 78.49 % of at 1000 µg/ml of protein denaturation using egg albumin. The presence of hypericin was screened by using HPLC at the retention time of 9.7 mins for hypericin in the methanolic extract and 9.8 min in hypericin standard. Thus, these findings suggest that *H. cordifolium* possesses significant anti-inflammatory potential, with hypericin as a key bioactive compound, supporting its therapeutic relevance in drug discovery.

KEYWORDS: Hypericin, *Hypericum cordifolium*, HPLC, phytochemical screening, Protein denaturation assay.

1- INTRODUCTION

Medicinal plants have served as a keystone of traditional medicine and contemporary pharmacology, offering a vast range of bioactive compounds with therapeutic potential. Over 50% of presently approved drugs are derived from natural sources, with plant-based molecules playing a vital role in treating cancer, infectious diseases, and inflammatory disorders. Among medicinal plants, the genus *Hypericum* comprises 490 species globally, with fifteen reported in Nepal.[1] One of its popular species, *Hypericum perforatum* being Native to Europe, North western Africa and western and Northen Asia, holds a significant place within the complementary and alternative medicine (CAM) system for treating conditions ranging from mild to severe depression, inflammatory, autoimmune diseases, burns, bacterial infections to wounds.[2] *H. perforatum* are well-recognized by the European Medicine Agency (EMA) under the Traditional Herbal Registration (THR) scheme and are licensed as medicines.[3] The aerial parts the plant contains many biologically active compounds, the most important are hypericin, a strong photosensitizer fluorescent red plant pigment, which causes apoptosis in tumor cells[4]; and hyperforin, the most abundant lipophilic compound is likely to account for the antidepressant action of the plant.[5] Previous research on *Hypericum perforatum* reports that the plant has antidepressant, antiviral, anti-inflammatory, antioxidant, antibacterial and anticancer properties.[6] Hypericin (I) and Hyperforin (II) are reported from several species of the genus *Hypericum* including the most traded species *H. perforatum*.[7],[8] Many species of this genus were reported to have biological active phytochemicals. Although, used as ethnic medicine by contemporary civilizations in various conditions such as burns, pain, inflammatory conditions, yet comprehensive pharmacognostic and bioactivity studies on other species remain limited.[9] Thus, this study aims to extract and identify one of the most potent compounds, (hypericin) from *H. cordifolium* species as well as unraveling its pharmacognostic attributes, phytochemical composition and in vitro anti-inflammatory activity.

2- MATERIALS AND METHODS

2.2 Extraction isolation and purification of *H. cordifolium* extracts

2.2.1 Collection of Plant Material

The plant materials were gathered from the Godawari area in the Kathmandu District, Bagmati Province, Nepal under the guidance of a botanist from the National Herbarium and Plant Laboratories (KATH), Department of Plant Resources. [GoN, Ministry of Forests and Environments, Nepal]. The collected materials were carefully identified and authenticated at the site and shed dried.

2.2.2 Pharmacognostic study of *H. cordifolium* extracts

The pharmacognostic study of *H. cordifolium* was conducted using the methods outlined in 'Quality Control Methods for Medicinal Plant Materials, WHO'.

2.2.3 Phytochemical Screening of *H. cordifolium* extracts

The dried plant was reduced to a coarse powder. About 200gms powdered form was then subjected to cold maceration in petroleum ether for 48 hours and then filtered. The filtrate was collected and the marc was again extracted with chloroform for 48 hours and subsequently with methanol. The filtrate was evaporated to dryness. The petroleum ether extract, chloroform extract and methanolic extract were subjected for phytochemical screening as outlined in 'The Practical Manuals on the Industrial Utilization of Medicinal and Aromatic Plants.[10]

3 Anti-inflammatory activity of *H. cordifolium* by protein denaturation assay using egg albumin

Anti-inflammatory activity was performed by taking 2ml of different concentration (1000 µg/ml, 800µg/ml, 400 µg/ml, 200 µg/ml and 100 µg/ml) of the methanolic extract.[11] Then, 0.2ml albumin (white part) from Hen's egg (centrifuged for 30 minutes) was mixed with different extract. Then 2.8ml phosphate buffer saline adjusted to pH6.6 was added in the mixture to make up volume to 5ml. It was then incubated at 37°C for 15 minutes. Afterwards, they were subjected for heating for 5 minutes at 70°C. After cooling the absorbance was taken at 660nm using UV spectrophotometer. Similarly, inhibitory activity of different concentration of diclofenac was taken as standard for positive control and methanol as a negative control. The method was triplicated to take mean absorbance value. The percentage inhibition was calculated as follows:

% reduction by test = (Absorbance of control−Absorbance of sample) × 100/Abs absorbance of control.

%reduction by standard = (Absorbance of control−Absorbance of sample) × 100/Absorbance of control.

Where At, As and Ac are absorbance of Test, Standard and Control respectively.

2.2.4 HPLC analysis System of *H. cordifolium* extracts

The hypericin standard was purchased from Ottokemi chemical suppliers, Mumbai, Maharashtra, India and prepared by dissolving in methanol in (0.5mg/ml) concentration. About 200 grams of powdered aerial parts was extracted with methanol by cold maceration process for 48 hours and then filtered and filtrate was collected. The analysis of *H. cordifolium* constituents were conducted using preparative HPLC. The mobile phase consisted of A: orthophosphoric acid (0.1%) in water and B: Acetonitrile in a ratio of 70:30, with a flow rate of 1mL/min and detection at a wavelength of 270 nm. The preparative HPLC (SHIMADZU, Model: LC-20 AP) equipped with SPD-M 40A PDA with wavelength between 190-800 nm, CBM 20A system controller, Fraction collector FRC-10A, C18 (10×250mm) column, and Lab Sol ADD PDA connect CS–ready software.

The sample was filtered through a 0.22 µm membrane filter. Samples and standards were injected systematically a run time of 30 minutes, and the column temperature was maintained at 25°C. Quantification of hypericin compound was achieved by constructing a calibration curve using the standard solutions.

3- RESULTS AND DISCUSSION

3.2 Pharmacognostic screening of *Hypericum cordifolium* constituents

3.2.1 Macroscopical studies

The details of the general appearance of the plant are described in (Table 1).

Table 1: The macroscopic studies of *H. cordifolium* species.

Leaves:	: Leaves are sessile, oblong-lanceolate acute, margin plane, shallowly cordate at base, blue green beneath
Inflorescence	Flowers stellate. Sepals free, lanceolateorrarely ovate or ovate-elliptic. Petals yellow, sometimes tinged red, spreading, narrowly obovate to oblanceolate, with apiculus acute, margin entire, glandular
Flowers	Flowers are yellow, 3.8 cm across, in many- flowered cluster; with prominent short sharp apical point; stamens as long as style, 2/3 as long as petals and 1.5 times as long as ovary

Organoleptic properties	Odor: odorless

3.2.2 Phytochemical screening of *H. cordifolium* constituents

Since, the phytochemical composition of plant extracts plays a crucial role in determining their medicinal properties and potential therapeutic applications. In this study, a comprehensive phytochemical screening of a plant extract was done using different solvents, namely methanol, chloroform, and petroleum ether (Table 2). The findings revealed the presence of terpenoids and steroids, flavonoids, glycosides, saponin, tannin, coumarin and reducing compounds exclusively in the methanolic extract, emphasizing the solvent-dependent extraction of these compounds. The absence of these bioactive constituents in chloroform and petroleum ether extracts suggests that these solvents may only be suitable for extracting specific constituents from the plant material under investigation. In support of this study *H. perforatum* extracts also had shown terpenes, including monoterpenes and sesquiterpenes. These compounds may contribute to the antitumor, anti-inflammatory, antibacterial, anti-inflammatory, antiviral antimalarial effects of the plant.[12]

Table 2: Represents the phytochemical screening of *H. cordifolium* extracts in petroleum ether, chloroform and methanol extract.

S. No	Tests	Pet. Ether Extract	Chloroform extract	Methanol extract
1	Alkaloids Mayer's reagent Wagner's reagent	 – –	 – –	 – –
2	Terpenoids and Steroids Salkowski's test	–	–	+
3	Glycosides Extract+NH4OH Molisch reagent	– – –	– – –	+ + –
4	Saponins	–	–	+
5	Flavonoids Shinoda test	–	–	+
6	Tannins Extract+FeCl3 Extract +Acetic acid Extract+HNO3	+	+	+
7	Coumarins	+	+	+
8	Carbohydrates	–	–	–
9	Reducing Compounds Fehling' test Benedict's test	 – –	 – –	 + +
10	Proteins	–	–	–

	Millon’s test			
11	Amino acid Ninhydrin test	–	–	–
	Volatile oil	_	_	_

3.3 Inhibition of albumin denaturation by *H. cordifolium* extract

Inflammation is the body's natural defense mechanism against injury, infection, or tissue damage, marked by redness, heat, swelling, pain, and disrupted physiological functions. It serves as a protective response triggered by physical trauma, harmful chemicals, or microbial agents. Protein denaturation occurs when proteins lose their secondary and tertiary structures due to external factors like strong acids, bases, concentrated salts, organic solvents, or heat. This structural change typically leads to the loss of the protein’s biological function. Importantly, protein denaturation is a well-established factor contributing to the onset of inflammation. As the species was used from centuries for the ailment of burns, pain and inflammatory conditions. So, the anti-inflammatory activity of *H. cordifolium* was accessed by protein denaturation assay in methanolic extract using egg albumin denaturation assay and compared with standard diclofenac.[11] The extracts of *H. cordifolium* had anti-inflammatory activity comparable with diclofenac standard as shown in graph % inhibition in protein in egg albumin. At the concentration of 1000µg/ml methanolic extract of *H. cordifolium* exhibited highest (78.49%) inhibition of protein denaturation when compared with diclofenac (83%). Similarly, the percentage inhibition of protein denaturation at concentration of at concentration of 800µg/ml, 400µg/ml, 200µg/ml and 100µg/ml (75.96%, 74.26%, 73.80%, 70%) (Figure 1). The anti-inflammatory activity is due to the presence of flavonoids, steroids and phenolic compounds. Various studies suggest that the anti-inflammatory activity of *Hypericum* species, including *Hypericum perforatum* (commonly known as St. John's Wort), is primarily attributed to several bioactive components, such as hypericin and pseudohypericin, these naphthodianthrone derivatives have demonstrated anti-inflammatory properties.[13] The other compounds such as flavonoids (e.g., quercetin, rutin and hyperoside)[14], phloroglucinols (e.g., hyperforin)[15], phenolic acids (e.g., chlorogenic acid, caffeic acid)[16] have also shown anti-inflammatory properties.

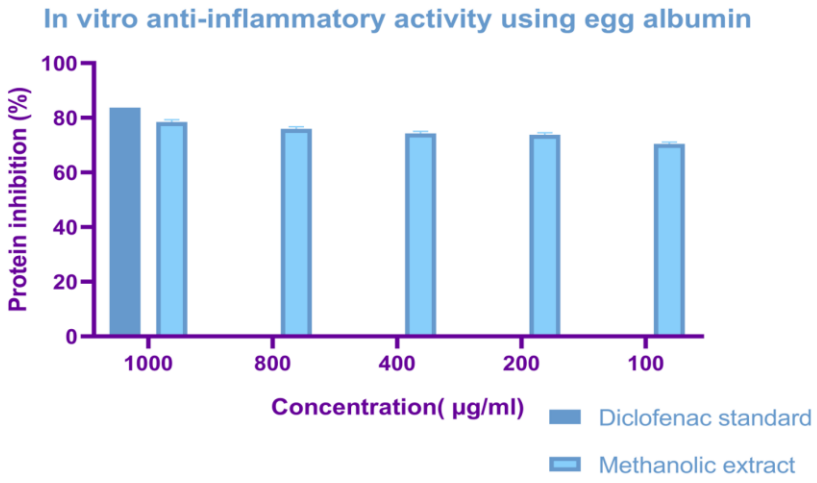

Figure 1: Effect of methanolic extractof *H. cordifolium* on protein denaturation in egg albumin.

3.2.3 Identification and separation of hypericin from *H. cordifolium* by Preparative HPLC

As naphthodianthrones (hypericin, pseudohypericin and protohypericin) are specific constituents of *Hypericum* species and are usually examined in all types of *Hypericum* species.[17] Multiple studies have revealed use of reversed-phase HPLC for the quantification of naphthodianthrones.[18] Also, Studies have shown, HPLC is widely used particularly for hypericin analysis, and it may replace all other analytical methods due to its fast analysis times and high sensitivity.[19] Hence, preparative HPLC aimed to isolate hypericin in a pure form from the complex matrix of phytochemicals present in the extract for detailed characterization.

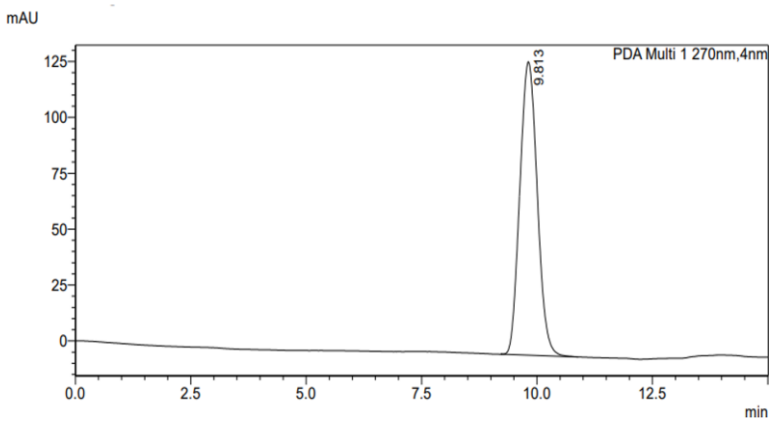

Figure 2: Identification of Peak of hypericin standard observed at 270nm with the retention time of 9.813 minutes.

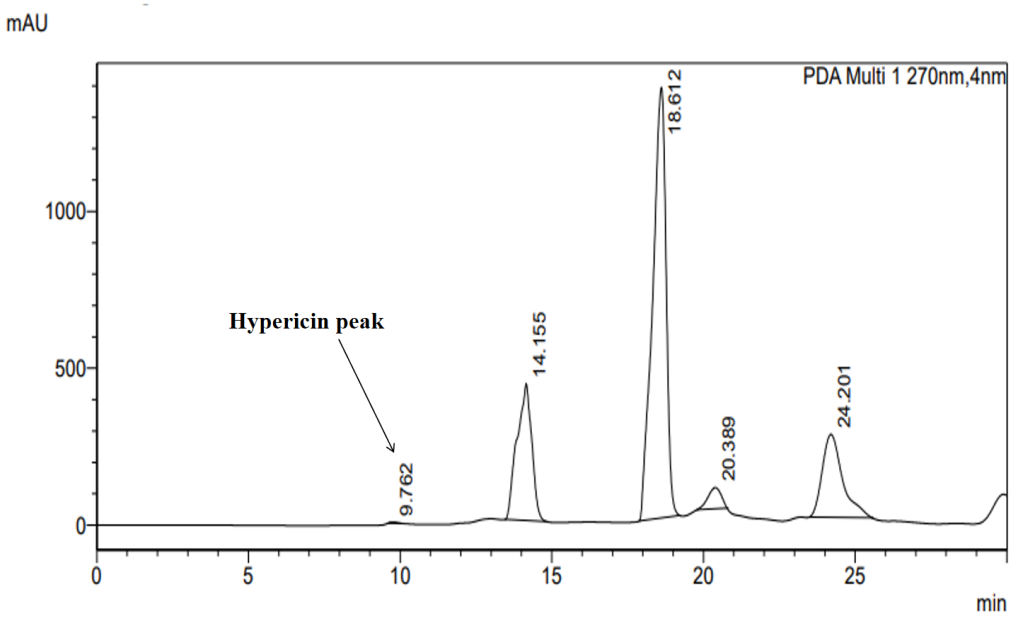

Figure 3: Identification of peak of hypericin from *H. cordifolium* extract at 270 nm at retention time of 9.762 minutes.

A distinct peak was observed at 270 nanometres with a flow rate of 1 ml/min and a c18 column having temperature of 30°C. The retention periods of the reference standard and H. cordifolium extract 9.786 (Figure 2) minutes and 9.8 (Figure 3) minutes, respectively provided a reliable standard for the identification and quantification of hypericin in the plant material. This result is consistent with the findings of[20] which established the dependability of UV detection at 270nm in the quantification of hypericin. In addition, similar outcomes were observed when employing a mobile phase having same composition of acetonitrile and 0.3% phosphoric acid (90:10, v/v) for the separation of integrating concurrent fluorescence and UV detection hypericin.[21] The results of our investigation align with those of[22], who similarly identified hypericin at same wavelength 270 nm utilising an RP-18 300 A column and a solvent system consisting of water, acetonitrile, methanol, and phosphoric acid.[21] C18 (octadecylsilyl) as stationary phase is the most remarkably used for *Hypericum* species However, other RP (reversed phase) stationary phase with lower hydrophobicity have been found for hypericin analysis, one of the example is C8 (octyl).[23] Stationary phase having full porosity can be used for naphthodianthrones, and monolith columns help to analyze the pseudohypericin and hypericin content rapidly with short retention time.[24] In support of our study hypericin was had a peak at a retention time of 12mins with the mobile phase consisting of ammonium acetate, methanol, and acetonitrile using Ag/AgCl as a reference electrode in the detector.[25] In contrast hypericin had a at a high retention time

(approximately 81.5 min) of the St. John's wort chromatograms at 590 nm. The difference in retention time is due to the choice of solvents used in mobile phase. The total hypericin content was found to be 0.115% likewise several *Hypericum* species were analysed for hypericin content which was found in between the range of (0.1 to 0.5%).[26] Similar kind of result was obtained in which hypericin content was (0.19-0.30) % in *H. perforatum* species.[27] Additionally, in support of our study, hypericin concentration in between (0.013% to 0.989%).[28] The application of the Prep HPLC method to the *H. cordifolium* extract enables the comparison of chromatographic profiles and confirms the presence of hypericin. The ability to isolate hypericin from the complex mixture of phytoconstituents in the plant extract opens avenues for in-depth structural elucidation, bioactivity assessments, and potential therapeutic applications.

4- CONCLUSION

Hypericum sp. has long been used as a medicinal plant in a variety of treatments, but it has lately acquired prominence in research due to its various properties. Additionally, one of the Nepalese endemic species, *H. cordifolium* explored for the secondary metabolites via phytochemical screening to ensure the presence of hypericin. Our study for the first time reported hypericin in *Hypericum cordifolium* species. Thus, it is not worthy to mention that hypericin was successfully identified, separated with the help of preparative HPLC technique from the endemic species of Nepal. So, we can conclude that preparative HPLC can be used on the industrial scale for the production of high purity hypericin. However, a final identification of substances is not possible using this method as other components could have the same retention time. The HPLC method only provides the information on the presence of that particular compound and helps to compare the chromatogram according to the literature data or the reference standard. Consequently, more precise methods, such as FTIR, NMR, and MS profiling are required.

Authorship contribution statement

SS conducted experiments and wrote the manuscript and prepared the figures. SS, MR, RC, VA and ASK analyzed the data. VM conceived and designed research. The final manuscript was reviewed and approved by all authors. The authors declare that all data were generated in-house and that no paper mill was used.

Declaration of competing interest

The authors declared no potential conflicts of interest.

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Data availability

Data will be made available on request.

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