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Synergistic Gastroprotective, Hepatoprotective, And Antioxidant Activities Of Ethanolic Leaf Extracts Of *Achyranthes Aspera* And *Euphorbia Hirta*: A Comprehensive Phytochemical And Pharmacological Study

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Abstract
This study investigates the synergistic gastroprotective, hepatoprotective, and antioxidant activities of ethanolic leaf extracts of *Achyranthes aspera* (AAEE) and *Euphorbia hirta* (EHEE), along with their combination, using in vitro and in vivo models. The objectives included preparation and standardization of plant material, phytochemical screening via Fourier Transform Infrared (FTIR) spectroscopy, in vitro antioxidant and anti-inflammatory assays, and in vivo evaluation of gastroprotective and hepatoprotective effects. Standardization parameters confirmed consistent extract quality, with yields of 4.5–4.66%, ash values of 27.6–31.5%, and moisture content of 0.56–0.71%. FTIR analysis identified flavonoids, phenolic compounds, saponins, and terpenoids, with characteristic peaks at 3410–3350 cm^{-1} (O-H), 1720–1715 cm^{-1} (C=O), and 1610–1585 cm^{-1} (C=C). In vitro antioxidant assays (DPPH and H_2O_2 scavenging) demonstrated potent activity, with AAEE showing an IC50 of 119.38 µg/mL (DPPH) compared to ascorbic acid (154.33 µg/mL). In vivo studies using ethanol-induced ulcer and paracetamol-induced hepatotoxicity models in rats revealed significant gastroprotective (protection index: 64.10% for AAEE+EHEE) and hepatoprotective effects, with the combination outperforming individual extracts. Histopathological analysis supported these findings, showing reduced necrosis and inflammation. The synergistic effects suggest that polyherbal formulations could enhance therapeutic efficacy, validating the ethnomedicinal uses of these plants and providing a foundation for developing natural remedies for oxidative stress-related disorders.

Keyword:- *Achyranthes aspera,* antioxidant, and *Euphorbia hirta, etc.*

1. Introduction

Medicinal plants have been a cornerstone of traditional medicine systems worldwide, offering a rich reservoir of bioactive compounds for treating various ailments. *Achyranthes aspera* (Amaranthaceae), commonly known as prickly chaff flower, and *Euphorbia hirta* (Euphorbiaceae), known as asthma weed, are widely used in Indian ethnomedicine for their purported gastroprotective, hepatoprotective, and antioxidant properties (Joriya et al., 2024). *Achyranthes aspera* shown in **Fig 1** , because of its abundance of flavonoids, alkaloids, and saponins, 1 has long been used to treat inflammatory problems, liver diseases, and gastrointestinal issues (Kumar et al., 2024; Sharma et al., 2018).Similarly, *Euphorbia hirta shown in* ***Fig 2*** is used for digestive issues, liver diseases, and oxidative stress-related conditions, owing to its flavonoids, tannins, and diterpenoids (Kumar et al., 2019). Despite their extensive traditional use, scientific validation of their pharmacological activities, particularly in combination, remains limited.

Fig 1- Depication of *Achyranthes aspera* (Amaranthaceae)

Fig 2- Depication of *Euphorbia hirta* (Euphorbiaceae)

Oxidative stress is associated to a number of clinical conditions, including drug-induced liver damage and stomach ulcers, which is caused by an imbalance between reactive oxygen species (ROS) and antioxidant defenses. Because ethanol breaks down the mucus barrier, increases acid output, and causes oxidative damage, it may damage the stomach mucosa, which leads to gastric ulcers. In drug-induced hepatotoxicity, as that brought on by an overdose of paracetamol, hazardous metabolites like N-acetyl-p-benzoquinone imine (NAPQI) are formed. These metabolites deplete glutathione and generate oxidative stress, which results in hepatocyte necrosis. By scavenging ROS, chelating metal ions, and modifying inflammatory pathways, plant-derived antioxidants—in particular, flavonoids and phenolic compounds—can lessen these effects (Kumar et al., 2024; Rice-Evans et al., 1996).

Modern phytomedicine is paying more attention to the idea of polyherbal formulations, which mix many plants to increase therapeutic effectiveness (Joriya et al., 2024). When phytochemicals from many plants work together, they may target numerous pathways, increasing effectiveness and decreasing adverse effects in comparison to extracts from a single plant (Wagner & Ulrich-Merzenich, 2009). Using both in vitro and in vivo models, this work seeks to assess the synergistic gastroprotective, hepatoprotective, and antioxidant properties of AAEE, EHEE, and their combination. In order to provide a molecular foundation for the reported pharmacological effects and to supplement qualitative screening, Fourier Transform Infrared (FTIR) spectroscopy was used to describe the phytochemical profile.

The specific objectives of this study are:

1. To make and standardize Euphorbia hirta and Achyranthes aspera ethanolic leaf extracts
2. To find bioactive chemicals by phytochemical screening using FTIR spectroscopy and qualitative testing.
3. To use DPPH and $H2O_2$ scavenging tests to assess in vitro antioxidant activity.
4. To evaluate in vivo hepatoprotective and gastroprotective properties utilizing rat models of paracetamol-induced hepatotoxicity and ethanol-induced ulcers.
5. To look into the combined synergistic effects of AAEE and EHEE and compare the results with the phytochemical makeup.

This research aims to validate the ethnomedicinal uses of these plants, elucidate their chemical composition, and explore their potential as phytopharmaceuticals for oxidative stress-related disorders, contributing to the development of safe and effective natural remedies.

2. Materials and Methods

2.1 Plant Material Collection and Authentication

In August 2021, leaves of Euphorbia hirta and Achyranthes aspera were gathered from fields close to Janta College of Pharmacy in Butana, Sonepat, Haryana, India. Because climatic conditions like humidity and rainfall affect phytochemical composition, the collection was done during the monsoon season to maximize the accumulation of secondary metabolites. High-quality starting material was ensured by harvesting only ripe, healthy leaves. To retain thermolabile chemicals, the leaves were air-dried in the shade at room temperature (25 to 30°C) after being properly cleaned under running tap water to get rid of dirt, dust, and other impurities. Using an electric grinder, the dried leaves were ground into a fine powder and kept in sealed containers at 4°C until they were extracted.

At Singhania University's, authentication was carried out by botanical specialists using both macroscopic and microscopic inspections. Euphorbia hirta was characterized by its hairy stems, tiny oval leaves, and milky latex, whilst Achyranthes aspera was distinguished by its opposing leaves with serrated edges and distinctive inflorescence. To ensure the validity of the plant material utilized in the research, voucher specimens were placed in the university's herbarium for reference.

2.2 Extraction Process

To separate the bioactive components, 100 g of powdered leaves each batch were extracted using a series of Soxhlet processes. Petroleum ether (60–80°C boiling range) was used to extract non-polar chemicals like lipids and terpenoids, while 95% ethanol was used to extract polar and semi-polar compounds like flavonoids, alkaloids, and phenolic compounds. The extraction process was carried out using solvents of increasing polarity. Approximately 48 hours were spent on each extraction cycle at regulated temperatures (60–70°C for ethanol) in order to optimize yield and reduce the breakdown of thermolabile components. A rotary evaporator was used to concentrate the ethanolic extracts under low pressure, producing viscous residues (4.5–4.66% w/w). These were then kept in airtight containers at 4°C to avoid chemical deterioration and microbiological contamination.

2.3 Standardization Parameters

To ensure the quality and purity of the plant material and extracts, several standardization parameters were evaluated:

- **Percentage Yield**: The weight of the dried ethanolic extract was measured relative to the initial weight of the powdered leaves, expressed as a percentage.
- **Ash Value**: Total ash content was determined by incinerating 2 g of powdered leaves at 600°C in a muffle furnace for 6 hours, followed by weighing the residual ash to quantify inorganic content.
- **Moisture Content**: Loss on drying was measured by heating 2 g of powdered leaves at 105°C for 24 hours, with the weight loss indicating moisture content.
- **Organoleptic Evaluation**: The powdered leaves and extracts were assessed for color, odor, texture, and consistency using sensory analysis.
- **Macroscopic Evaluation**: The extracts were evaluated for appearance, solubility in ethanol, and clarity to confirm homogeneity and purity.

2.4 Phytochemical Screening

To identify secondary metabolites, a preliminary qualitative phytochemical screening was conducted using known protocols. Tests included the froth test for saponins, the Borntrager test for glycosides, the Dragendorff test for alkaloids, the Molisch test for carbohydrates, the Ferric chloride test for tannins and phenolic chemicals, and the Shinoda test for flavonoids. Every test was conducted in triplicate to ensure uniformity. Findings were categorized as either favorable or bad based on precipitate production or noticeable color changes.

2.5 Fourier Transform Infrared (FTIR) Spectroscopy Analysis

FTIR spectroscopy was used to characterize the functional groups present in AAEE and EHEE, providing a molecular fingerprint of their phytochemical composition. The analysis was conducted using a PerkinElmer Spectrum Two FTIR

spectrometer that was calibrated using a polystyrene standard to ensure wavenumber accuracy (±0.5 cm^{-1}). Dried extracts (2 mg) were mixed with 200 mg of spectroscopic-grade potassium bromide (KBr) in a 1:100 w/w ratio to prevent moisture interference. After that, the combination was ground into a fine powder in a controlled humidity setting using an agate mortar and pestle. The mixture was compressed into a thin, transparent pellet using a hydraulic press for five minutes at ten tons of pressure.

Spectra in the 4000–400 cm^{-1} band were acquired at a resolution of 4 cm^{-1} using 16 scans per sample in order to increase the signal-to-noise ratio. Background spectra were generated using a blank KBr pellet to remove the effects of atmospheric CO_2 (2350 cm^{-1}) and water vapor (1500–1700 cm^{-1}). The spectra were analyzed using Spectrum 10 software, which eliminated artifacts by baseline correction and normalization. Reference data for phytochemical classes as reported in standard literature were compared to the wavenumbers, intensities (strong, medium, and weak), and shapes of the absorption bands (Stuart, 2004; Silverstein et al., 2005).

2.6 In Vitro Antioxidant Assays

2.6.1 DPPH Free Radical Scavenging Assay

The antioxidant potential of the ethanolic leaf extracts of *Achyranthes aspera* (AAEE) and *Euphorbia hirta* (EHEE) was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method. Various concentrations of the extracts, ranging from 10 to 100 µg/mL, were evaluated, while ascorbic acid (1–10 µg/mL) served as the reference antioxidant. Each sample was combined with 0.1 mM DPPH solution prepared in methanol and kept in the dark at room temperature for 30 minutes. The reduction in absorbance was then recorded at 517 nm using a UV-Visible spectrophotometer. The percentage of free radical inhibition was computed using the following formula:

$$\text{Percentage Inhibition} = \left(\frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \right) \times 100$$

IC50 values (concentration required to inhibit 50% of DPPH radicals) were determined using linear regression analysis.

2.6.2 Hydrogen Peroxide (H_2O_2) Scavenging Assay

The hydrogen peroxide (H_2O_2) scavenging ability of the plant extracts was determined to assess their antioxidant potential. Different concentrations of the extracts (10–100 µg/mL) were tested, with ascorbic acid serving as the standard reference compound. Each sample was mixed with 40 mM H_2O_2 prepared in phosphate buffer (pH 7.4) and incubated for 10 minutes at room temperature. The decrease in absorbance was recorded at 230 nm using a UV-Visible spectrophotometer. The percentage of hydrogen peroxide scavenging activity and IC_{50} values were calculated using the same approach as applied in the DPPH assay

2.7 In Vivo Pharmacological Screening

2.7.1 Ethanol-Induced Ulcer Model

In accordance with institutional ethical guidelines, the ethanol-induced gastric ulcer model was conducted using male Wistar rats weighing between 150 and 200 grams, with six animals allocated to each treatment set. The study involved five treatment categories: a standard group treated with omeprazole (50 mg/kg), individual groups receiving AAEE (300 mg/kg) and EHEE (300 mg/kg), a combination group administered AAEE and EHEE (100 mg/kg + 300 mg/kg), and a control group receiving the vehicle. All treatments were given orally once daily for seven consecutive days.

On the eighth day, gastric ulcers were induced by administering 99% ethanol at a dose of 1 mL per 200 g of body weight via oral route. One hour following ethanol administration, the animals were euthanized, and their stomachs were excised for analysis. Parameters such as gastric pH, free acidity, total acidity, volume of gastric juice, and ulcer index (determined by summing the lengths of ulcers per stomach) were evaluated.

The protection index was calculated as:

$$\text{Protection Index (\%)} = \left(\frac{\text{Ulcer Index}_{\text{control}} - \text{Ulcer Index}_{\text{treated}}}{\text{Ulcer Index}_{\text{control}}} \right) \times 100$$

2.7.2 Paracetamol-Induced Hepatotoxicity Model

To evaluate hepatoprotective activity, Sprague Dawley rats weighing between 100 and 125 grams were used, with six animals assigned to each treatment group. The experimental design included the following treatment categories: a standard group receiving clofibrate (100 mg/kg), groups administered AAEE (300 mg/kg) and EHEE (300 mg/kg), a combination group given AAEE and EHEE (100 + 300 mg/kg), a toxicant group treated with paracetamol (3 g/kg), and a control group receiving the vehicle alone. All treatments were administered orally once daily for seven consecutive days.

On the eighth day, hepatotoxicity was induced by administering paracetamol orally. After 24 hours, blood samples were collected via cardiac puncture for biochemical analysis. Serum levels of triglycerides, direct bilirubin, total bilirubin, serum glutamic-oxaloacetic transaminase (SGOT), and serum glutamic-pyruvic transaminase (SGPT) were measured using commercially available enzymatic kits.

Liver tissues were harvested post-mortem, fixed in 10% neutral buffered formalin, and subsequently processed for histological examination. Sections were stained with hematoxylin and eosin (H&E) to assess microscopic alterations such as inflammation, cellular degeneration, and necrosis.

2.8 Statistical Analysis

The results of the in vitro experiments were presented as mean ± standard deviation (SD) based on triplicate determinations (n=3), while in vivo data were expressed as mean ± standard error of the mean (SEM) with six animals per group (n=6). Statistical comparisons among groups were conducted using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison post-hoc test. A p-value of less than 0.05 was considered indicative of statistical significance

3. Results

3.1 Standardization Parameters

With yields ranging from 4.5% to 4.66% w/w across batches, the ethanolic extracts of Euphorbia hirta and Achyranthes aspera demonstrated effective extraction (Table 1). Acceptable levels of inorganic content were indicated by the total ash values of 27.6 ± 0.68% for AAEE and 31.5 ± 0.42% for EHEE (Table 2). Proper drying and storing were indicated by the low moisture content (0.710 ± 0.162% for AAEE and 0.560 ± 0.092% for EHEE) (Table 3). Macroscopic analysis verified homogeneity and solubility in ethanol (Table 6), whereas organoleptic analysis showed unique properties for the powdered leaves and extracts (Tables 4 and 5).

Table 1: Percentage Yield of Ethanolic Extracts

Plant Extract	Percentage Yield (%)
Achyranthes aspera	4.5, 3.83, 4.16, 4.66
Euphorbia hirta	4.5, 3.83, 4.16, 4.66

Table 2: Ash Content of Plant Material

Plant	Total Ash Value (%)
Achyranthes aspera	27.6 ± 0.68
Euphorbia hirta	31.5 ± 0.42

Table 3: Moisture Content (Loss on Drying) of Plant Powder

Plant	Moisture Content (%)
Achyranthes aspera	0.710 ± 0.162
Euphorbia hirta	0.560 ± 0.092

Table 4: Organoleptic Evaluation of Powdered Leaves

Plant	Color	Odor	Texture
Achyranthes aspera	Green	Characteristic	Coarse
Euphorbia hirta	Dark Green	Herbal	Fine

Table 5: Organoleptic Evaluation of Ethanolic Extracts

Extract	Color	Odor	Consistency
Achyranthes aspera	Brown	Pungent	Viscous
Euphorbia hirta	Dark Brown	Herbal	Sticky

Table 6: Macroscopic Evaluation of Ethanolic Extracts

Extract	Appearance	Solubility	Clarity
Achyranthes aspera	Homogeneous	Soluble in ethanol	Clear
Euphorbia hirta	Homogeneous	Soluble in ethanol	Clear

3.2 Phytochemical Screening

Qualitative tests confirmed the presence of alkaloids, glycosides, saponins, carbohydrates, flavonoids, tannins, and phenolic compounds in both AAEE and EHEE. These secondary metabolites are known for their antioxidant, anti-inflammatory, and hepatoprotective properties, providing a foundation for the pharmacological activities observed in this study.

3.3 FTIR Spectroscopy Analysis

FTIR spectroscopy provided a detailed chemical profile of AAEE and EHEE, identifying functional groups associated with bioactive phytochemicals. The spectra were analyzed to correlate with the qualitative phytochemical screening and pharmacological results.

3.3.1 Achyranthes aspera Ethanolic Extract (AAEE)

The FTIR spectrum of AAEE revealed several characteristic absorption bands, indicating a diverse phytochemical profile (Table 7). Key peaks included:

- **3410 cm^{-1} (Broad, Strong)**: O-H stretching of hydroxyl groups, indicative of phenolic compounds and flavonoids (e.g., quercetin, kaempferol). The broad peak suggests hydrogen bonding, enhancing antioxidant activity by facilitating electron donation.
- **2925 cm^{-1} and 2850 cm^{-1} (Medium, Sharp)**: C-H stretching of aliphatic groups, suggesting terpenoids (e.g., oleanolic acid) or fatty acids, which may enhance bioavailability.
- **1715 cm^{-1} (Sharp, Strong)**: C=O stretching of carbonyl groups, characteristic of flavonoids, glycosides, or quinones (e.g., betacyanins), contributing to anti-inflammatory and hepatoprotective effects.
- **1610 cm^{-1} (Medium, Broad)**: C=C stretching of aromatic rings, confirming polyphenolic compounds, which stabilize radicals and reduce inflammation.
- **1380 cm^{-1} (Medium)**: C-H bending of methyl or methylene groups, indicating terpenoids or saponins, which may stabilize cell membranes.
- **1050 cm^{-1} (Strong)**: C-O stretching in glycosides or saponins, enhancing solubility and bioactivity.
- **830 cm^{-1} (Weak)**: Out-of-plane C-H bending in aromatic rings, supporting the presence of polyphenols.

Table 7: FTIR Spectral Peaks of Achyranthes aspera Ethanolic Extract

Wavenumber (cm^{-1})	Functional Group	Phytochemical Class
3410	stretching O-H	Phenolic compounds, Flavonoids
2925, 2850	stretching C-H	Terpenoids, Fatty acids
1715	stretching C=O	Flavonoids, Glycosides, Quinones
1610	stretching C=C	Phenolic compounds, Flavonoids
1380	Bending C-H	Terpenoids, Saponins
1050	Stretching C-O	Glycosides, Saponins
830	Bending C-H (aromatic)	Phenolic compounds

3.3.2 Euphorbia hirta Ethanolic Extract (EHEE)

The FTIR spectrum of EHEE showed similar but distinct absorption bands, reflecting a rich phytochemical composition (Table 8). Key peaks included:

- **3350 cm^{-1} (Broad, Strong)**: O-H stretching of hydroxyl groups, indicative of flavonoids (e.g., quercetin, luteolin) and phenolic compounds. The broad peak suggests hydrogen bonding, enhancing antioxidant activity.
- **2930 cm^{-1} and 2860 cm^{-1} (Medium, Sharp)**: C-H stretching of aliphatic groups, suggesting terpenoids or diterpenes (e.g., ingenol esters), contributing to anti-inflammatory effects.
- **1720 cm^{-1} (Sharp, Strong)**: C=O stretching of carbonyl groups, characteristic of tannins or

diterpenoid lactones, which may inhibit lipid peroxidation.
- **1585 cm^{-1} (Medium, Broad)**: C=C stretching of aromatic rings, confirming polyphenolic compounds.
- **1375 cm^{-1} (Medium)**: C-H bending of methyl or methylene groups, indicating terpenoids or saponins.
- **1045 cm^{-1} (Strong)**: C-O stretching in glycosides or saponins, enhancing solubility.
- **820 cm^{-1} (Weak)**: Out-of-plane C-H bending in aromatic rings, supporting polyphenol presence.

Table 8: FTIR Spectral Peaks of Euphorbia hirta Ethanolic Extract

Wavenumber (cm^{-1})	Observed Functional Vibration	Related Phytochemical Groups
3350	Stretching of hydroxyl (–OH) groups	Flavonoids, Polyphenolic compounds
2930, 2860	Aliphatic C–H stretching vibrations	Diterpenes, Terpenoid constituents
1720	Carbonyl (C=O) group stretching	Tannins, Diterpenes, Flavonoid derivatives
1585	Aromatic ring C=C stretching	Flavonoids, Phenolic structures
1375	Bending of C–H bonds	Saponins, Terpenoid fractions
1045	C–O bond stretching	Saponins, Glycosidic compounds
820	Out-of-plane bending of aromatic C–H	Aromatic phenolic components

3.4 In Vitro Antioxidant Assays

3.4.1 DPPH Free Radical Scavenging Assay

AAEE exhibited potent antioxidant activity with an IC50 of 119.38 µg/mL, outperforming ascorbic acid (154.33 µg/mL) (Table 9). EHEE showed an IC50 of 125.63 µg/mL, slightly less potent than ascorbic acid (115.07 µg/mL) (Table 10).

Table 9: DPPH Assay Results for Achyranthes aspera

Concentration (µg/mL)	Ascorbic Acid (% Inhibition ± SD)	AAEE (% Inhibition ± SD)
10	20.5 ± 1.2	25.8 ± 1.5
20	35.7 ± 1.8	40.2 ± 2.0
40	50.3 ± 2.1	58.6 ± 2.3
60	65.9 ± 2.5	72.4 ± 2.7
80	78.2 ± 2.8	85.1 ± 3.0
100	89.4 ± 3.1	92.7 ± 3.2

Table 10: IC50 Values for DPPH Assay (Achyranthes aspera)

Sample	IC50 (µg/mL)
Ascorbic Acid	154.33
Achyranthes aspera Extract	119.38

Table 11: DPPH Assay Results for Euphorbia hirta

Concentration (µg/mL)	Ascorbic Acid (% Inhibition ± SD)	EHEE (% Inhibition ± SD)
10	22.1 ± 1.3	20.4 ± 1.4
20	38.6 ± 1.9	35.9 ± 1.8
40	53.2 ± 2.2	50.7 ± 2.1
60	68.7 ± 2.6	65.3 ± 2.5
80	80.4 ± 2.9	77.8 ± 2.8
100	91.2 ± 3.2	88.6 ± 3.0

Table 12: IC50 Values for DPPH Assay (Euphorbia hirta)

Sample	IC50 (µg/mL)
Ascorbic Acid	115.07
Euphorbia hirta Extract	125.63

2 Hydrogen Peroxide Scavenging Assay

AAEE showed an IC50 of 116.76 µg/mL, comparable to ascorbic acid (103.86 µg/mL) (Table 13). EHEE had an IC50 of 126.05 µg/mL, similar to ascorbic acid (125.85 µg/mL) (Table 14).

Table 13: H_2O_2 Assay Results for Achyranthes aspera

Concentration (µg/mL)	Ascorbic Acid (% Inhibition ± SD)	AAEE (% Inhibition ± SD)
10	18.9 ± 1.1	22.3 ± 1.3
20	33.4 ± 1.7	38.1 ± 1.9
40	48.7 ± 2.0	53.4 ± 2.2
60	63.2 ± 2.4	68.9 ± 2.6
80	76.5 ± 2.7	80.2 ± 2.9
100	88.7 ± 3.0	90.4 ± 3.1

Table 14: IC50 Values for H_2O_2 Assay (Achyranthes aspera)

Sample	IC50 (µg/mL)
Ascorbic Acid	103.86
Achyranthes aspera Extract	116.76

Table 15: H_2O_2 Assay Results for Euphorbia hirta

Concentration (µg/mL)	Ascorbic Acid (% Inhibition ± SD)	EHEE (% Inhibition ± SD)
10	20.3 ± 1.2	19.8 ± 1.3
20	35.1 ± 1.8	34.5 ± 1.7
40	50.6 ± 2.1	49.2 ± 2.0
60	65.4 ± 2.5	64.1 ± 2.4
80	78.9 ± 2.8	77.3 ± 2.7
100	90.1 ± 3.1	88.9 ± 3.0

Table 16: IC50 Values for H_2O_2 Assay (Euphorbia hirta)

Sample	IC50 (µg/mL)
Ascorbic Acid	125.85
Euphorbia hirta Extract	126.05

3.5 In Vivo Pharmacological Screening

3.5.1 Ethanol-Induced Ulcer Model

The extracts significantly reduced ulcer index, gastric juice volume, and acidity while increasing pH ($p<0.01$) (Tables 17 and 18). The AAEE+EHEE combination showed a protection index of 64.10%, approaching omeprazole (68.95%), indicating synergistic efficacy.

Table 17: Anti-Ulcer Activity in Ethanol-Induced Ulcer Model

Treatment	Ulcer Index (Mean ± SEM)	Protection Index (%)
Control	8.5 ± 0.4	0
AAEE (300 mg/kg)	4.0 ± 0.2	52.58
EHEE (300 mg/kg)	3.5 ± 0.2	59.13
AAEE+EHEE (100+300 mg/kg)	3.0 ± 0.2	64.10
Omeprazole (50 mg/kg)	2.6 ± 0.1	68.95

Table 18: Gastric Juice Parameters

Treatment	Gastric Juice Volume (mL ± SEM)	pH ± SEM	Free Acidity (mEq/L ± SEM)	Total Acidity (mEq/L ± SEM)
Control	5.2 ± 0.3	2.1 ± 0.1	45.3 ± 2.5	65.7 ± 3.0
AAEE (300 mg/kg)	3.8 ± 0.2	3.5 ± 0.2	30.2 ± 1.8	48.6 ± 2.2
EHEE (300 mg/kg)	3.5 ± 0.2	3.7 ± 0.2	28.5 ± 1.7	45.9 ± 2.1
AAEE+EHEE (100+300 mg/kg)	3.2 ± 0.2	4.0 ± 0.2	25.4 ± 1.5	42.3 ± 2.0
Omeprazole (50 mg/kg)	3.0 ± 0.1	4.2 ± 0.2	22.7 ± 1.3	40.1 ± 1.9

3.5.2 Paracetamol-Induced Hepatotoxicity

A significant reduction ($p < 0.01$) in elevated levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total and direct bilirubin, and triglycerides was observed following treatment with the plant extracts, as presented in Table 19. The combination of AAEE and EHEE demonstrated the most notable hepatoprotective effect, closely approximating the activity of clofibrate. Histopathological analysis further corroborated these findings, showing reduced hepatic necrosis, inflammation, and fatty degeneration in treated animals, particularly in those receiving the combination therapy

Table 19: Biochemical Parameters in Paracetamol-Induced Hepatotoxicity

Treatment	SGOT (IU/L ± SEM)	SGPT (IU/L ± SEM)	Direct Bilirubin (mg/dL ± SEM)	Total Bilirubin (mg/dL ± SEM)	Triglycerides (mg/dL ± SEM)
Control	45.2 ± 2.1	40.3 ± 1.9	0.3 ± 0.02	0.8 ± 0.04	90.5 ± 4.2
Paracetamol (3 g/kg)	180.7 ± 8.5	165.4 ± 7.8	1.5 ± 0.08	2.8 ± 0.12	150.3 ± 6.5
AAEE (300 mg/kg)	80.4 ± 3.8	75.6 ± 3.5	0.6 ± 0.03	1.2 ± 0.06	110.2 ± 5.0
EHEE (300 mg/kg)	78.9 ± 3.7	73.2 ± 3.4	0.5 ± 0.03	1.1 ± 0.05	108.7 ± 4.9
AAEE+EHEE (100+300 mg/kg)	65.3 ± 3.0	60.8 ± 2.8	0.4 ± 0.02	0.9 ± 0.04	95.4 ± 4.3
Clofibrate (100 mg/kg)	60.1 ± 2.7	55.7 ± 2.5	0.3 ± 0.02	0.8 ± 0.04	92.3 ± 4.1

4. Discussion

The findings show that AAEE and EHEE have strong hepatoprotective, gastroprotective, and antioxidant properties, and that they work well together. With yields of 4.5–4.66%, ash values of 27.6–31.5%, and low moisture content (0.56–0.71%), the standardization criteria validated the extracts' quality and consistency, guaranteeing dependability for pharmacological investigations.

4.1 Phytochemical Profile and FTIR Analysis

Alkaloids, glycosides, saponins, carbohydrates, flavonoids, tannins, and phenolic chemicals were found in both extracts using qualitative phytochemical screening, which is in line with their ethnomedical use (Harborne, 1998). The existence of functional groups linked to these phytochemicals was confirmed by the complete molecular profile obtained from FTIR spectroscopy. The outcomes of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and hydrogen peroxide (H_2O_2) radical scavenging assays suggest that hydroxyl functional groups—identified at 3410 cm^{-1} in *Achyranthes aspera* ethanolic extract (AAEE) and 3350 cm^{-1} in *Euphorbia hirta* ethanolic extract (EHEE)—play a crucial role in antioxidant activity. These groups facilitate hydrogen atom donation, thereby aiding in the neutralization of reactive oxygen species (ROS), as reported by Rice-Evans et al. (1996). These peaks' wide character indicates hydrogen bonding, which improves the stability and effectiveness of flavonoids such as luteolin in EHEE and quercetin and kaempferol in AAEE.

According to Kumar et al. (2019), the carbonyl groups (1715 cm^{-1} for AAEE and 1720 cm^{-1} for EHEE) indicate flavonoids, glycosides, and tannins, which are known to suppress lipid peroxidation and alter inflammatory pathways. By neutralizing NAPQI in the paracetamol model, the greater carbonyl peak in EHEE indicates a higher concentration of tannins or diterpenoids, which may enhance its hepatoprotective activity (Jaeschke et al., 2012). The gastroprotective benefits shown in the ethanol-induced ulcer model are supported by polyphenolic chemicals, which stabilize radicals and decrease inflammation, as confirmed by the aromatic C=C stretching (1610 cm^{-1} for AAEE, 1585 cm^{-1} for EHEE) (Borrelli & Izzo, 2000). Saponins are suggested by the glycosidic C-O linkages (1050 cm^{-1} for AAEE and 1045 cm^{-1} for EHEE), which may strengthen the stomach mucus barrier and lessen damage from ethanol. Terpenoids, which improve bioavailability and regulate inflammatory pathways, are shown by the aliphatic C-H stretching (2925–2850 cm^{-1}) and bending (1380–1375 cm^{-1}) peaks. The chemical composition and its pharmacological significance are validated by the FTIR findings, which are consistent with earlier research on Euphorbia hirta (Kumar et al., 2019) and Achyranthes aspera (Sharma et al., 2018).

4.2 Antioxidant Activity

The DPPH and H_2O_2 assays confirmed the potent antioxidant activity of both extracts. AAEE's lower IC50 in the DPPH assay (119.38 µg/mL vs. 154.33 µg/mL for ascorbic acid) indicates a higher content of electron-donating compounds, likely flavonoids and phenolic acids, as supported by the strong O-H peak at 3410 cm^{-1}. EHEE's comparable performance in the H_2O_2 assay (126.05 µg/mL vs. 125.85 µg/mL) suggests efficacy against peroxides, which are critical in oxidative stress-related damage (Halliwell & Gutteridge, 1999). The FTIR-identified hydroxyl and carbonyl groups facilitate radical scavenging and metal chelation, key mechanisms for antioxidant activity.

4.3 Gastroprotective Activity

AAEE, EHEE, and their combination substantially decreased ulcer index, gastric juice volume, and acidity while raising pH ($p<0.01$) in the ethanol-induced ulcer model. Synergistic effects were shown by the AAEE+EHEE combination's protection index of 64.10%, which was close to that of omeprazole (68.95%). Saponins strengthen the stomach mucus barrier, tannins inhibit acid output via their astringent qualities, and flavonoids probably scavenge ROS caused by ethanol (Borrelli & Izzo, 2000). Glycosidic linkages detected by FTIR facilitate the presence of saponins, which improve mucosal defense.

4.4 Hepatoprotective Activity

The extracts substantially decreased increased SGOT, SGPT, bilirubin, and triglyceride levels ($p<0.01$) in the paracetamol-induced hepatotoxicity model, with the AAEE+EHEE combination exhibiting near-normal values. Reduced necrosis,

inflammation, and fatty degeneration were verified by histopathological investigation, especially in the combo group. The FTIR-identified carbonyl and hydroxyl groups support the hepatoprotective effects, which are ascribed to flavonoids and phenolic substances neutralizing NAPQI and increasing glutathione levels (Jaeschke et al., 2012). By preventing lipid peroxidation, the greater carbonyl peak in EHEE indicates a larger tannin concentration, which might increase its effectiveness.

4.5 Synergistic Effects

In both in vivo models, the AAEE+EHEE combination performed better than each extract alone, suggesting synergy. This might be the consequence of complementing phytochemical profiles, where the tannins in EHEE reduce inflammation while the flavonoids in AAEE boost antioxidant capacity. According to Wagner and Ulrich-Merzenich (2009), the FTIR findings indicate that the combination of hydroxyl, carbonyl, and glycosidic groups targets many pathways, such as ROS scavenging, membrane stability, and enzyme inhibition. The potential of polyherbal formulations to enhance therapeutic results is shown by the synergistic effects.

4.6 Implications and Future Directions

The research supports the traditional applications of Euphorbia hirta and Achyranthes aspera in medicine and highlights how well they work together to treat conditions linked to oxidative stress. Future study will be guided by the chemical foundation for their bioactivity provided by the FTIR analysis. Important instructions consist of:

1. Compound Isolation: To identify and measure active chemicals such as quercetin, oleanolic acid, or diterpenoid lactones, bioactivity-guided fractionation using HPLC-MS is used.
2. Mechanistic Studies: Using molecular docking and enzyme tests, researchers can clarify how flavonoids and tannins interact with inflammatory mediators like cyclooxygenase or antioxidant enzymes like superoxide dismutase.
3. Synergy Analysis: To measure the synergistic interactions between AAEE and EHEE, isobologram analysis or combination index computations are used.
4. Pharmacokinetic Studies: Use the solubility-enhancing glycosidic groups found by FTIR to assess absorption, distribution, metabolism, and excretion patterns in order to maximize therapeutic administration.
5. Clinical Trials: Create standardized polyherbal formulations for liver diseases and peptic ulcers by conducting safety and effectiveness trials in people.

5. Conclusion

Strong antioxidant, gastroprotective, and hepatoprotective properties are shown by the ethanolic leaf extracts of Achyranthes aspera and Euphorbia hirta, and their combined effects are synergistic. The hydroxyl, carbonyl, aromatic, glycosidic, and aliphatic groups that support the pharmacological actions of flavonoids, phenolic compounds, saponins, and terpenoids were validated by FTIR analysis. While in vivo investigations confirmed their therapeutic potential against ethanol-induced ulceration and paracetamol-induced hepatotoxicity, in vitro antioxidant tests (DPPH and H_2O_2) shown effectiveness on par with or better than ascorbic acid. The improved effectiveness of the AAEE+EHEE combination demonstrates the importance of polyherbal formulations in boosting efficacy via synergistic interactions. These discoveries provide the ethnomedical usage of these plants a scientific basis and open the door to the creation of natural treatments for illnesses linked to oxidative stress, which may find utility in the creation of phytopharmaceuticals.

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