Journal of Research in Pharmacy

Research Article

www.jrespharm.com

Pharmacognostic evaluation and HPTLC quantification of rutin in *Adina cordifolia* leaf with profiling of anti-inflammatory, and antioxidant activities

Rakesh Surappa ANJANEYA [1] *, Guno Sindhu CHAKRABORTY [1]

[1] Department of Pharmacognosy, Parul Institute of Pharmacy & Research, Parul University, Vadodara, Gujarat, 391760 India.

* Corresponding Author. E-mail: rakesh.sagmipsr@gmail.com (R.S.A.); Tel. ++91-998-632 93 32.

Received: 25 September 2024 / Revised: 24 December 2024/ Accepted: 29 December 2024

ABSTRACT: The Rubiaceae family includes the medicinally significant species *Adina cordifolia* (Roxb.) Hook. f. ex-Brandis, traditionally used to treat conditions such as inflammation, fever, wounds, and parasitic infections. This study investigates the phytochemical profile, pharmacognostic properties, and bioactive potential of *Adina cordifolia* (ACF) leaf and bark extracts. Emphasis was placed on evaluating antioxidant and anti-inflammatoryactivities and determining rutin and tannic acid levels using High-Performance Thin-Layer Chromatography (HPTLC).Pharmacognostic analysis involved morphological and microscopic studies of ACF leaves, aiding botanical characterization. Soxhlet extraction was performed using various solvents, with ethanol yielding the highest extractive values for leaves (31.5%) and bark (29.8%). Phytochemical screening identified carbohydrates, flavonoids, tannins, saponins, alkaloids, and triterpenoids in the extracts, with ethanol and butanol showing the highest concentrations. HPTLC analysis quantified rutin, while antioxidant activity was assessed through the DPPH assay. The anti-inflammatory potential was evaluated using protein denaturation and HRBC membrane stabilization techniques. HPTLC analysis confirmed the presence of rutin in all leaf extracts, with the butanolic extract showing the highest concentration (0.43%), while tannic acid was absent in the leaf extracts. The butanolic extract exhibited strong antioxidant activity, with an IC50 value of 36.90 µg/ml. Ethanolic extracts had the highest tannin (14.4%) and polyphenol (5.2%) content, correlating with significant bioactivity. Anti-inflammatory assays further highlighted the therapeutic potential of ACF extracts. These findings substantiate its traditional uses and underscore its relevance in modern pharmacological research.

KEYWORDS: *Adina cardifolia*; rutin; tannic acid; HPTLC; fractionation; anti-inflammatory; antioxidant.

1. INTRODUCTION

Known by many as "Kadam" or "Haldu," Adina cordifolia (Roxb.) Hook. f. ex-Brandis is a well-known species in the Rubiaceae family that is extensively dispersed throughout the Indian subcontinent and Southeast Asia. This herb has historically been widely utilized in ethno medicine to treat a wide range of ailments, such as fever, inflammation, wounds, and skin disorders [1]. *Adina cordifolia* (ACF) has a rich phytochemical profile that includes flavonoids, alkaloids, glycosides, and tannins, which is largely responsible for its therapeutic efficacy [2]. Flavonoid glycoside rutin is one of the main bioactive substances found in ACF. This substance is well known for having strong antioxidant qualities that allow it to scavenge free radicals and shield biological systems from oxidative stress [3]. Additionally, rutin significantly reduces inflammation by blocking enzymes like cyclooxygenase and lipoxygenase, important intermediaries in pathways leading to inflammation [4]. Rutin's various pharmacological actions highlight how crucial it is to precisely quantify and standardize it in herbal medicines made from ACF.

A critical phase in the standardization of medicinal plants is pharmacognostic assessment. It entails a thorough examination of the plant material's morphological, anatomical, and physicochemical properties. Such assessments are essential for guaranteeing the genuineness, quality, and purity of herbal medications, especially in light of the expanding issue of adulteration and replacement in the herbal market [5]. Comprehensive pharmacognostic studies are required for ACF in order to differentiate it from comparable species and to set guidelines for quality control.

How to cite this article: Anjaneya RS, Chakraborty GS. Pharmacognostic evaluation and HPTLC quantification of rutin in *Adina cordifolia* leaf with profiling of anti-inflammatory, and antioxidant activities. J Res Pharm. 2025; 29(5): 2151-2164.

ISSN: 2630-6344

https://doi.org/10.12991/jrespharm.1766404

For accurate quantification of bioactive chemicals in plant materials, sophisticated analytical techniques are necessary in addition to pharmacognostic assessments. The efficacy of High-Performance Thin-Layer Chromatography (HPTLC) in the separation, identification, and quantification of phytochemicals has made it a preferred technique [6]. Several benefits come with HPTLC, such as ease of use, affordability, and the capacity to examine multiple samples at once. HPTLC offers a strong and dependable technique for quantifying rutin in ACF, guaranteeing the medicinal grade of the plant extract is consistent [7].

Plants are important for healing because they contain compounds that are particularly strong and affect human physiology. Major population globally rely on herbal remedy [8,9]**.** Hepato-defensive [10], along with other natural qualities, have been isolated from several locations. These include moderating, combating diabetes, being hostile to amoebic, antiulcer, combating nociceptive, antibacterial, and anticancer. This spice has been utilized in the past to cure a variety of illnesses, including conjunctivitis, barrenness, bacterial infection, fever, cold/hack, toothache, stiffness, congestion, and other ailments [11,12]**.** Beyond its conventional applications, ACF has a wide range of medicinal possibilities.

It has been demonstrated that ACF extracts alter pro-inflammatory cytokines, which lowers inflammation and opens up new possibilities for the creation of anti-inflammatory drug therapies [13]. Furthermore, ACF's antioxidant activity enhances its therapeutic potential by reducing oxidative stress, which is a major contributor to the aetiology of numerous chronic illnesses, such as cancer, heart disease, and neurological disorders [14,15]. The goal of the current work is to quantify rutin using HPTLC and do a thorough pharmacognostic evaluation of ACF leaves. In addition, the study aims to characterize the leaf extract's antioxidant, anthelmintic, and anti-inflammatory properties.

Current studies are investigating its antioxidant, and anti-inflammatory properties. With the prevalence of chronic inflammatory disorders on the rise, the anti-inflammatory activity is especially noteworthy. This will support the extract's prospective uses in modern medicine and provide traditional practices a scientific foundation. This strategy will guarantee the consistent use of ACF in herbal formulations and help establish it as a useful resource in pharmacological research.

2. RESULTS AND DISCUSSION

2.1 Macro-morphological and microscopic evaluation

The leaves are simple, opposite, and arranged in a decussate pattern. They have stipules that are interpetiolar, obovate, foliaceous, and shed early, measuring 10-12 x 7 mm. The petiole is stout, pubescent, and varies in length from 4-10 cm. The leaf blade is either orbicular, ovate, or broadly ovate, typically measuring 10-20 x 10-20 cm, with a heart-shaped base and a pointed apex. The leaf margins are smooth, with the upper surface being glabrous and the lower surface pubescent, giving the leaf a papery texture. The leaves feature 5-7 main nerves originating from the base, with 3-5 pairs of lateral, pinnate, and prominent nerves. The intercostal veins are prominent and arranged in a ladder-like pattern, with domatia also present. The microscopic evaluation of ACF leaf reveals distinct anatomical features critical for its identification and characterization. The transverse section of the ACF leaf, shows well-defined vascular bundles consisting of xylem and phloem, along with palisade cells and unicellular covering trichrome. The powder microscopic images, highlights the presence of key leaf tissues, including mesophyll, epidermis, and covering trichrome. Notably, the stomatal analysis indicates a stomatal number of 100 per square millimetre, with a stomatal index approximately valued at 6.06. These findings contribute to the comprehensive understanding of the leaf's microstructural properties, aiding in its pharmacognostic evaluation. Additionally, the powder microscopy of ACF bark, demonstrates the presence of phloem fibres and cork cells, further supporting the detailed characterization of this plant material.

2.2 Extraction and Fractionation of Chemical Constituents from Plant Materials

Using ethanol as the solvent, a Soxhlet device was used to extract the bark and leaves of ACF **(Figure 1)**. After the extraction, different solvents were used to calculate the percentage yield of different fractions from the bark and leaf, as indicated in (Table 1).

With an ACF leaf extract yield of 31.5% w/w and a bark extract yield of 29.80% w/w, the alcoholic extract produced the greatest fraction of all the solvents examined. By comparison, the yields from the petroleum ether extract were much lower, yielding 1.23% w/w for the leaf and 0.70% w/w for the bark. For the leaf and bark, the chloroform extracts produced yields of 1.84% and 1.60% w/w, respectively. The yields of the ethyl acetate fraction were 3.76% w/w for the bark and 3.49% w/w for the leaf. Moderate yields were observed in the n-butanol extract, with 14.24% w/w for the leaf and 9.48% w/w for the bark. These findings

suggest that, as compared to the other solvents examined, ethanol is a more efficient solvent for removing bioactive chemicals from ACF bark and leaf.

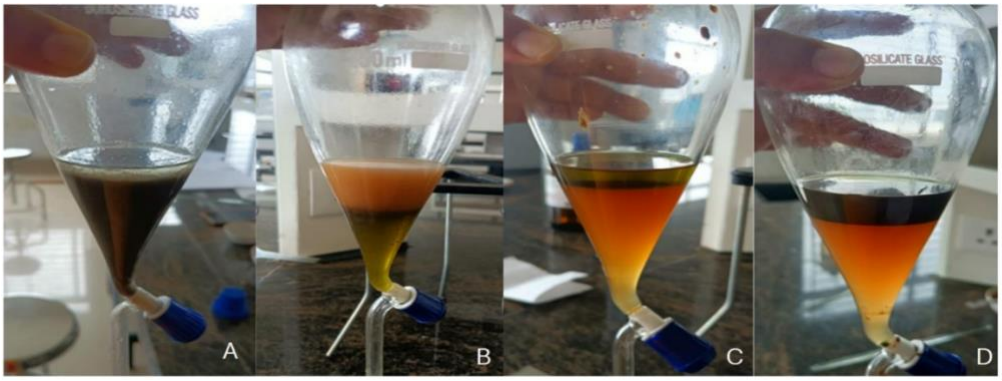

Figure 1. Sequential Fractionation of *Adina cordifolia* Extracts, (A) Petroleum Ether Fraction, (B) Chloroform Fraction, (C) Ethyl Acetate Fraction, (D) n-Butanol Fraction

Table 1. Physical characteristics and percentage yield of *Adina cordifolia* leaf and bark extracts across different solvent fractions

Extract	Leaf		Bark	
	Color & consistency	% W/W yield	Color & consistency	% W/W yield
Alcoholic extract	Greenish brown (soft extract)	31.5%	Dark brown (soft extract)	29.80%
Petroleum ether (fraction-1)	Yellowish (sticky)	1.23%	Yellowish (sticky)	0.70%
Chloroform (fraction-2)	Light yellow (sticky)	1.84%	Light yellow (sticky)	1.60%
Ethyl acetate (fraction-3)	Light brown (dry residue)	3.49%	Light brown (dry residue)	3.76%
N-Butanol (fraction-4)	Dark brown (dry residue)	14.24%	Dark brown (dry residue)	9.48%

2.3 Phytochemical screening

As shown in (Table 2), the chemical analysis of ACF leaf and bark extracts revealed a variety of phytoconstituents in various solvent extracts. Numerous experiments, including Molisch's and Benedict's, verified that there were large amounts of carbohydrates present in the alcoholic and butanol extracts. Mayer's and Wagner's experiments showed that the alcoholic and chloroform extracts contained the highest concentrations of alkaloids. Positive results in the ferric chloride, Shinoda, and foam tests indicated the presence of tannins, flavonoids, and saponins in high concentrations in the alcoholic, ethyl acetate, and butanol extracts. Furthermore, using the Liberman-Burchard and Salkowski tests, steroids and triterpenes were detected in the petroleum ether and chloroform extracts. These findings emphasize the potential medicinal uses of the bioactive chemicals found in ACF leaf and bark by providing a thorough characterization of them.

Table 2. Phytochemical screening of *Adina cordifolia* leaf and bark extracts and their fractions

Chemical Constituents	Tests	AE	EAE	BE	CE	PE
Carbohydrate	1. Molisch's test	+++	---	+	---	---
	2. Benedict's test	+++	---	+	---	---
	3. Fehling's test	+	---	---	---	---
	4. Barford's test	-	---	---	---	---
Proteins	1.Millon's test	---	---	---	---	---
	2.Biuret test	---	---	---	---	---
	3.Ninhydrin test	---	---	---	---	---
Alkaloids	1.Mayer's test	+	---	---	+	---
	2.Wagner's test	+++	---	---	---	---
	3.Dragendorf's test	---	---	---	+	---

	4.Hager's test	---	---	---	---	---
	1. Modified Borntrager's	---	---	---	---	---
Glycosides	2. Legal's Test	---	---	---	---	---
	3. Balget Test	---	---	---	---	---
	1.Ferric chloride test	+++	+++	+++	---	---
Tannins	2.Lead acetate test	+	+	++	---	---
	3.Gelatin test	+	+	+	---	---
	1. Shinoda Test	+++	+++	+++	---	---
Flavonoids	2. Ferric chloride test	+++	+++	+++	---	---
	3. Mineral acid test	+++	+++	+++	---	---
	4. Lead- acetate test	+++	+++	+++	---	---
Steroids and triterpenes	1. Liberman-Burchard's	---	---	---	+	+
	2. Salkowski's Test	---	---	---	+	+
Saponins	3. Foam test	++	+	+++	---	---

AE: Alcoholic Extract, EAE: Ethyl Acetate Extract, BE: Butanol Extract, CE: Chloroform Extract, PE: Petroleum Ether Extract ("+" indicates the presence of the constituent, "---" indicates absence, and "+++" indicates strong presence)

2.3.1 Determination of Total Flavonoid Content

Using UV spectroscopy, the total flavonoid concentration of ACF leaf extracts was estimated, with particular attention paid to the ethanolic, butanolic, and ethyl acetate extracts. With an average of 2.5% w/w, the butanolic extract indicated the highest flavonoid content, according to the results. The extract made from ethyl acetate came next, with an average flavonoid concentration of 2.2% w/w. With an average of 2.0% w/w, the ethanolic extract had the lowest flavonoid content (Figure 2A). These results imply that, in comparison to the other extracts examined, the butanolic extract of ACF leaves is especially rich in flavonoids.

2.3.2 Determination of Total Tannin Content

Using UV spectroscopy, the total tannin content of ACF leaf extracts was ascertained, with particular attention paid to ethanolic, butanolic, and ethyl acetate extracts. With an average tannin level of 14.4% w/w, the study showed that the ethanolic extract had the highest tannin content. The butanolic extract came next, with an average tannin content of 12.4% w/w. With an average of 9.4% w/w, the ethyl acetate extract exhibited the lowest tannin content (Figure 2B). According to these findings, the ethanolic extract has the highest tannin content of all the extracts examined, suggesting that it may be used as a source of compounds high in tannins.

2.3.3 Determination of total Polyphenols Content

Using UV spectroscopy, the number of total polyphenols in ACF leaf extracts was estimated by looking at ethanolic, butanolic, and ethyl acetate extracts. With an average polyphenol level of 5.2% w/w, the ethanolic extract was found to have the highest polyphenol content. The butanolic extract, with an average weight-to-weight ratio of 4.6%, came next. With an average of 4.2% w/w, the ethyl acetate extract had the lowest polyphenol content (Figure 2C). These results reveal that the ethanolic extract has a higher polyphenol content than the other extracts, which may be useful for applications that call for high polyphenol concentrations.

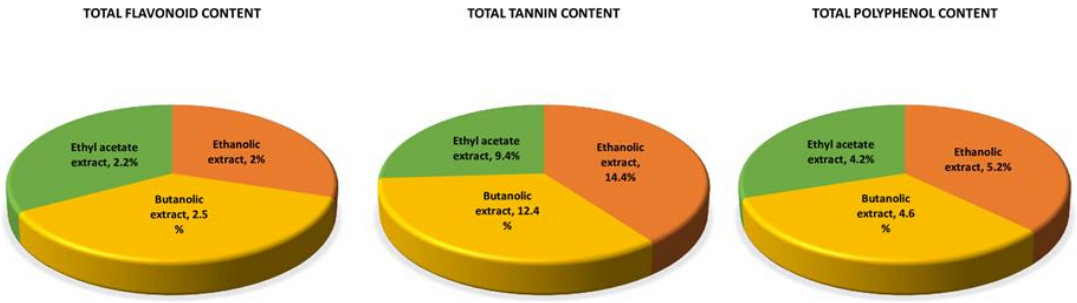

Figure 2. Total Flavonoid Content (A) Total Tannin Content (B) Total polyphenol Content (C) in *Adina cordifolia* leaf extracts.

2.4 HPTLC identification and quantification of extract:

This work used the High-Performance Thin-Layer Chromatography (HPTLC) method to identify, fingerprint, and quantify (where applicable) rutin and tannic acid in ACF leaf and bark extracts (Figure 3).

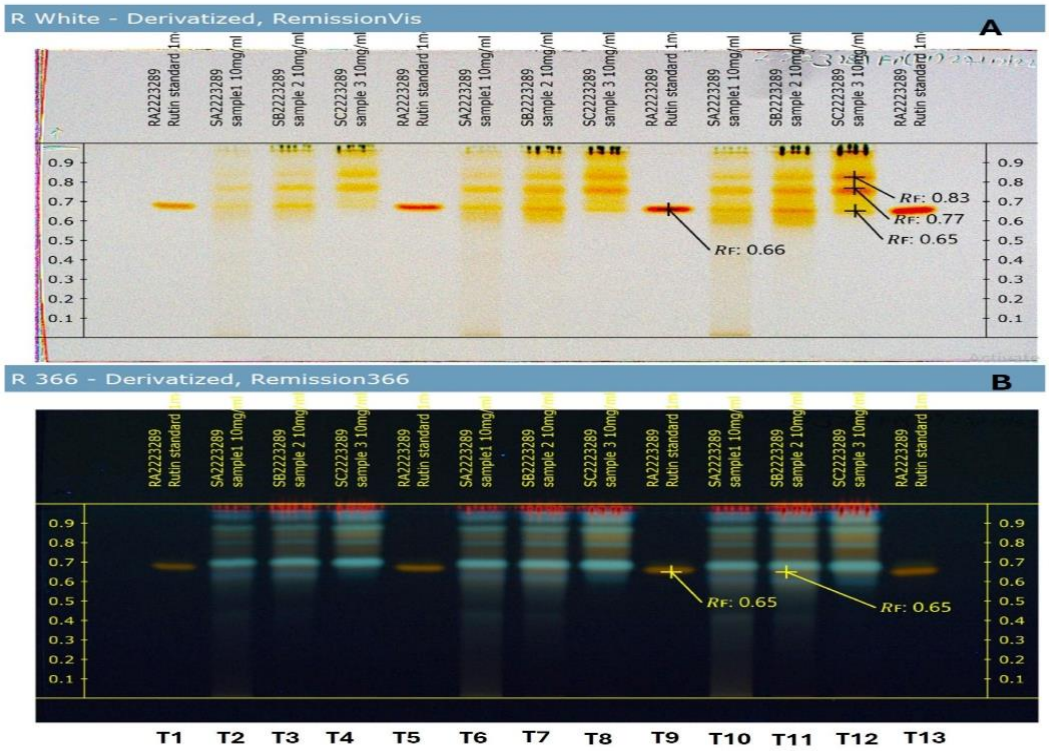

Figure 3. Derivatization under A) White light B) under 366 nm

Three bark extracts (alcoholic extract, butanol fraction, and ethyl acetate fraction) and three leaf extracts (alcoholic extract, butanol fraction, and ethyl acetate fraction) were among the six samples that were analyzed. The samples were made at a concentration of 10 mg/ml in methanol, and then they were ultra-centrifuged and sonicated for 15 minutes. Whatman Paper No. 1 was used as the stationary phase, which was Aluminium TLC silica gel 60 F254 by Merck, with a developing distance of 70 mm from the plate's lower edge and a saturation duration of 20 minutes. For the purpose of separating rutin and tannic acid, the mobile phase was composed of ethyl acetate, formic acid, acetic acid, and water in a ratio of 100:11:11:26. Vanillin Sulfuric Acid Reagent (VSR reagent) was used for tannic acid and Natural Product Reagent (NP reagent) for rutin throughout the derivatization process (Figure 4).

A thorough comprehension of the chemical profiles and contents in the bark and leaf extracts was made possible by the HPTLC analysis. Following derivatization with NP reagent, fingerprint analysis of the leaf extracts showed a noticeable yellow band that corresponded to rutin with an Rf value of 0.65 in all three samples, demonstrating the presence of rutin in each extract. Rutin concentrations of 0.25% in the ethanolic extract, 0.43% in the butanolic extract, and 0.35% w/w in the ethyl acetate extract were obtained using single-point calibration with a Linear-1 regression model. After derivatization (Figure 5) with VSR reagent, tannic acid was not found in any of the leaf samples, indicating that it was not present in these extracts.

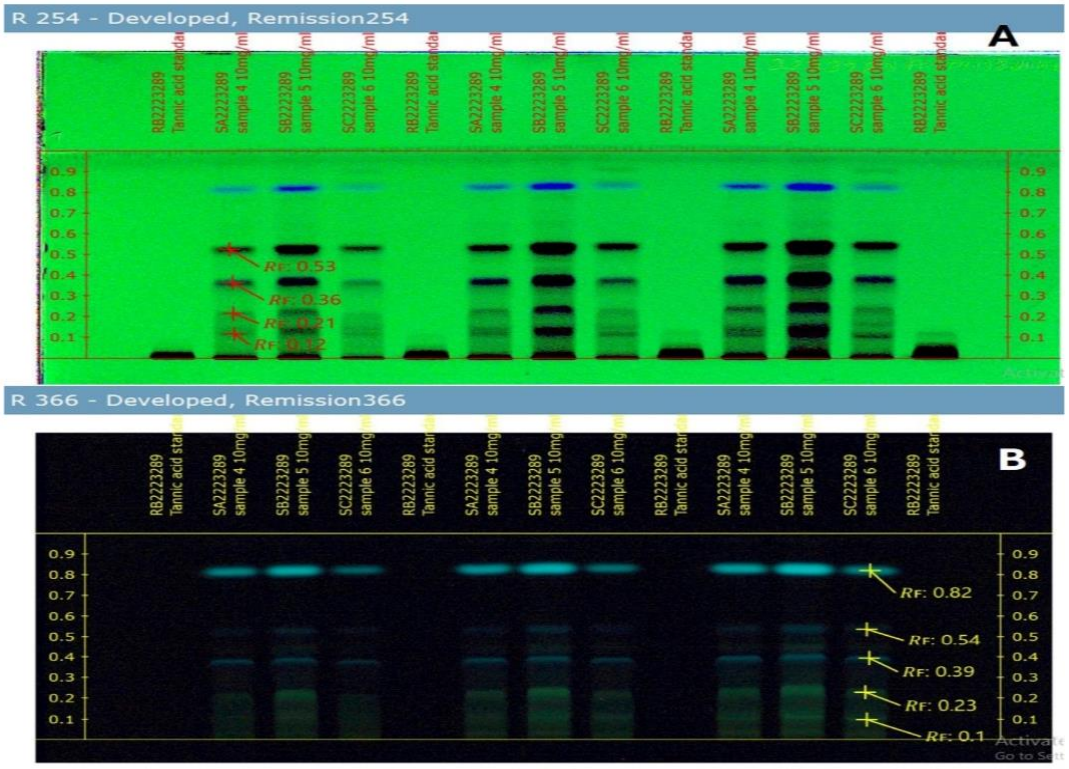

Figure 4. Fingerprinting at 254 and 366 nm

Figure 6 displays the fingerprint graphs and HPTLC spectra and Figure 7 shows the linearity single point calibration for rutin at 540 nm. HPTLC fingerprinting was done on the bark extracts at both 254 and 366 nm.

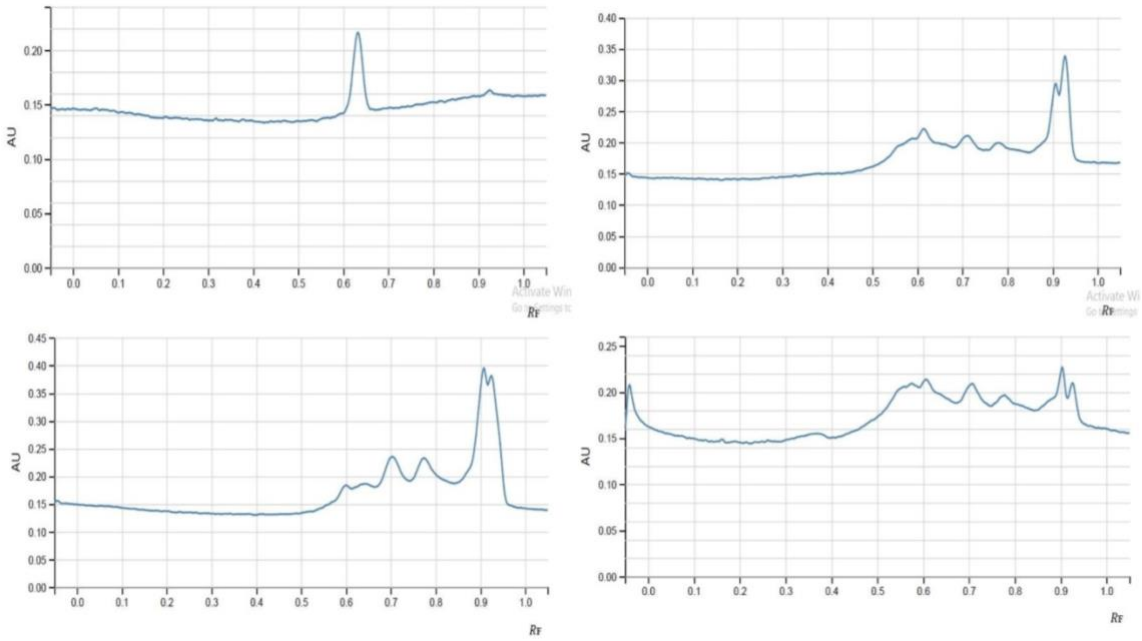

Figure 5. HPTLC spectrum of *Adina cordifolia* at 540 nm, I) Standard Rutin II) Ethanolic extract III) Butanolic extract IV) Ethyl acetate extract.

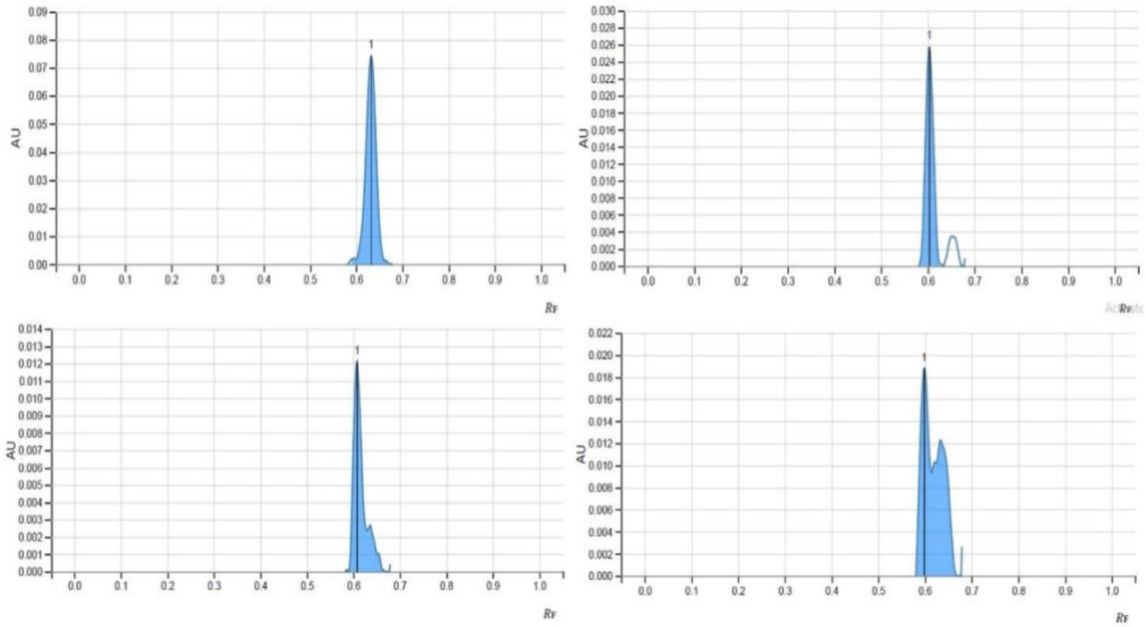

Figure 6. HPTLC fingerprint spectrum of *Adina cordifolia* at 540 nm I) Standard Rutin II) Ethanolic extract III) Butanolic extract IV) Ethyl acetate extract.

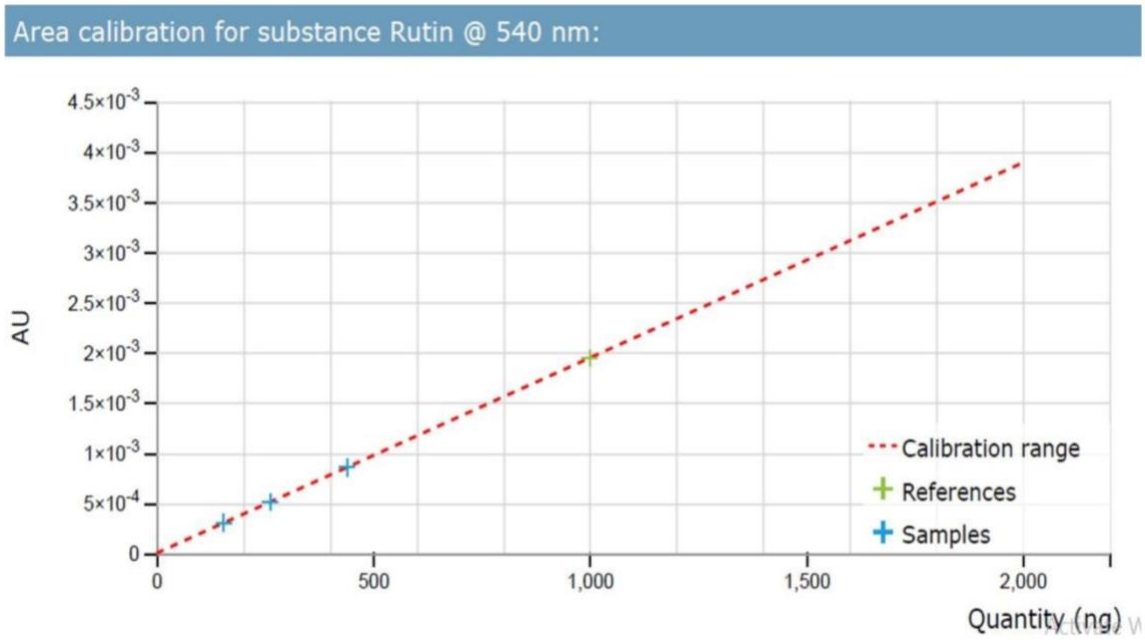

Figure 7. Single point calibration for Rutin at 540 nm

Four prominent bands were detected at 254 nm, with Rf values of 0.12, 0.21, 0.36, and 0.53, suggesting the existence of different chemicals. Five prominent bands having Rf values of 0.10, 0.23, 0.39, 0.54, and 0.82 were seen at 366 nm, indicating the presence of additional ingredients in the bark extracts. The successful identification and isolation of rutin in the leaf extracts was validated by the yellow-colored bands that were compact, crisp, and high-resolution following derivatization [34,35]. Several components were further validated by derivatization and subsequent HPTLC analysis of the ACF bark extract at both 254 and 366 nm. In addition to effectively identifying rutin, the investigation revealed the whole chemical fingerprint of the bark and leaf extracts, emphasizing the leaf extracts' lack of tannic acid. The plant's valuable botanical features and chemical composition were brought to light by the pharmacognostic evaluation and HPTLC identification of ACF (R) Hook, which helped with the plant's taxonomical classification, authentication, and

quality control. These results highlight how important HPTLC is to the standardization and quality control of ACF.

2.5 Method validation parameters

The developed HPTLC method was validated for linearity, LOD, LOQ, accuracy, and precision, following ICH guidelines (Table 3). Six serial dilutions of each analyte were prepared in triplicate to construct calibration curves, plotting average peak area against concentration using least squares regression. To determine the linearity range of standard rutin, six bands (1.0–6.0 µL) were applied to the HPTLC plate, and a peak area vs. concentration graph was created. The linearity varied between 0.5 to 5.5 ng/ml. The accuracy of mean recoveries is found in the range of 96.5% to 100.6%

Table 3. Method validation of Rutin using HPTLC

Parameters	Results
Linearity range	0.5-5.5 ng/ml
Correlation coefficient (R2 ± SD)	R= 0.9801
LOD	0.00079 ng
LOQ	0.0031 ng
Specificity	Specific
RSD (%) of Intraday precision (n = 3)	1.49 %
RSD (%) of Inter day precision (n = 3)	0.81 %

2.6 Antioxidant activity

Using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, the antioxidant activity of ACF leaf extracts was thoroughly assessed in ethanolic, butanolic, and ethyl acetate extracts. The concentration-dependent increase in Radical Scavenging Activity (% RSA) exhibited by each extract underscores their potential as organic antioxidants. The percentage of RSA in the ethanolic extract ranged from 32.73% at 40 µg/ml to 46.82% at 200 µg/ml, indicating moderate antioxidant activity. Across the investigated concentrations, the IC50 values—which show the concentration needed to block 50% of DPPH radicals—rose from 3.21 µg/ml to 48.92 µg/ml. This indicates that although the ethanolic extract possesses antioxidant qualities, at greater doses it becomes more effective at scavenging radicals (Table 3). The butanolic extract also shown notable antioxidant activity; at 40 µg/ml, the % RSA values increased to 23.95%, and at 200 µg/ml, they reached 44.21%. Additionally, the IC50 values increased from 4.24 µg/ml to 36.90 µg/ml, showing a strong dose-response relationship [36,34].

Table 4. Radical Scavenging Activity (%RSA) and IC50 Values of *Adina cordifolia* Leaf Extracts

Concentration (µg/ml)	Ethanolic extract		Butanolic extract		Ethyl acetate extract	
	% RSA	IC50	% RSA	IC50	% RSA	IC50
40	32.73	3.21	23.95	4.24	16.88	4.63
80	35.23	14.64	30.00	12.41	24.38	11.20
120	39.55	26.06	33.16	20.57	30.94	17.77
160	42.05	37.49	38.95	28.73	37.19	24.34
200	46.82	48.92	44.21	36.90	40.94	30.91

This extract is a strong contender for lowering oxidative stress since it has a stronger anti-free radical effect than the ethanolic extract. Although the ethyl acetate extract demonstrated antioxidant activity as well, its total efficacy in scavenging free radicals was marginally lower than that of the other two extracts. The IC50 values increased from 4.63 µg/ml to 30.91 µg/ml, while the percentage RSA varied from 16.88 % at 40 µg/ml to 40.94% at 200 µg/ml. Its antioxidant capacity is noteworthy; however, it performs marginally worse than the butanolic extract. Because bioactive components like flavonoids and polyphenols are important for radical scavenging, variations in their solubility and concentration can explain the variance in antioxidant efficacy across these extracts. The ethyl acetate extract, while having a strong antioxidant capacity, may contain less of these active ingredients, resulting in a relatively lower percentage of RSA and higher IC50 values. In contrast, the butanolic extract may be more effective because of its superior ability to

extract these chemicals. All things considered; these results highlight how crucial solvent selection is to enhancing plant extracts' antioxidant potential.

2.7 Anti-inflammatory activity

2.7.1 Human red blood cell (HRBC) membrane stabilization method

The ability of different extracts to shield red blood cells from hemolysis was measured in order to assess their anti-inflammatory effectiveness using the HRBC membrane stabilization method. The benchmark drug, aspirin, showed up to 79.97% protection at 1000 µg/ml. While the n-butanol extract offered up to 60.44% protection at the highest dosage, the ethanolic extract shown moderate action, with a maximum protection of 68.15%. At 1000 µg/ml, the ethyl acetate extract showed impressive stability, attaining 71.23% protection. These findings suggest that all extracts have concentration-dependent anti-inflammatory qualities, with the plant extract with the highest efficacy being ethyl acetate [37,38].

2.7.2 Protein denaturation method

Using a heat-induced protein denaturation method, the % inhibition of protein denaturation for various extracts and aspirin (standard) was determined. The inhibition exhibited by aspirin was dose-dependent, rising from 6.38% at 62.5 µg/ml to 63.83% at 1000 µg/ml. At 1000 µg/ml, the ethanol extract showed a noteworthy inhibition of 70.57%, but the n-butanol extract showed the highest level of inhibition at 77.30%. With a high of 64.89%, the ethyl acetate extract demonstrated moderate activity. These findings suggest that by preventing protein denaturation, the extracts have significant anti-inflammatory qualities.

3. CONCLUSION

The rich therapeutic and phytochemical potential, particularly in antioxidant, anti-inflammatory, and anthelmintic applications, was highlighted in the present research on ACF leaf and bark extracts. Pharmacognostic evaluations, including macro-morphological and microscopic analyses, provide essential information for accurate identification and quality control, helping distinguish ACF from adulterants. Phytochemical screening identified significant levels of flavonoids, tannins, alkaloids, and saponins, especially in ethanol and butanol extracts. HPTLC fingerprinting confirmed rutin as a primary bioactive in leaf extracts, although tannic acid was either absent or minimal. Analytical validation parameters were established for rutin quantification, showing a linearity range from 0.5-5.5 ng/ml with a correlation coefficient ($R^2 \pm SD$) of 0.9801. The limit of detection (LOD) and limit of quantification (LOQ) were determined to be 0.00079 ng and 0.0031 ng, respectively, indicating high sensitivity. The method demonstrated specificity, with intraday precision (RSD %) of 1.49% (n = 3), ensuring reliable reproducibility. Antioxidant activity assays showed that all extracts effectively scavenged free radicals, with the butanol extract demonstrating the highest activity, likely due to a high concentration of phenolic and flavonoid compounds. Both the HRBC membrane stabilization and protein denaturation assays supported the anti-inflammatory potential of the extracts, with the ethyl acetate and butanol extracts showing strong effects. These findings suggest that ACF's bioactive components could aid in developing anti-inflammatory treatments by modulating pro-inflammatory cytokines. In summary, this study supports ACF's traditional medicinal use and suggests its potential for modern therapeutic applications. Further research is essential to isolate and characterize the active compounds to ensure consistent therapeutic efficacy.

4. MATERIALS AND METHODS

4.1 Chemicals and Reagents

CHD Chemicals in Mumbai, MS, India is where we got all of the analytical grade chemicals.

4.2 Collection, identification and authentication of plant material

The Western Ghats, namely the Shimoga district, are where the bark and leaves of ACF were gathered. Dr. Halleshi C., an assistant professor in the botany department of Davangere University in Davangere, verified the authenticity of the plant. After a thorough washing, the fresh bark and leaves were dried in the shade. Before being utilized for additional analysis, the dried samples were first ground into a powder using a laboratory mixer grinder set at a high speed for five minutes. They were then kept in a container that was tightly sealed for a whole day.

4.3 Evaluation of macro morphology and microscopic features

Samples of freshly picked leaves and bark were examined morphologically and contrasted with industry standards [16]**.** Using normal pharmacognostic procedures, the leaves and bark were also inspected for a variety of microscopic parameters, including as histology, powder microscopy, stomatal number, stomatal index, and fiber length determination [17]**.**

4.4 Chemical constituent extraction and fractionation from plant materials

Ethanol was used as the solvent in a Soxhlet system to extract the chemical components from the plant material. In order to evaporate the solvent, the final extract was immediately transferred from the round-bottom flask (RBF) into a China dish and submerged in water. The extract was kept at room temperature in a China dish or an airtight container after the solvent had completely evaporated. A first fractionation was carried out in order to investigate the active ingredients in the extract in greater detail. In this procedure, the polar components were separated from the less polar ones using solvents with increasing polarity, petroleum ether, chloroform, ethyl acetate, and butanol. With great care, each solvent layer was extracted, evaporated in a China dish with a water bath, and labeled with the corresponding fraction [18].

4.5 Screening for phytochemicals

Several chemical constituents, such as carbohydrates, proteins, alkaloids, glycosides, tannins, flavonoids, steroids, terpenoids, and saponins, were found through a phytochemical analysis of the total ethanolic extract and different fractions of ACF bark and leaf [19,20].

4.6 Calculating the Overall Flavonoid Content

The process entails making both a standard and sample solution in order to assess the flavonoid content relative to rutin. One milliliter of rutin solution and one milliliter of aluminum chloride reagent should be pipetted into a ten-milliliter volumetric flask for the standard. Then, ethanol is added to the capacity to make it to 10 ml. Measure the standard solution's (S) optical density at 410 nm in relation to a reagent blank after precisely 15 minutes.

1 milliliter of aluminum chloride is dissolved in 10 milliliters of ethanol to create the reagent blank. Pipette one milliliter of the test solution into a ten-milliliter volumetric flask, then add one milliliter of the aluminum chloride reagent and stir. The vessel is similarly filled with ethanol to a capacity of 10 ml, and precisely 15 minutes after the addition of the reagent, the optical density of the test solution (T) is measured at 410 nm. The following formula [21] is used to determine the flavonoid content, which is given as % w/w of flavonoids:

$$\frac{\textbf{Abs of sample (T)}}{\textbf{Abso of std (S)}} \times \frac{\textbf{Wt of std (mg)}}{\textbf{100}} \times \frac{\textbf{5}}{\textbf{100}} \times \frac{\textbf{Vol of std (reaction)}}{\textbf{Vol of sample (reaction)}} \times \frac{\textbf{Total vol of sample}}{\textbf{Wt of sample (mg)}} \times \%\textbf{Purity (Std)}$$

4.7 Determination of Total Tannin Content

To estimate total tannin content, the procedure varies based on tannin concentration. For samples with less than 5% tannins, weigh about 0.1 g of the sample and reflux with 50 ml of purified water at 100°C for 1 hour. After cooling, decant the extract into a 100 ml volumetric flask, make up the volume with water, filter, and discard the first 25 ml of filtrate. For samples with more than 5% tannins, use 100 ml of water, decant into a 500 ml flask, and discard the first 50 ml of filtrate. For the test, pipette 0.2 ml of the test solution into a 10 ml volumetric flask, add 1.0 ml each of potassium ferricyanide and ferric chloride, dilute with water, and measure absorbance at 720 nm (A1). For the standard, use 1.0 ml of the standard solution and measure absorbance (A2) [22]**.** The percentage of total tannins, expressed as tannic acid, is calculated using the formula:

$$\frac{\boldsymbol{A1}}{\boldsymbol{A2}} \times \frac{\boldsymbol{W1}}{\boldsymbol{V1}} \times \frac{\textbf{5}}{\textbf{100}} \times \frac{\textbf{1}}{\textbf{10}} \times \frac{\boldsymbol{V2}}{\boldsymbol{W2}} \times \frac{\textbf{10}}{\textbf{0.2}} \times \%\boldsymbol{Purity\ of\ Standard}$$

where A1 is the sample absorbance, A2 is the standard absorbance, W1 is the weight of the standard (mg), W2 is the weight of the sample (mg), V1 is the standard volume (100 ml), and V2 is the sample volume (100 ml).

4.8 Calculating the total amount of polyphenols

Precisely balance out 1 g of the powdered test material into a 250 ml flask with a flat bottom, then add 150 ml of clean water to get an idea of the overall polyphenol content. After 30 minutes of refluxing the mixture in a boiling water bath at 97±2°C, cool it under running water and let the residue settle. Repeat the extraction procedure with 30 ml of distilled water until the extract turns colorless, then transfer the dissolved extract to a 250 ml volumetric flask. After rinsing the flask, gather the washings in the volumetric flask and dilute with purified water to a level of 250 ml. Let the residue settle, then pass the liquid through Whatman No. 1 filter paper. Discard the first 50 milliliters of the filtrate, and then dilute the liquid. Once the residue has had time to settle, strain the liquid through Whatman No. 1 filter paper. Discard the first 50 milliliters of the filtrate, and then dilute the remaining 5 milliliters to 25 milliliters with purified water for lab analysis. Transfer 2.0 ml of the test solution into a 25 ml volumetric flask using a pipette. Then, add 1.0 ml of Folin & Ciocalteu's phenol reagent and 10.0 ml of purified water. After adding the reagent, dilute to 25.0 ml with sodium carbonate solution, and measure the absorbance (A1) at 760 nm precisely 30 minutes later. Using the same procedures as for the test solution, 2.0 ml of the working standard solution is pipetted into a 25 ml volumetric flask to create the standard solution. At 760 nm, calculate the absorbance (A2) [23]. The following formula is used to determine the percentage content of total polyphenols, expressed as pyrogallol or tannic acid:

$$\frac{A1}{A2} \times \frac{W1}{V1} \times \frac{5}{100} \times \frac{2}{25} \times \frac{V2}{W2} \times \frac{25}{5} \times \frac{25}{2} \times \%Purityof\ Standard$$

4.9 Identification and quantification using HPTLC [24-26]

Anchrome Private Ltd., Mumbai, received the extract and fractionated samples of ACF leaves and bark for HPTLC fingerprint analysis. LABSERVER Ver 3.002 software was used for the analysis. Sample preparation involved dissolving 10 mg of the sample in 1 ml of methanol, sonicating the mixture for 15 minutes, and then centrifuging the mixture. A standard Rutin solution was made with 0.1 mg/ml of methanol as the concentration. Aluminum plates precoated with TLC silica gel 60 F254 (Merck, Cat. No. 1.05554.0007) made up the stationary phase. Using filter paper liner (Whatman Paper No. 1), the developing distance was set at 70 mm from the lower border of the plate, with a saturation time of 20 minutes. Ethyl acetate, formic acid, acetic acid, and water were combined in the mobile phase in the following ratio: 100:11:11:26 (v/v/v/v). Following development, a natural product reagent was applied to the plate as a derivatizing agent, and an image was taken at a wavelength of less than 366 nm. The compound was quantified by comparison of peak area and linear regression. It was obtained from calibration curve of pure reference compound. Each experiment was performed in triplicate, with the resulting peak area data utilized to determine the limit of detection (LOD), limit of quantification (LOQ), as well as accuracy and precision.

4.10 Method Validation

The HPTLC method was validated following guidelines from the International Conference on Harmonization (ICH). Validation parameters included assessments of linearity, precision, limit of detection (LOD), limit of quantification (LOQ), and peak area purity for rutin and tannic acid [27,28].

4.11 Calibration curves, LOD and LOQ

To construct calibration curves, six concentrations (50–2000 ng/band) of rutin standards were applied in triplicate using specified chromatographic conditions. Calibration curves were generated by plotting peak area against concentration, characterized by slope, y-intercept, and correlation coefficient. Linearity was evaluated through linear regression analysis. These curves enabled analyte concentration determination in Ethanolic extract, Butanolic extract and Ethyl acetate extract. System sensitivity was assessed via LOD, LOQ, and analytical sensitivity (AS), with AS calculated as the ratio of residual standard deviation (σ) to the slope (S). LOD and LOQ were determined using the formulas LOD = 3 × AS and LOQ = 10 × AS [29].

4.12 Analysis of Analyte Recovery, Precision, and Accuracy

Relative recoveries were evaluated using the standard addition method, with precision determined as %RSD for peak area ratios in intra-day and inter-day runs. Triplicate HPTLC analyses were performed on spiked samples, and percent recovery was obtained from calibration curves. Accuracy was calculated by comparing the measured analyte concentration to the known spiked amount [30,31].

4.13 Anti-oxidant activity

The plant extracts capacity to scavenge free radicals (DPPH) was assessed through the application of appropriate modifications to a standard procedure. Methanol was used to create stock solutions of the extracts at 40, 80, 120, 160, and 200 µg/ml. For the test, 1 milliliter of each sample solution was mixed with 4 milliliters of a 0.1 milligram methanolic DPPH solution, and the mixture was left to react for 30 minutes at room temperature in the dark.

The reference standard was ascorbic acid, while the blank and positive controls were methanol and DPPH, respectively. A double-beam UV-visible spectrophotometer was used to detect absorbance at 517 nm. Using the appropriate equation, the percentage inhibition was computed, and a non-linear regression approach was used to extract the IC50 values from the plot of the percentage inhibition vs concentration. The findings were presented as mean values (n = 3) ± standard deviation [32].

4.14 Anti-inflammatory activity

4.14.1 Stabilization of the membrane of human red blood cells (HRBC)

Using the HRBC membrane stabilization method, blood from healthy individuals who had not used NSAIDs for two weeks was combined with sterile Elsevier solution and centrifuged to determine in vitro anti-inflammatory activity. After using iso saline to wash the packed cells, a 10% v/v suspension was made. 0.5 ml of different extract concentrations (62.5-1000 µg/ml) or aspirin were mixed with phosphate buffer, hypo saline, and HRBC solution for the experiment. This mixture was then incubated at 37°C for 30 minutes. At 560 nm, the amount of hemoglobin in the supernatant was measured. Membrane protection was computed using the following formula [33], whereas hemolysis was determined using pure water as the 100% hemolysis control.

$$\textbf{Percentage protection} = \left(1 - \frac{\textbf{optical density of test}}{\textbf{optical density of control}}\right) \times 100$$

4.14.2 Protein denaturation method

To estimate in vitro anti-inflammatory activity through the inhibition of protein denaturation, a reaction mixture was prepared with 0.2 ml of egg albumin, 2.8 ml of phosphate-buffered saline (PBS, pH 6.4), and 2 ml of extract at final concentrations of 62.5, 125, 250, 500, and 1000 µg/ml. A control was set up using an equal volume of double-distilled water. The mixtures were incubated at 37±2°C for 15 minutes, followed by heating at 70°C for 5 minutes. After cooling, the absorbance was measured at 660 nm, using the vehicle as a blank. Aspirin at similar concentrations served as the reference drug. The percentage inhibition of protein denaturation was calculated using the formula:

$$\textbf{Percentage protection} = \left(\frac{\textbf{Absorbance of test} - 1}{\textbf{Absorbance of control}}\right) \times 100$$

Acknowledgements: Thankful to Parul Institute of Pharmacy & Research, Parul University for providing the research facility.

Author contributions: Concept- RSA, Design- RSA, Supervision-Resources- GSC, Data Collection- RSA & GSC Analysis- RSA, GSC, Literature Search- GSC, Writing & Critical Reviews- RSA & GSC.

Conflict of interest statement: The authors declared no conflict of interest in the manuscript.

REFERENCES

[1] Nandkarni KM. Indian Materia Medica (Vol. 1). Bombay Popular Prakashan. (2009)

[2] Negi S, Negi A, Pokriyal A, Tripathi G. Pharmacognostical investigation of "Adina cordifolia (Roxb.) Brandis" Family-Rubiaceae, collected from areas of Haldwani District Nainital. J Pharmacogn Phytochem. 2023;12(1):309-315. https://doi.org/10.22271/phyto.2023.v12.i1d.14591.

[3] Janbaz KH, Saeed SA, Gilani AH. Protective effect of rutin on paracetamol-and CCl4-induced hepatotoxicity in rodents. Fitoterapia. 2002 Dec 1;73(7-8):557-563. https://doi.org/10.1016/j.fitote.2014.03.002

[4] Ghasemzadeh A, Jaafar HZ, Rahmat A. Antioxidant activities, total phenolics and flavonoids content in two varieties of Malaysia young ginger (*Zingiber officinale* Roscoe). Molecules. 2010;15(6):4324-4333. https://doi.org/10.3390/molecules15064324

[5] Trease GE, & Evans WC. Pharmacognosy (16th ed.). Elsevier Health Sciences, 2009.

[6] Wagner H, & Bladt S. Plant Drug Analysis: A Thin Layer Chromatography Atlas (2nd ed.). Springer-Verlag, 2009. https://doi.org/10.1007/978-3-662-02398-3

[7] Kumar S, Kumar D, Singh, N. HPTLC analysis and validation of rutin in *Melia azedarach* leaf and stem extracts. J Pharm Biomed Anal. 2012;56(2): 311-315. https://doi.org/10.1016/j.jpba.2011.12.003

[8] Yu M, Gouvinhas I, Rocha J, Barros AI. Phytochemical and antioxidant analysis of medicinal and food plants towards bioactive food and pharmaceutical resources. Sci Rep. 2021; 11(1): 10041. https://doi.org/10.1038/s41598-021-89437-4
[9] Taleuzzaman M, Jain P, Verma R, Iqbal Z, Mirza MA. Eugenol as a potential drug candidate: A review. Curr Topic Med Chem. 2021;21(20):1804-1815. http://dx.doi.org/10.2174/1568026621666210701141433
[10] Sharma A, Sangameswaran B, Jain V, Saluja MS. Hepatoprotective activity of *Adina cordifolia* against ethanol induce hepatotoxicity in rats. Int Curr Pharm J. 2012; 1(9): 279-284. https://doi.org/10.3329/icpj.v1i9.11619
[11] Parham S, Kharazi AZ, Bakhsheshi-Rad HR, Nur H, Ismail AF, Sharif S, RamaKrishna S, Berto F. Antioxidant, antimicrobial and antiviral properties of herbal materials. Antioxidants. 2020;9(12):1309. https://doi.org/10.3390/antiox9121309.
[12] Hernando-Amado S, Coque TM, Baquero F, Martínez JL. Defining and combating antibiotic resistance from One Health and Global Health perspectives. Nat Microbiol. 2019;4(9):1432-1442. https://doi.org/10.1038/s41564-019-0503-9
[13] Kaur G, Hamid H, Ali A, Alam MS, Athar M. Antiinflammatory evaluation of alcoholic extract of galls of *Quercus infectoria*. J Ethnopharmacol. 2004;90(2-3):285-292. https://doi.org/10.1016/S0378-8741(03)00199-8
[14] Sannigrahi S, Mazumder UK, Pal, D. Antioxidant activities of methanol extract of *Adina cordifolia* (Roxb.) leaves. Pharmacol Online. 2010; 2: 454-460.
[15] Qiao Q, Ye M, Wu C, Wang J, Liu Q, Tao J, Zhang L, Feng Z. Analysis of leaf morphology variation and genetic diversity via SRAP markers for near-threatened plant *Acer truncatum*. Glob Ecol Conserv. 2022;33: e01980. https://doi.org/10.1016/j.gecco.2021.e01980
[16] Raypa P, Verma AK, Tewari S, Dubey A. Analysis of medicinally important phytocompounds from *Adina cordifolia* leaves. Int J Curr Microbiol App Sci. 2018; 7(11): 3007-3019 https://doi.org/10.20546/ijcmas.2018.711.345
[17] Cos P, Vlietinck AJ, Berghe DV, Maes L. Anti-infective potential of natural products: How to develop a stronger in vitro 'proof-of-concept'. J Ethnopharmacol. 2006;106(3):290-302. doi.org/10.1016/j.jep.2006.04.003
[18] Shaikh SS, Bawazir AS, Yahya BA. Phytochemical, histochemical and in vitro antimicrobial study of various solvent extracts of *Costus speciosus* (j. koenig) sm. and *Costus pictus* d. don. Turk J Pharm Sci. 2022;19(2):145-152. https://doi.org/10.4274/tjps.galenos.2021.02058.
[19] Rodríguez-Valdovinos KY, Salgado-Garciglia R, Vázquez-Sánchez M, Álvarez-Bernal D, Oregel-Zamudio E, Ceja-Torres LF, Medina-Medrano JR. Quantitative analysis of rutin by HPTLC and in vitro antioxidant and antibacterial activities of phenolic-rich extracts from *Verbesina sphaerocephala*. Plants. 2021;10(3):475. https://doi.org/10.3390/plants10030475
[20] Prakash V, Saxena S, Gupta S, Saxena AK, Yadav R, Singh SK. Preliminary phytochemical screening and biological activities of *Adina cardifolia*. J Microb Biochem Technol. 2015;7:1. http://dx.doi.org/10.4172/1948-5948.1000178
[21] Tahia F, Sikder MA, Al-Mansur MA, Rashid MA. Bioactivities of *Adina cordifolia* (Roxb.) Hook. f.-growing in Bangladesh. Bangladesh J Bot. 2019;48(2):307-313. https://doi.org/10.3329/bjb.v48i2.47672.
[22] Hossain MA, Hossain MS, Fatema K, Siddique BA, Sikder H, Sarker MS, Jain P. An Evaluation on antioxidant activity, total phenolic and total flavaonoid contents of extracts from *Adina cordifolia* (Roxb.). Am J Plant Sci. 2015;6(05):633. http://dx.doi.org/10.4236/ajps.2015.65068
[23] Zhang P, Wu Q, Wang Y, Huang Y, Xie M, Fan L. Rapid detection of tannin content in wine grapes using hyperspectral technology. Life. 2024;14(3):416. https://doi.org/10.3390/life14030416
[24] Oliveira LM, Bevilaqua CM, Costa CT, Macedo IT, Barros RS, Rodrigues AC, Camurça-Vasconcelos AL, Morais SM, Lima YC, Vieira LS, Navarro AM. Anthelmintic activity of *Cocos nucifera* L. against sheep gastrointestinal nematodes. Vet Parasitol. 2009;159(1):55-59. https://doi.org/10.1016/j.vetpar.2008.10.018
[25] Taleuzzaman M, Imam SS, Gilani SJ. Quantitative Determination of thymoquinone in *Nigella sativa* and its nano formulation using validated stability indicating HPTLC densiometric method. Int Curr Pharm J. 2017;6(10):53-60. https://doi.org/10.3329/ICPJ.V6I10.35897.
[26] Ghoshal DS, Kharat SN, Godbole SA. HPTLC Quantification and estimation of rutin and kaempferol using aerial parts of *Ixora javanica* (blume) dc. and *Ixora barbata* roxb. ex. sm. Res J Pharm Technol. 2022;15(10):4533-4541. https://doi.org/10.52711/0974-360X.2022.00761.
[27] Dalu AP, Zagare VS, Avchar PE, Kadam MP, Ingole AS, Nagrik SU, Patil PA. A pharmacological potential of *Adina cordifolia*. J Drug Deliv Ther. 2021;11(2-S):132-135. https://doi.org/10.22270/jddt.v11i2-S.4652
[28] Tomar V, Beuerle T, Sircar D. A validated HPTLC method for the simultaneous quantifications of three phenolic acids and three withanolides from *Withania somnifera* plants and its herbal products. J Chromatogr B. 2019;1124:154-160. https://doi.org/10.1016/j.jchromb.2019.06.009
[29] Taleuzzaman M. Limit of blank (LOB), limit of detection (LOD), and limit of quantification (LOQ). Org Med Chem. Int J. 2018;7(5):127-131. https://doi.org/10.19080/OMCIJ.2018.07.555722.
[30] Amir M, Mujeeb M, Ahmad S, Akhtar M, Ashraf K. Design expert-supported development and validation of HPTLC method: An application in simultaneous estimation of quercetin and rutin in *Punica granatum*, *Tamarindus indica* and *Prunus domestica*. Pharm Method. 2013;4(2):62-67. http://dx.doi.org/10.1016/j.phme.2013.12.004
[31] Huria N, Saraf A, Saraf A. Estimation of rutin from Morus species using validated HPTLC method. Int J Pharm Sci. 2024;2(3):565-577. https://doi.org/10.5281/zenodo.10954737

[32] Xu J, Zhu LY, Shen H, Zhang HM, Jia XB, Yan R, Li SL, Xu HX. A critical view on spike recovery for accuracy evaluation of analytical method for medicinal herbs. J Pharm Biomed Anal. 2012;62:210-215. https://doi.org/10.1016/j.jpba.2011.12.034

[33] Pessoa LM, Morais SM, Bevilaqua CM, Luciano JH. Anthelmintic activity of essential oil of *Ocimum gratissimum* Linn. and eugenol against *Haemonchus contortus*. Vet Parasitol. 2002;109(1-2):59-63. https://doi.org/10.1016/S0304-4017(02)00253-4

[34] Rodríguez-Valdovinos KY, Salgado-Garciglia R, Vázquez-Sánchez M, Álvarez-Bernal D, Oregel-Zamudio E, Ceja-Torres LF, Medina-Medrano JR. Quantitative analysis of rutin by HPTLC and in vitro antioxidant and antibacterial activities of phenolic-rich extracts from *Verbesina sphaerocephala*. Plants. 2021;10(3):475. https://doi.org/10.3390/plants10030475

[35] Ferreira LE, Castro PM, Chagas AD, França SC, Beleboni RO. In vitro anthelmintic activity of aqueous leaf extract of *Annona muricata* L.(Annonaceae) against *Haemonchus contortus* from sheep. Exp Parasitol. 2013;134(3):327-332. https://doi.org/10.1016/j.exppara.2013.03.032

[36] Baral P, Dubey A, Tewari S, Vasmatkar P, Verma AK. Total polyphenolic contents and antioxidant activity of leaf, bark and root of *Adina cordifolia* Benth. & Hook. J Pharm Chem Biol Sci. 2016;4(3):394-401.

[37] de Veras BO, de Oliveira JR, de Menezes Lima VL, Navarro DM, de Oliveira Farias JC, de Medeiros Moura GM, da Silva JW, de Assis CR, Gorlach-Lira K, de Assis PA, de Souza Barbosa JI. The essential oil of the leaves of *Verbesina macrophylla* (Cass.) SF Blake has antimicrobial, anti-inflammatory and antipyretic activities and is toxicologically safe. J Ethnopharmacol. 2021; 265:113248. https://doi.org/10.1016/j.jep.2020.113248

[38] Lee KJ, Oh YC, Cho WK, Ma JY. Antioxidant and anti-inflammatory activity determination of one hundred kinds of pure chemical compounds using offline and online screening HPLC assay. Evid-Based Complement Altern Med. 2015;2015(1):165457. https://doi.org/10.1155/2015/165457