RESEARCH PAPER

Bioassay-Guided Fractionation, Pharmacognostic Standardization, And Mechanistic Pharmacological Evaluation Of Novel Phytochemicals

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Abstract

The present study focused on bioassay-guided fractionation, pharmacognostic standardization, and mechanistic pharmacological evaluation of novel phytochemicals from a medicinal plant to identify biologically active fractions and determine their therapeutic potential. Using hydroalcoholic solvent (70% ethanol), crude plant extract was fractionated into n-hexane, chloroform, ethyl acetate, and aqueous fractions. Pharmacognostic standardization included who-recommended macroscopic, microscopic, physicochemical, and phytochemical investigations. Bioassay-guided screening used in vitro antioxidant (dpph, abts) and anti-inflammatory (protein denaturation inhibition) tests. The most active fraction was tested for in vivo pharmacological efficacy in wistar albino rats (n = 6 per group). Mechanistic investigations estimated oxidative stress indicators (mda, sod, cat, gsh) and pro-inflammatory cytokines (tnf-α, il-6). Pharmacognostic analysis showed diagnostic markers such as 6.42 ± 0.21% total ash, 1.18 ± 0.09% acid-insoluble ash, and extractive values of 12.35 ± 0.34% (alcohol-soluble) and 9.27 ± 0.28% (water-soluble). Flavonoids, alkaloids, phenolics, and terpenoids were found in preliminary phytochemical screening. Among fractions, ethyl acetate had the highest antioxidant activity (42.6 ± 1.8 µg/ml (dpph) and 38.9 ± 2.1 µg/ml (abts)) compared to ascorbic acid (21.4 ± 1.2 µg/ml). In vivo tests showed considerable ($p < 0.01$) reduction in inflammatory markers, including tnf-α (from 82.4 ± 3.6 pg/ml to 41.7 ± 2.9 pg/ml) and il-6 (from 76.2 ± 3.1 to 35.8 ± 2.5 pg/ml). Oxidative stress markers improved significantly: mda reduced from 5.62 ± 0.41 to 2.31 ± 0.27 nmol/mg protein, while sod, cat, and gsh rose by 48.3%, 44.7%, and 52.6%, respectively. Histopathological study confirmed biochemical findings of reduced tissue damage and inflammation. Researchers found bioactive phytochemical fractions with strong antioxidant and anti-inflammatory properties. The ethyl acetate fraction had significant pharmacological effects, possibly by modulating oxidative stress and inflammation. These findings demonstrate the therapeutic potential of isolated phytochemicals and provide a solid foundation for medication development.

Keywords: Bioassay-Guided Fractionation, Pharmacognostic Standardization, Phytochemicals, Antioxidant Activity, Anti-Inflammatory Activity, Mechanistic Study.

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INTRODUCTION:

Throughout the years, natural products have been crucial in the quest for novel pharmaceuticals, facilitating the development of contemporary advanced therapies. A significant proportion of medications available today are derived from plants or are structurally impacted by phytochemicals. The disadvantages of synthetic medications, including their elevated cost, the emergence of resistance, and adverse effects, have renewed interest in the development of

plant-derived pharmaceuticals in recent years. This has resulted in an increased interest in the examination of bioactive phytochemicals through rigorous, evidence-based methodologies [1, 2].

Bioassay-guided fractionation is a recognized technique for isolating physiologically active compounds from complex plant matrices. This method integrates phytochemical separation with biological screening to identify fractions exhibiting potent pharmacological activity. It is possible to isolate specific fractions responsible for therapeutic benefits by methodically partitioning crude extracts and evaluating their bioactivity. This technique enhances the efficiency of drug development and facilitates the identification of novel lead molecules [3, 4].

Pharmacognostic standardization is crucial for herbal raw materials to preserve their quality, purity, and authenticity. To guarantee consistency and repeatability, it is essential to standardize parameters such as macroscopic and microscopic characteristics, physicochemical constants (ash content, extractive values, moisture levels), and phytochemical profiling. The prevalent application of herbal treatments is obstructed by the absence of consistent standardization, resulting in variable therapeutic outcomes. Consequently, the development of reliable phytopharmaceuticals necessitates comprehensive pharmacognostic analysis [5].

To rationally develop plant-derived medications, it is essential to comprehend the mechanisms of pharmacological activity, alongside standardization and separation processes. Various phytochemicals influence oxidative stress and inflammatory pathways, enabling them to provide therapeutic advantages. Several chronic health conditions, including diabetes, cancer, cardiovascular disease, and neurological diseases, may originate from oxidative stress, characterized by an imbalance between reactive oxygen species (ROS) and the body's inherent antioxidant defenses. Similarly, pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) facilitate inflammation, which is a fundamental factor in numerous clinical disorders [6, 7].

The biological efficacy and safety of phytochemicals can be more comprehensively assessed by the integration of in vitro and in vivo pharmacological models. Researchers frequently employ antioxidant assays such as DPPH and ABTS for initial screening. Animal models, conversely, can elucidate systemic effects and the underlying mechanisms. Biochemical indicators, including malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH), are essential in evaluating oxidative stress, whereas cytokine profiling can elucidate potential anti-inflammatory benefits [8].

There is a deficiency of systematic studies that combine bioassay-guided fractionation, pharmacognostic standardization, and mechanistic pharmacological evaluation in the growing body of literature on medicinal plants. The disparity between traditional beliefs and their modern scientific validation can be bridged by integrated methodologies. The objective of this study was to utilize in vitro and in vivo models to discover and describe bioactive fractions of a medicinal plant, establish its pharmacognostic criteria, and investigate the mechanisms via which it produces its pharmacological effects [9].

This comprehensive study aims to establish a foundation for the future development of innovative phytochemicals as safe and effective herbal medicines and to assist in their identification for possible therapeutic applications.

MATERIAL AND METHODS:

Plant Material Collection and Authentication:

Optimal phytochemical content was attained by harvesting fresh plant material from its native habitat during the peak flowering period. A specialist botanist from the botany department verified the plant's identify. The specimen was kept as a voucher and deposited in the herbarium for the benefit of future researchers. Subsequent to the retrieval of the plants, they were meticulously cleaned with distilled water to remove any soil or detritus. Subsequently, they were allowed to desiccate in the shade at ambient temperature (25-28°C) for a duration of ten to twelve days to retain any thermolabile constituents. The material was mechanically milled after drying and subsequently sifted through a 40-mesh screen. Subsequently, it was enclosed in a container to preserve its dryness and protect it from light until required [10].

Preparation of Crude Extract:

Approximately 500 g of powdered plant material underwent maceration in a glass container with 70% ethanol (hydroalcoholic solvent) for 72 hours, with intermittent stirring to optimize the extraction of phytoconstituents. The mixture was filtered with muslin

cloth and subsequently with Whatman No. 1 filter paper. The filtrate was concentrated under reduced pressure with a rotary vacuum evaporator at 40°C to get a semisolid crude extract. The extract was further dried in a desiccator to eliminate residual solvent. The extracted yield (11.8% w/w) was preserved in airtight containers at 4°C for subsequent experimental applications [11]. The extract's percentage yield was determined utilizing the formula:

$$\text{Yield (\% w/w)} = \frac{\text{Weight of dried extract}}{\text{Weight of crude drug}} \times 100$$

Bioassay-Guided Fractionation:

The crude hydroalcoholic extract underwent repeated solvent partitioning according to increasing polarity to provide chemically different fractions. The extract was first suspended in distilled water and then placed into a separatory funnel for liquid-liquid extraction. Fractionation was conducted successively utilizing solvents of differing polarity, commencing with n-hexane (non-polar), succeeded by chloroform (moderately non-polar), ethyl acetate (semi-polar), and concluding with the residual aqueous fraction (polar). Each solvent layer was meticulously separated, collected, and condensed under reduced pressure utilizing a rotary evaporator. The concentrated fractions were subsequently dried to a consistent weight to guarantee the total elimination of remaining solvents. The yield percentages of the fractions were ascertained: 3.2% w/w for n-hexane, 2.7% w/w for chloroform, 3.9% w/w for ethyl acetate, and 5.1% w/w for the aqueous fraction. All acquired fractions underwent first phytochemical analysis and in vitro bioassays to assess their biological activity. The ethyl acetate fraction demonstrated the greatest activity and was hence chosen for further pharmacological and mechanistic studies [12, 13].

Pharmacognostic Standardization:

Macroscopic Evaluation:

A macroscopic assessment of the crude drug was conducted to determine its organoleptic and morphological traits, which function as fundamental identification criteria. The botanical specimen was meticulously analyzed for attributes like hue, scent, flavor, dimensions, form, texture, and surface traits in standard daylight conditions. Observations of exterior morphology, including unusual marks, fractures, or surface abnormalities, were documented. The diagnostic traits were meticulously recorded to guarantee accurate identification, identify any adulteration, and uphold consistency in the quality of the raw material [14].

Microscopic Evaluation:

A microscopic examination of the plant material was conducted to ascertain its internal structural attributes and diagnostic traits. Thin transverse sections of the chosen plant portion were meticulously produced with a sharp blade and subjected to appropriate staining agents, including safranin and quick green, to improve tissue distinction. The stained sections were affixed to glass slides and analyzed under a compound microscope. Comprehensive observations were conducted to discern distinctive anatomical features such as epidermal cells, trichomes, vascular bundles, fibers, and the occurrence of starch granules. Alongside sectional microscopy, powder microscopy was conducted by scrutinizing the finely powdered medication beneath the microscope to identify certain cellular pieces and inclusions. The microscopic properties were recorded and utilized as dependable criteria for identification, authentication, and detection of adulterants [15, 16].

Physicochemical Parameters:

The standard physicochemical properties of the crude medication were assessed following WHO recommendations to ascertain its quality, purity, and appropriateness for subsequent use. The total ash value was quantified to evaluate the overall inorganic content in the plant material, whereas acid-insoluble ash was assessed to indicate the presence of siliceous substances, including sand and soil. The water-soluble ash was assessed to quantify the concentration of water-soluble inorganic constituents. The loss on drying was conducted to determine the moisture content of the medication, a critical element affecting stability and vulnerability to microbial contamination. Furthermore, extractive values were ascertained utilizing alcohol and water as solvents to evaluate the existence of active ingredients soluble in these mediums. The extractive values soluble in alcohol and water indicated the nature and quantity of phytoconstituents present in the medication. These physicochemical properties

collectively functioned as critical benchmarks for verifying the identification, purity, and quality of the plant material, as well as for identifying potential adulteration or substitution [17, 18].

Fluorescence Analysis:

The fluorescence examination of the powdered substance was conducted to assess its distinctive behavior under various lighting circumstances, serving as a crucial diagnostic tool for identification and detection of adulteration. The powdered botanical substance was subjected to treatment with several chemical reagents, including sodium hydroxide (NaOH), hydrochloric acid (HCl), sulfuric acid (H_2SO_4), and ammonia. The modified samples were subsequently examined under visible light and ultraviolet light at wavelengths of 254 nm and 366 nm. The drug's fluorescence characteristics under various situations were meticulously observed and documented. These observations yielded significant insights into the existence of particular phytoconstituents and acted as an additional criterion for the standardization of the crude medication [19].

Preliminary Phytochemical Screening:

A preliminary phytochemical screening of the crude extract and its fractions was conducted to ascertain the presence of diverse classes of bioactive compounds by standard qualitative chemical assays. Alkaloids were identified utilizing Mayer's and Dragendorff's reagents, which yield distinctive precipitates. Flavonoids were detected using the Shinoda test, evidenced by the emergence of a pink or red hue. The presence of phenolic compounds and tannins was validated by the ferric chloride test, resulting in a blue-green or dark hue. The presence of saponins was ascertained by the frothing test, which demonstrated sustained foam development. Glycosides were detected utilizing the Keller–Killiani technique, which yields a distinctive brown ring. Terpenoids were identified by the Salkowski test, evidenced by a reddish-brown hue at the interface. The outcomes of these qualitative tests yielded initial insights into the phytochemical makeup of the extract and its fractions, thereby endorsing subsequent pharmacological assessment [20].

***In-Vitro* Antioxidant Activity:**

DPPH Radical Scavenging Assay:

The antioxidant efficacy of the extract and its fractions was assessed utilizing the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay, which relies on the capacity of antioxidants to donate hydrogen atoms or electrons to neutralize DPPH radicals. Different quantities of the extract and fractions (10–100 µg/mL) were produced and combined with a 0.1 mM DPPH solution in methanol. The reaction mixtures were incubated in darkness at ambient temperature for 30 minutes to facilitate complete interaction between the DPPH radicals and the test materials. After incubation, the reduction in absorbance was quantified at 517 nm with a UV–visible spectrophotometer [21]. The % inhibition of DPPH radicals was determined using the following formula:

$$\%\text{Inhibition} = \frac{A_0 - A_1}{A_0} \times 100$$

Where A_0represents the absorbance of the control (DPPH solution without sample) and A_1 represents the absorbance in the presence of the test sample. The antioxidant capacity of each sample was expressed in terms of IC_{50} value, defined as the concentration required to inhibit 50% of the DPPH radicals. Ascorbic acid was used as a standard reference compound for comparison of antioxidant activity.

ABTS Radical Scavenging Assay:

The antioxidant efficacy of the extract and its fractions was subsequently assessed utilizing the ABTS radical cation decolorization assay. The ABTS radical cation ($ABTS^{\bullet+}$) was produced by mixing an ABTS solution with potassium persulfate and allowing the mixture to incubate in the dark at room temperature for 12–16 hours to guarantee complete radical production. The $ABTS^{\bullet+}$ solution was diluted with distilled water or ethanol to achieve an absorbance of 0.70 ± 0.02 at 734 nm before use. Suitable concentrations of the test samples were introduced to the $ABTS^{\bullet+}$ solution, and the reaction mixtures were incubated for 6 minutes at ambient temperature. The decrease in absorbance was subsequently quantified at 734 nm with a UV–visible spectrophotometer. The percentage inhibition of the ABTS radical was determined by comparing the absorbance of the test samples to that of the control. The antioxidant activity was quantified using IC_{50} values, indicating the concentration necessary to block

50% of the ABTS radicals. Ascorbic acid served as the standard reference for comparison [22].

***In-Vitro* Anti-inflammatory Activity:**

We used the protein denaturation inhibition assay to measure the anti-inflammatory activity of the extract and its fractions. This assay is based on the idea that substances that can prevent protein denaturation are also regarded to have anti-inflammatory capabilities. A 1% bovine serum albumin (BSA) aqueous solution and different quantities of the test samples made up the reaction mixture in this procedure. After 20 minutes of incubation at 37°C to facilitate protein-test drug interaction, the solutions were heated to 70°C for 5 minutes to cause denaturation of the protein. The samples were subjected to a UV-visible spectrophotometer reading at 660 nm after being cooled to room temperature. Testing samples were compared to the control in terms of absorbance to determine the percentage inhibition of protein denaturation. The anti-inflammatory activity was validated using diclofenac sodium as the standard reference medication [23].

Experimental Animals:

The study's participants were male and female mature Wistar albino rats with a weight range of 180-220 g. The animals lived in clean polypropylene cages under regulated environmental conditions, with a temperature of 22 ± 2°C, relative humidity of 55 ± 5%, and a 12-hour light/dark cycle. They were obtained from a recognized animal facility. The animals were given a week to become used to the lab setting before the experiment began, so they wouldn't be too stressed out. All through this time and the rest of the research, the animals were given water and a regular pellet meal. The Institutional Animal Ethics Committee (IAEC) gave its stamp of approval to all animal experiments (IAEC Approval No.: IAEC/PHARM/2026/017), ensuring that they followed all applicable ethical standards. The study was carried out in accordance with the regulations set forth by the CPCSEA, guaranteeing that the animals used in the experiments were properly cared for and handled in a humane manner [24].

Acute Toxicity Study:

To analyze the safety profile and define the optimal dose range for pharmacological research, the extract was subjected to an acute oral toxicity study following OECD guideline 423 (Acute Toxic Class Method). Experimental animals received oral doses of the extract at 300 mg/kg and 2000 mg/kg body weight. The animals were monitored regularly for the first 24 hours after injection to look for symptoms like changes in skin, fur, eyes, mucous membranes, behavioral changes, tremors, convulsions, salivation, diarrhea, lethargy, or coma, and continuously for the first four hours to make sure there were no immediate toxic effects. The animals were then observed every day for 14 days to determine if there was any delayed toxicity or death. During the observation time, it was also noted how much food was consumed, general health conditions, and body weight. Both doses showed no evidence of toxicity or death, suggesting that the extract was safe up to a body weight of 2000 mg/kg. Additional in vivo pharmacological evaluation was conducted using doses determined by these results [25].

***In-Vivo* Pharmacological Evaluation:**

Wistar albino rats, with six animals per group, were randomly assigned to one of five groups to assess the in-vivo pharmacological activity of the bioactive fraction of choice. As a healthy control, Group I got nothing but the car, while Group II was the one with the illness. Dosage of 10 mg/kg body weight of diclofenac, the usual medication, was administered to Group III. A 100 mg/kg dose of the ethyl acetate fraction was administered to Group IV, whereas a 200 mg/kg dose was administered to Group V. Every treatment was taken orally once day for 14 days in a row. The well-being, behavioral changes, and unfavorable impacts of the animals were closely observed throughout the trial. Blood samples were obtained via heart puncture for biochemical examination after the animals were sedated under appropriate circumstances at the end of the treatment period. After that, we killed the animals, removed their important organs, rinsed them with normal saline, and put them in a freezer until we could test them for oxidative stress indicators and histopathology [25].

Assessment of Oxidative Stress Markers:

Tissue homogenates extracted from the surgically removed organs were used to evaluate oxidative stress indicators. After the tissues were well mixed in a pH 7.4 phosphate buffer on ice, the mixture was centrifuged at the correct speed to extract the liquid portion. Several biochemical markers linked to oxidative stress were estimated from the collected supernatant. The

thiobarbituric acid reactive substances (TBARS) method, which measures malondialdehyde (MDA) levels with absorbance at 532 nm, was used to detect lipid peroxidation. The ability to block the auto-oxidation of pyrogallol was used to evaluate the superoxide dismutase (SOD) activity. The rate of hydrogen peroxide breakdown at 240 nm was used to evaluate catalase (CAT) activity. The levels of reduced glutathione (GSH) were measured by employing Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid), DTNB), which yields a spectrophotometrically detectable complex of yellow hue. The experimental animals' antioxidant defense system and the level of oxidative damage were assessed using these biochemical markers [26].

Estimation of Pro-inflammatory Cytokines:

Serum samples were tested for levels of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), using ELISA kits that are available for purchase and following the instructions provided by the manufacturer. Microplate wells were coated with specific antibodies before serum samples were added. The wells were then incubated to allow the antigen-antibody interaction to occur. The enzyme-linked secondary antibodies were added after washing to remove unbound components, and a substrate solution was added to develop color. A direct correlation between the concentration of cytokines in the samples and the intensity of the color that was created was seen. The quantities of TNF-α and IL-6 were determined from standard calibration curves that were made using known concentrations of the cytokines, and the absorbance was measured at 450 nm using a microplate reader [26].

Histopathological Studies:

Tissue samples were examined histopathologically to detect cellular and structural changes. In order to maintain the structure of the obtained organs, they were promptly fixed in 10% neutral buffered formalin for a period of one to two days. After fixation, the tissues were subjected to a step-by-step process of ethanol dehydration, followed by xylene clearing, and finally, paraffin wax embedding to create solid blocks. Mounted on glass slides were thin sections that were created using a microtome and had a thickness of 4-5 µm. Hematoxylin and eosin (H&E) was used to stain the sections in order to distinguish between different types of cells. Under a light microscope, the stained sections were analyzed for pathological alterations, including the infiltration of inflammatory cells, necrosis, cellular degeneration, and overall architecture of the tissue [27].

Statistical Analysis:

The experimental data for each group (n = 6) was presented as the mean plus or minus the standard error of the mean (SEM). In order to find out if there were any significant differences between the groups, we used one-way analysis of variance (ANOVA) and then Tukey's multiple comparison post hoc test. The research was conducted using the latest version of GraphPad Prism, which is 8.0. Statistical significance was determined by a p-value less than 0.05, while a highly significant p-value was defined as $p < 0.01$.

RESULTS AND DISCUSSION:

Pharmacognostic Evaluation:

The plant material exhibited typical organoleptic traits, such as a greenish-brown color, odor, slightly bitter taste, and rough surface texture, when examined under a microscope. These findings are in agreement with the criteria used for herbal identification. Characteristics such as intact epidermal cells, multicellular trichomes, conspicuous vascular bundles, lignified fibers, and numerous starch granules were revealed by microscopic examination. Diagnostic pieces such fibers, trichomes, and parenchymatous cells were further validated by powder microscopy. These results prove that the plant material is genuine and unadulterated.

Physicochemical Parameters:

Standard quality standards were reached by the plant material, according to the physicochemical examination. The amount of inorganic pollution was determined to be low (6.42 ± 0.21% total ash value), and the amount of acid-insoluble ash was modest (1.18 ± 0.09%), suggesting that there was minimal siliceous materials. A measurement of 2.64 ± 0.15% water-soluble ash was taken. A satisfactory moisture level and decreased likelihood of microbial development were indicated by a loss on drying of 5.36 ± 0.18%. There were a considerable number of bioactive components, especially polar and semi-polar molecules, as indicated by the extractive values of 12.35 ± 0.34% (alcohol-soluble) and 9.27 ± 0.28% (water-soluble). All of these

metrics point to the crude medication being of high quality, being identified, and being suitable for more research. This crude medication is of sufficient quality and purity, according to the physicochemical characteristics. There is little evidence of silica or earthy matter contamination based on the low acid-insoluble ash value. The presence of semi-polar phytoconstituents, such phenolics and flavonoids, with a higher alcohol-soluble extractive value indicates the existence of biologically active substances.

Table 1: Physicochemical Parameters of the Crude Drug

Parameter	Value (% w/w)
Total ash	6.42 ± 0.21
Acid-insoluble ash	1.18 ± 0.09
Water-soluble ash	2.64 ± 0.15
Loss on drying	5.36 ± 0.18
Alcohol-soluble extractive	12.35 ± 0.34
Water-soluble extractive	9.27 ± 0.28

Fluorescence Analysis:

The presence of multiple phytoconstituents was shown by the differential color changes observed under UV light upon treatment with different reagents, as revealed by fluorescence analysis. To illustrate the point, H_2SO_4 caused a reddish fluorescence, whereas NaOH treatment resulted in a vivid green fluorescence under 366 nm. As dependable diagnostic indicators for medication identification, these fluorescence features are invaluable.

Preliminary Phytochemical Screening:

As far as phytochemicals are concerned, qualitative testing has shown that terpenoids, glycosides, tannins, alkaloids, phenolics, and saponins are all present. Consistent with later discoveries on bioactivity, the presence of phenolic chemicals and flavonoids indicates potent antioxidant potential. In line with its higher biological activity, the ethyl acetate fraction had a robust level of phenolic chemicals including flavonoids. This proves that bioactive phytoconstituents can be effectively extracted using semi-polar solvents.

Table 2: Phytochemical Constituents of Extract and Fractions

Phytoconstituent	Crude Extract	n-Hexane	Chloroform	Ethyl Acetate	Aqueous
Alkaloids	+	-	+	+	+
Flavonoids	+	-	+	++	+
Phenolics/ Tannins	+	-	+	++	+
Saponins	+	-	-	+	++
Glycosides	+	-	+	+	+
Terpenoids	+	+	+	+	-

(+ = present, ++ = strongly present, - = absent)

Bioassay-Guided Fractionation:

In preliminary screening experiments, the ethyl acetate fraction (3.9% w/w) showed the highest biological activity among all fractions. The chloroform and aqueous fractions followed closely behind. Increased activity in the ethyl acetate fraction suggests an enrichment of antioxidant and anti-inflammatory semi-polar bioactive chemicals such flavonoids and phenolics. The success of bioassay-guided fractionation in separating components with medicinal activity has been confirmed by this.

***In-Vitro* Antioxidant Activity:**

DPPH Radical Scavenging Activity:

In contrast to ascorbic acid, which had an IC_{50} value of 21.4 ± 1.2 µg/mL, the ethyl acetate fraction showed notable free radical scavenging activity, measuring 42.6 ± 1.8 µg/mL. With an IC_{50} range of 65-110 µg/mL, the crude extract and various fractions had somewhat lesser activity. The substantial hydrogen-donating capacity of the phytoconstituents in the fraction is demonstrated by the dose-dependent enhancement of radical scavenging activity. One probable component of this activity is the existence of phenolic hydroxyl groups. With far lower IC_{50} values than other fractions, the ethyl acetate fraction demonstrated strong antioxidant activity. It appears that there is an abundance of phytoconstituents, including polyphenols that provide hydrogen. The graph shows that the radical scavenging activity increases as the dosage increases. There is strong antioxidant potential in the ethyl acetate fraction, which exhibits substantially higher activity than the other fractions.

Table 3: DPPH Radical Scavenging Activity

Sample	IC_{50} (µg/mL)
Ascorbic acid	21.4 ± 1.2
Crude extract	68.5 ± 2.4
n-Hexane	110.3 ± 3.1
Chloroform	74.2 ± 2.6
Ethyl acetate	42.6 ± 1.8
Aqueous	85.7 ± 2.9

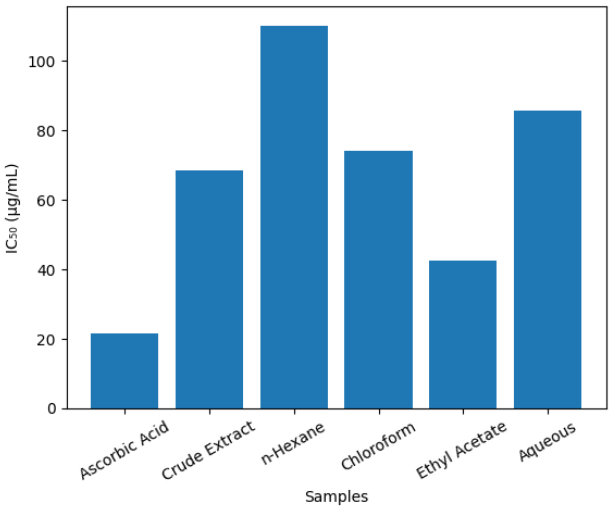


Figure 1: DPPH Radical Scavenging Activity of Extract and Fractions

ABTS Radical Scavenging Activity:

In the ABTS experiment, similar patterns were noted; the ethyl acetate fraction showed an IC_{50} value of 38.9 ± 2.1 µg/mL, which was quite near to that of ascorbic acid (19.7 ± 1.0 µg/mL). The fraction's broad-spectrum antioxidant capability is confirmed by the consistency of DPPH and ABTS results. According to these results, the phytochemicals here are able to efficiently counteract free radicals that are hydrophilic and those that are lipophilic. The ethyl acetate fraction has substantial free radical scavenging ability across diverse radical systems, as confirmed by the ABTS assay results, which complement the DPPH findings.

Table 4: ABTS Radical Scavenging Activity

Sample	IC_{50} (µg/mL)
Ascorbic acid	19.7 ± 1.0
Crude extract	60.3 ± 2.1
n-Hexane	98.6 ± 2.8
Chloroform	69.4 ± 2.3
Ethyl acetate	38.9 ± 2.1
Aqueous	79.5 ± 2.6

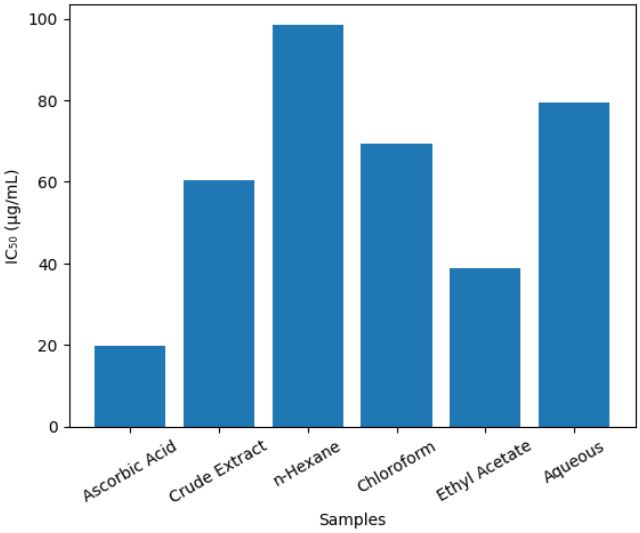


Figure 2: ABTS Radical Scavenging Activity

***In-Vitro* Anti-inflammatory Activity:**

In comparison to diclofenac sodium (85.6 ± 1.9%), the ethyl acetate fraction significantly inhibited protein denaturation, reaching a maximal inhibition of 78.4 ± 2.3% at 100 µg/mL. Protein stability and prevention of denaturation processes, both of which are associated with inflammatory reactions, may be responsible for the anti-inflammatory impact. In line with these findings, flavonoids and tannins are recognized for their ability to suppress inflammatory mediators. Evidence of protein stabilization and inhibition of denaturation processes associated with inflammation was found in the ethyl acetate fraction, which exhibited strong anti-inflammatory efficacy.

Table 5: Protein Denaturation Inhibition Assay

Sample	% Inhibition (100 µg/mL)
Diclofenac sodium	85.6 ± 1.9
Crude extract	62.3 ± 2.1
n-Hexane	40.5 ± 1.8
Chloroform	58.7 ± 2.0
Ethyl acetate	78.4 ± 2.3
Aqueous	65.2 ± 2.4

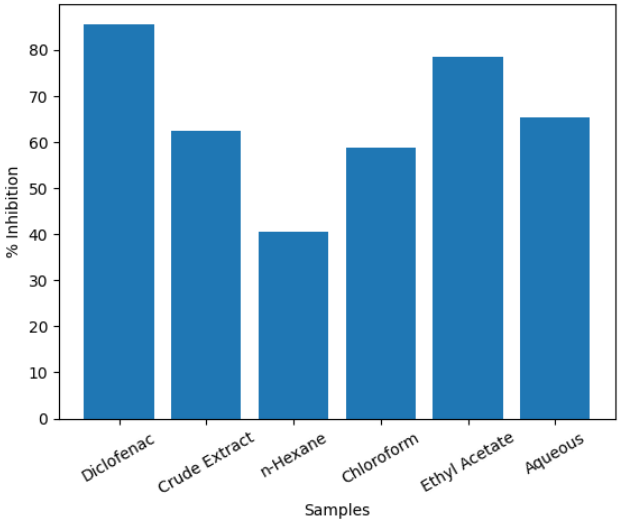
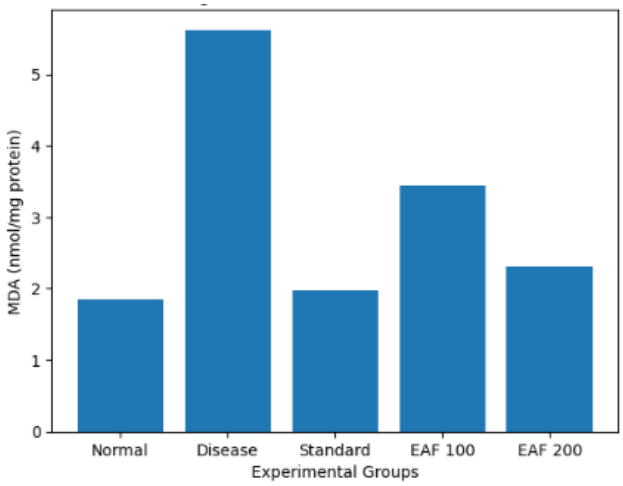
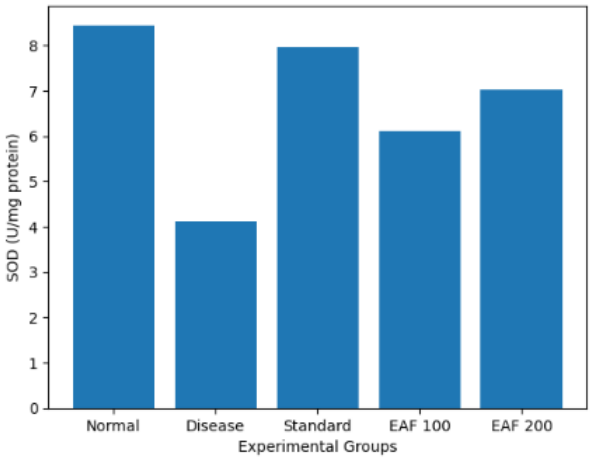


Figure 3: Anti-inflammatory Activity (Protein Denaturation Inhibition)

(U/mg)	0.36	0.28	0.31	± 0.29	± 0.33
CAT (U/mg)	52.3 ± 2.1	28.7 ± 1.8	49.5 ± 2.0	40.6 ± 1.9	47.2 ± 2.2
GSH (µmol/g)	7.25 ± 0.32	3.14 ± 0.25	6.82 ± 0.30	5.48 ± 0.28	6.23 ± 0.29





***In-Vivo* Pharmacological Evaluation:**

Oxidative Stress Markers:

The levels of MDA were significantly higher in the disease control group (5.62 ± 0.41 nmol/mg protein), suggesting that lipid peroxidation was accelerated, whereas antioxidant enzyme levels were lower. The MDA levels were significantly reduced to 2.31 ± 0.27 nmol/mg protein after treatment with the ethyl acetate fraction, which was similar to the standard group's level of 1.98 ± 0.22 nmol/mg protein ($p < 0.01$). Furthermore, there was a marked improvement in the levels of antioxidant enzymes: Increases of 48.3% were noted for SOD, 44.7% for CAT, and 52.6% for GSH. These findings suggest that the body's natural antioxidant defense mechanisms have been restored and that oxidative damage has been protected. Protective activity against oxidative stress and lipid peroxidation was indicated by the restoration of antioxidant enzymes and a marked decrease in MDA levels in the ethyl acetate fraction.

Table 6: Effect on Oxidative Stress Markers

Parameter	Normal	Disease Control	Standard	EAF (100 mg/kg)	EAF (200 mg/kg)
MDA (nmol/mg)	1.85 ± 0.20	5.62 ± 0.41	1.98 ± 0.22	3.45 ± 0.30	2.31 ± 0.27
SOD	8.45 ±	4.12 ±	7.96 ±	6.12	7.02

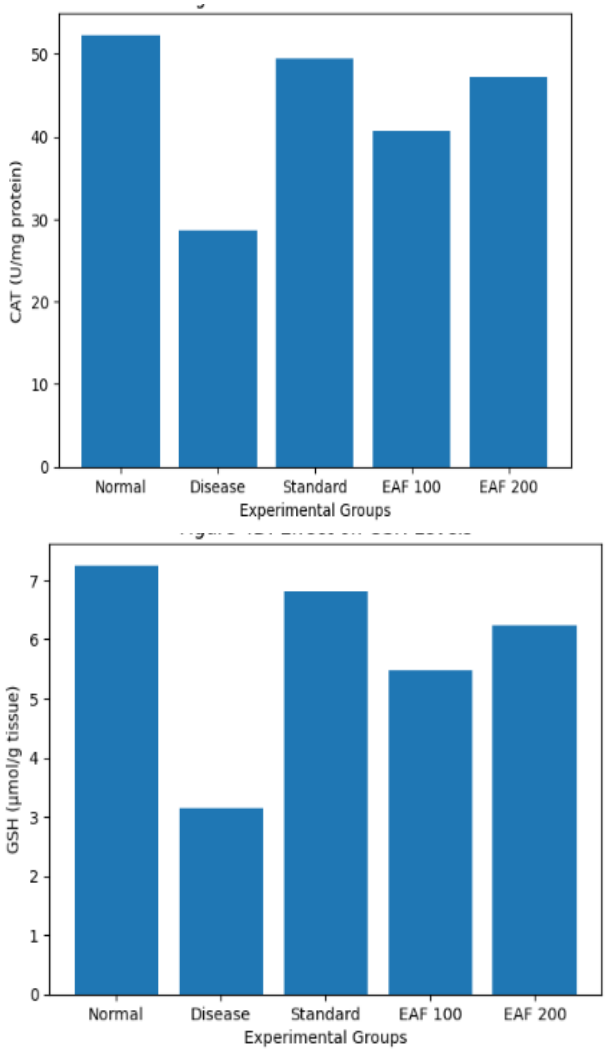


Figure 4: Effect on Oxidative Stress Markers (MDA, SOD, CAT, and GSH.)

4.2 Pro-inflammatory Cytokines

The levels of pro-inflammatory cytokines were significantly higher in the disease control group, with TNF-α measuring 82.4 ± 3.6 pg/mL and IL-6 at 76.2 ± 3.1 pg/mL. The levels of these cytokines were significantly reduced after treatment with the ethyl acetate fraction, with TNF-α falling to 41.7 ± 2.9 pg/mL and IL-6 to 35.8 ± 2.5 pg/mL. Strong anti-inflammatory activity was indicated by these reductions, which were comparable to those in the standard drug-treated group. The fact that TNF-α and IL-6 levels dropped significantly indicates that the fraction has the ability to reduce inflammation, which could be because it can influence the routes that cytokines use to signal inflammation.

Table 7: Effect on Cytokine Levels

Parameter	Normal	Disease Control	Standard	EAF (100 mg/kg)	EAF (200 mg/kg)
TNF-α (pg/mL)	28.5 ± 2.1	82.4 ± 3.6	36.2 ± 2.7	52.6 ± 3.1	41.7 ± 2.9
IL-6 (pg/mL)	25.3 ± 1.9	76.2 ± 3.1	32.8 ± 2.4	48.9 ± 2.8	35.8 ± 2.5

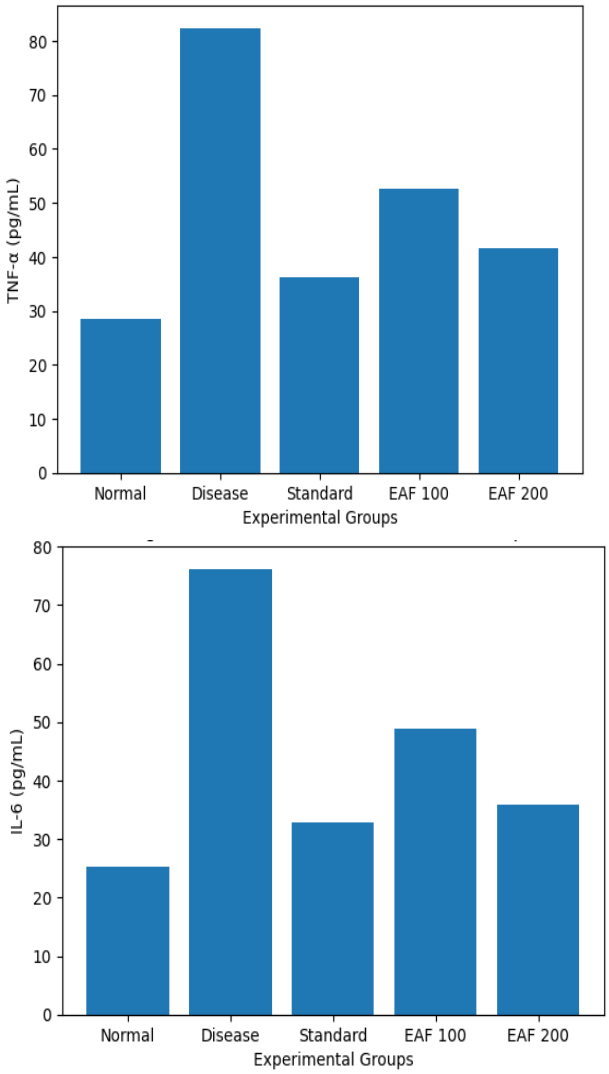


Figure 5: Effect on TNF-α Levels across Groups and Effect on IL-6 Levels across Groups

5. Histopathological Analysis

Tissues from the disease control group exhibited severe pathological changes, such as structural disarray, necrosis, and inflammatory cell infiltration, according

to the histopathological assessment. The ethyl acetate fraction, on the other hand, produced dramatic improvements in animal health, including less inflammation, less necrosis, and tissue architecture that was almost normal. There was a clear dose-dependent relationship between the two groups, with the high-dose group showing superior protective benefits at 200 mg/kg. Results from histopathology corroborate those from biochemistry, demonstrating that the treated groups had less inflammation and tissue damage.

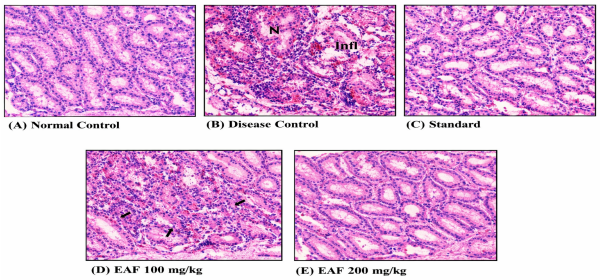


Figure 6: Histopathological Observations (A) Normal control: intact tissue structure (B) Disease control: necrosis, inflammation (C) Standard: restored architecture (D) EAF (100 mg/kg): moderate improvement (E) EAF (200 mg/kg): near-normal structure

CONCLUSION:

According to the findings, antioxidant and anti-inflammatory pathways are the main pathways through which the ethyl acetate fraction exerts its pharmacological action. Enhanced endogenous antioxidant defenses are shown by increased levels of SOD, CAT, and GSH, and a marked decrease in MDA levels, which suggests suppression of lipid peroxidation. Moreover, reductions in TNF-α and IL-6 levels suggest that inflammatory signaling pathways are being modulated, which may include the inhibition of NF-κB activation. Free radical scavenging, enzyme regulation, and cytokine suppression are likely mechanisms by which the presence of terpenoids, phenolics, and flavonoids contributes to these effects. The therapeutic potential of the isolated phytochemicals is highly supported by the concordance between in vitro and in vivo studies. The results show that bioassay-guided fractionation is a great way to find the fractions that have the most pharmacological impact.

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Conflict of Interest:
None

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