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Phytochemical Screening, Antioxidant Activity, And Evaluation of Hepatoprotective Potential of a Polyherbal Formulation of Picrorhiza Kurroa, Phyllanthus Niruri, And Fermented Allium Sativum Against CCl_4 And Paracetamol-Induced Hepatotoxicity in Rats

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KEYWORDS

Picrorhiza kurroa

Allium sativum

Phyllanthus niruri

Carbon tetrachloride

Hepatoprotective activity

Marker enzymes

Paracetamol

ABSTRACT:

Background: Picrorhiza kurroa, Phyllanthus niruri, and fermented Allium sativum have been traditionally used for their hepatoprotective properties. However, their combined efficacy in a polyherbal solution (PHS) remains underexplored.

Methods: The extracts of the crude plant powder were screened for phytochemicals and antioxidant activity. A polyherbal solution (PHS) was formulated in a 1:1:1 ratio and evaluated for hepatoprotective efficacy in Wistar rats using carbon tetrachloride (CCl4) and paracetamol-induced hepatotoxicity models. Biochemical markers (SGOT, SGPT, ALP, DB, LDH) and histopathological analyses were performed. Silymarin (50 mg/kg) served as the standard.

Results: The polyherbal solution (PHS) comprising Picrorhiza kurroa, Phyllanthus niruri, and fermented Allium sativum exhibited significant hepatoprotective and antioxidant activity. Phytochemical screening confirmed the presence of key bioactives including flavonoids, phenols, tannins, and alkaloids. Quantitative analysis showed high total phenolic (237.29 mg GAE/g) and flavonoid content (120.38 mg QE/g) in the combined extract. PHS demonstrated strong radical scavenging effects across DPPH, ABTS, NO, and SOD assays with low IC_{50} values, alongside robust iron-chelating and lipid peroxidation inhibition. Enzymatic assays showed enhanced catalase, peroxidase, and GSH levels, particularly in the high-dose group. Acute toxicity tests indicated PHS is safe up to 5000 mg/kg. In vivo, PHS significantly restored altered liver function biomarkers (ALP, AST, ALT, GGT, TBil, TP, ALB) and improved histopathology in both CCl_4 and PCM-induced hepatotoxicity, especially at 400 mg/kg, confirming a dose-dependent hepatoprotective effect.

Conclusion: The synergistic action of phytoconstituents in the polyherbal formulation confers strong antioxidant and hepatoprotective effects, supporting its potential as an effective therapeutic option.

1. Introduction

The liver is an extremely important organ that plays a crucial role in managing various body functions such as metabolism, secretion, and storage; damage from toxic agents can have serious consequences[1]. Despite great advances in liver research recently, liver issues are increasing, with jaundice and hepatitis being two key disorders that contribute to high mortality rates[2]. Many herbal remedies have been employed to both prevent and treat a range of diseases, including those affecting the liver[3]. Phytochemicals like diterpene lactones, triterpenes, alkaloids, carotenoids, saponins, flavonoids, and polyphenolic compounds have demonstrated liver-

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protecting effects in studies on cell cultures and animal models[4]. Herbal formulations made from several herbs contain a complex mix of chemicals, and these multi-component blends might help prevent or treat liver conditions thanks to their synergistic actions[5]. Picroside and kutkoside, the active compounds from the roots and rhizomes of Picrorhiza kurroa (commonly called "Kutki" or "Kutaki"), have long been used to treat liver disorders[6]. Phyllanthin, a potent hepatoprotective lignan found in Phyllanthus niruri (also known as "gale of the wind"), has been a well-established herbal remedy for jaundice and other liver diseases[7]. S-allyl cysteine, an important sulfur-containing compound present in fermented Allium sativum (Black garlic), has been shown to offer protection against acute liver injury[8]. Since these plants contain various active compounds of different natures, combining their extracts might deliver better liver protection than using them individually, which is why the mixture was tested for its protective effects against liver toxicity induced by carbon tetrachloride and paracetamol in rats.

2. Materials and methods

2.1 Collection of Crude plant powder

The crude plant powder of Picrorhiza kurroa (Scrophulariaceae) – Rhizomes, Phyllanthus niruri (Euphorbiaceae) – Leaves or Aerial parts, and Allium sativum (Amaryllidaceae) – Bulb or cloves, were procured from Yucca Enterprises Private Limited.

2.2 Extraction of plants

2.2.1 Picrorhiza kurroa

2 g of crude plant powder were extracted with methanol by sonication at 120 MHz and 35 ± 1°C for varying durations (20–40 minutes). Following filtration and solvent evaporation, the dried extract was obtained. The optimal extraction time was determined to be 36 minutes[9].

2.2.2 Phyllanthus niruri

50 g of crude plant powder was extracted with 500 ml of n-hexane using a Soxhlet apparatus for several hours. The extract was filtered, and solvent was evaporated under reduced pressure to obtain a dry residue[10].

2.2.3 Fermented Allium sativum

5 g of crude plant powder were extracted with 150 mL of 50% ethanol or distilled water at 60°C for 90 minutes. The extract was filtered and concentrated using a rotary vacuum evaporator at 50°C to preserve heat-sensitive bioactive compounds like S-Allyl Cysteine (SAC). The final extract was weighed to determine the extraction yield gravimetrically[11].

3. Chemicals and Reagents

Analytical grade chemicals were used in the study (Sigma Chemicals Co. U.S.A., Merck India Ltd., Mumbai and Loba Chemie Pvt. Ltd., Mumbai) - Methanol, n-Hexane, Ethanol (EtOH), Liquid paraffin, Carbon tetrachloride (CCl_4), Paracetamol (PCM), Ascorbic acid, Potassium persulfate, ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), DPPH (2,2-Diphenyl-1-picrylhydrazyl), Nitroblue tetrazolium (NBT), Sodium nitroprusside, Griess reagent, Hydrogen peroxide (H_2O_2), Thiobarbituric acid (TBA), Ferrous sulfate ($FeSO_4$), Ferrozine, EDTA (Ethylenediaminetetraacetic acid), TPTZ (2,4,6-tripyridyl-s-triazine), Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), Ammonium molybdate, Sodium phosphate, Sulfuric acid, DTNB (5,5′-Dithiobis(2-nitrobenzoic acid)), Gum acacia, Formalin (10% neutral buffered).

4. In Vitro Analysis

4.1 Qualitative Phytochemical Screening

The plants extracts underwent qualitative chemical testing to identify various phytochemicals present, following standard methodologies outlined by Harbone, Trease and Evans, and Sofowora [12–14].

4.2 Quantitative Estimation

Total phenolic and flavonoid contents were estimated using the Folin-Ciocalteu and aluminum chloride colorimetric methods, respectively, with results expressed as mg gallic acid equivalents (GAE/g) and mg quercetin equivalents (QE/g) based on standard calibration curves[15].

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4.3 Antioxidant Analysis of Plant Extracts

The antioxidant potential of Picrorhiza kurroa, Phyllanthus niruri, fermented Allium sativum, and their combined extract was assessed using twelve in vitro assays, categorized into radical scavenging, metal chelation, total antioxidant capacity (TAC), and enzymatic antioxidant activity to evaluate different antioxidant pathways comprehensively.

4.3.1 Radical Scavenging Activity Assays:

Radical scavenging assays evaluated the extracts' ability to neutralize reactive oxygen species (ROS) and reactive nitrogen species (RNS) and protect biomolecules.

a. DPPH (2,2-Diphenyl-1-picrylhydrazyl) Assay:

Free radical neutralization was measured at 517 nm using ascorbic acid as a standard; IC50 values were calculated [16].

b. ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) Assay:

Antioxidant activity was assessed via $ABTS^{\bullet+}$ decolorization at 734 nm; IC50 values were determined using ascorbic acid [17] .

c. SOD (Superoxide Dismutase) Mimetic Activity Assay:

Superoxide inhibition was assessed by the reduction of nitroblue tetrazolium (NBT) formazan at 562 nm under light exposure; IC50 values were calculated[16].

d. NO (Nitric Oxide) Scavenging Assay:

Nitric oxide neutralization was measured using the Griess reagent at 540 nm after sodium nitroprusside incubation; IC50 values were determined[16].

e. H_2O_2 (Hydrogen Peroxide) Scavenging Assay:

Hydrogen peroxide neutralization was measured at 230 nm after incubation with extracts; IC50 values were calculated [16].

f. LPO (Lipid Peroxidation Inhibition) Assay:

Lipid peroxidation inhibition was evaluated via malondialdehyde (MDA)-thiobarbituric acid (TBA) complex formation at 532 nm; results were compared against ascorbic acid[16].

4.3.2 Metal Chelating Activity:

Metal chelation assessed the extracts' ability to bind ferrous ions (Fe^{2+}), preventing the generation of harmful hydroxyl radicals (•OH) through the Fenton reaction.

a. Iron Chelating Assay:

The binding of ferrous ions to ferrozine was measured at 562 nm; EDTA (Ethylenediaminetetraacetic Acid) was used as a positive control[16].

4.3.3 Total Antioxidant Capacity (TAC) Assays:

Total Antioxidant Capacity (TAC) assays evaluated the overall antioxidant profile based on electron and hydrogen atom transfer mechanisms.

a. FRAP (Ferric Reducing Antioxidant Power) Assay:

Fe^{3+} was reduced to Fe^{2+}-TPTZ (2,4,6-Tris(2-pyridyl)-S-triazine) complex, which was measured at 593 nm; absorbance was compared with ascorbic acid standards[16].

b. Phosphomolybdenum Assay:

Molybdenum (VI) (Mo(VI)) was reduced to Molybdenum (V) (Mo(V)) in the presence of antioxidants, forming a green complex measured at 695 nm; IC50 values were determined[18].

4.3.4 Enzymatic Antioxidant Activity Evaluations:

Enzymatic antioxidant activity evaluated the modulation of key antioxidant enzymes.

a. Catalase (CAT) Activity Evaluation:

Catalase decomposes hydrogen peroxide (H_2O_2) into oxygen and water, preventing hydroxyl radical formation. This activity was monitored at 240 nm; one unit of catalase decomposes 1 µmole of H_2O_2 per minute at pH 7.0 and 25 °C[19].

b. Peroxidase (POD) Evaluation:

Peroxidase promotes the oxidation of pyrogallol using hydrogen peroxide (H_2O_2) as an electron acceptor, forming a colored compound measured at 420 nm; one enzyme unit produces 1.0 mg of purpurogallin in 20 s[19].

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c. Reduced Glutathione (GSH) Content Evaluation:

The GSH levels were determined using DTNB (5,5′-Dithiobis (2-nitrobenzoic acid)), with absorbance measured at 412 nm. Results were expressed as µM GSH/g extract[20].

4.4 Formulation of Polyherbal Solution (PHS)

A total of 100 mg of combined plant extracts (Picrorhiza kurroa, Phyllanthus niruri, and fermented Allium sativum) in a 1:1:1 ratio was weighed and transferred to a sterile beaker. Distilled water (50% of the final volume) was added, and the mixture was stirred until partially dissolved. Acacia gum (0.5% w/v) was gradually incorporated under continuous stirring to enhance viscosity, followed by the addition of citric acid (0.05–0.1% w/v) to adjust the pH for better formulation stability. Honey (0.5% w/v) was used as a natural preservative. The mixture was then diluted to the final volume with distilled water. Homogenization was achieved by probe sonication at 40% amplitude for 10 minutes, with intermittent cooling to prevent thermal degradation and ensure uniform particle distribution. The final polyherbal solution (PHS) was transferred to a labeled container and stored at 4°C.

Table 1. Formulation of Polyherbal Solution of Combined Extract

Component	Quantity / Concentration	Function
Combined Plant Extract (*Picrorhiza kurroa*, *Phyllanthus niruri*, fermented *Allium sativum*)	100 mg per dose	Therapeutic agent for treatment
Acacia Gum	0.5% w/v	Viscosity Enhancer
Honey	0.5% w/v	Natural preservative
Citric Acid	0.05–0.1% w/v	pH adjuster and mild preservative
Distilled Water	q.s. to final volume	Solvent/Vehicle

4.5 Evaluation Parameters of Polyherbal Solutions (PHS)

The polyherbal formulations were assessed for a range of attributes, such as aesthetic properties, pH levels, viscosity, flow rate, particle size, and density[21].

4.6 In Vivo Study

4.6.1 Animals and Experimental Conditions

Adult Albino Wistar rats (Rattus norvegicus) aged 12 to 16 weeks, weighing 150–250 g, and of either sex, were obtained from the National Institute of Biosciences, Pune. The rats were acclimatized to laboratory conditions for one week before the experiment. They were housed under standard conditions (temperature 23 ± 2°C, 60–70% humidity, and a 12-hour light/dark cycle) and provided standard rodent pellet diet and water ad libitum. The rats were divided into groups (six rats per group) based on the experimental design. The protocol was approved by the Institutional Animal Ethics Committee (IAEC).

4.6.2 Acute Oral Toxicity

An acute toxicity test of the extract was conducted according to OECD 423 Guidelines. Prior to dosing, animals were fasted overnight, with water provided ad libitum. After fasting, the animals were weighed, and the test substance was administered. Food was withheld for 3 to 4 hours post-administration. Three animals were used for each phase of the study. The initial dose was selected from one of four predetermined levels: 50, 300, 2000 mg/kg body weight, within the limit dose of 5000 mg/kg body weight[22].

4.6.3 Selection of Doses

Acute oral toxicity of the Polyherbal Solution (PHS) was evaluated using the Acute Toxic Class Method,

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following OECD Guideline 423. No toxicity or mortality was observed at a dose of 5000 mg/kg, indicating that the LD_{50} of PHS is greater than 5000 mg/kg and confirming its safety for oral use. Based on this, doses were selected to assess the hepatoprotective efficacy of PHS. A high dose of 400 mg/kg (1/12.5th of the LD_{50}) was chosen, followed by a medium dose of 200 mg/kg (1/25th of the LD_{50}) and a low dose of 100 mg/kg (1/50th of the LD_{50}).

4.6.4 Method for Carbon Tetrachloride-Induced Hepatotoxicity

In this study, six groups of six rats each were used. The Polyherbal Solution (PHS) and standard drug (silymarin) were freshly prepared in gum acacia solution, while carbon tetrachloride (CCl_4) was prepared in liquid paraffin.

- **Group I** – Normal Control
- **Group II** – Disease (Negative) Control: CCl_4 (1 mL/kg)
- **Group III** – Standard (Positive) Control: Silymarin (50 mg/kg)
- **Group IV** – Test Low Dose: PHS (100 mg/kg)
- **Group V** – Test Medium Dose: PHS (200 mg/kg)
- **Group VI** – Test High Dose: PHS (400 mg/kg)

PHS and silymarin were administered once daily for three consecutive days. On day four, the respective treatments were repeated, followed two hours later by CCl_4 administration (except Group I). Six hours post-CCl_4, PHS and silymarin were re-administered. On day five, 24 hours after the last CCl_4 dose, blood was collected via the retro-orbital plexus under light anesthesia, and the animals were sacrificed. Liver tissues were excised, washed with ice-cold saline, blotted dry, and weighed. Body weight was recorded on day one and day five, and relative liver weight was calculated as the percentage of liver weight to body weight. A portion of liver tissue was preserved in formalin for histopathological examination.

4.6.5 Method for Paracetamol-Induced Hepatotoxicity

In this study, six groups of six rats each were used. The Polyherbal Solution (PHS) and standard drug (silymarin) were freshly prepared in gum acacia solution, while paracetamol was prepared in distilled water.

- **Group I** – Normal Control: Distilled water orally
- **Group II** – Disease (Negative) Control: Paracetamol (1000 mg/kg) orally
- **Group III** – Standard (Positive) Control: Silymarin (50 mg/kg) orally
- **Group IV** – Test Low Dose: PHS (100 mg/kg) orally
- **Group V** – Test Medium Dose: PHS (200 mg/kg) orally
- **Group VI** – Test High Dose: PHS (400 mg/kg) orally

PHS and silymarin were administered once daily for three consecutive days. On day four, the respective treatments were repeated, followed two hours later by paracetamol administration (except Group I). Six hours post-paracetamol, PHS and silymarin were re-administered. On day five, 24 hours after the last paracetamol dose, blood was collected via the retro-orbital plexus under light anesthesia, and the animals were sacrificed. Liver tissues were excised, washed with ice-cold saline, blotted dry, and weighed. Body weight was recorded on day one and day five, and relative liver weight was calculated as the percentage of liver weight to body weight. A portion of liver tissue was preserved in formalin for histopathological examination.

4.6.6 Assessment of liver function test

Liver function was evaluated using biochemical markers, including Alkaline Phosphatase (ALP), Serum Glutamate Pyruvate Transaminase (SGPT or ALT), Aspartate Aminotransferase (AST), Total Protein (TP), Total Bilirubin (TBil), Gamma-Glutamyl Transferase (GGT), and Albumin (ALB). Elevated levels of ALP, SGPT, AST, TBil, and GGT in toxin-treated groups indicated hepatocellular damage and bile flow obstruction, while reduced TP and ALB levels suggested impaired protein synthesis. Treatment with the Polyherbal Solution (PHS) significantly restored these parameters, demonstrating its hepatoprotective activity.

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4.6.7 Histopathological Examination

Liver slices were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 6 µm thickness. The sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope. Photomicrographs were captured, and tissue alterations such as fibrosis, fatty infiltration, centrilobular necrosis, and lymphocyte infiltration were evaluated[23].

4.7 Statistical analysis:

Data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test, using GraphPad Prism version 10.4.1. A p-value of < 0.05 was considered statistically significant.

5. Results

5.1 In Vitro Findings

5.1.1 Identification tests for Major constituents

Table 2: Phytochemical Screening of Plant Extracts

Types of Phytochemical Constituents	Extract of Picrorhiza kurroa	Extract of Phyllanthus niruri	Extract of Allium sativum
Alkaloids	-	+	+
Tannins	+	+	+
Cardiac Glycosides	+	-	-
Saponins	-	+	-
Flavonoids	+	+	+
Terpenoids	+	+	-
Carbohydrates	-	-	+
Proteins	-	-	+
Glycosides	+	-	+
Phenols	+	+	+
Steroids	+	-	-
Coumarins	+	+	-
Sterols	-	+	-

5.1.2 Quantitative Estimation

Table 3: The table displays the quantitative phytochemical analysis for Picrorhiza kurroa, Phyllanthus niruri, Fermented Allium sativum, and Combined Extract, highlighting Total Phenolic Content (TPC) in mg GAE/g and Total Flavonoid

Content (TFC) in mg QE/g according to standard calibration curves.

Quantitative Phytochemical Analysis	Plant Extracts	Results (mg/g)
1. **Total Phenolic Content (TPC):**	Picrorhiza kurroa	99.96 ± 5.34 mg GAE/g
	Phyllanthus niruri	98.29 ± 4.05 mg GAE/g

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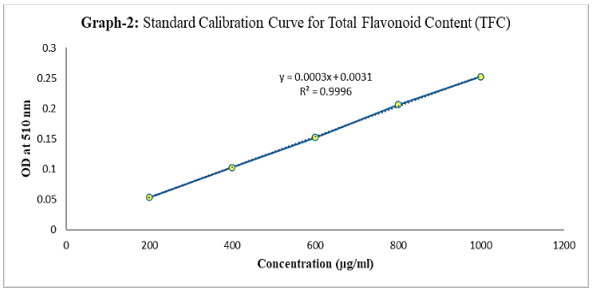
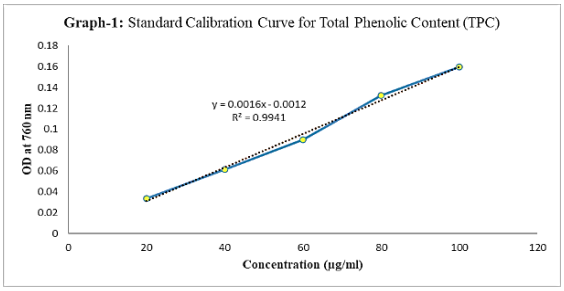
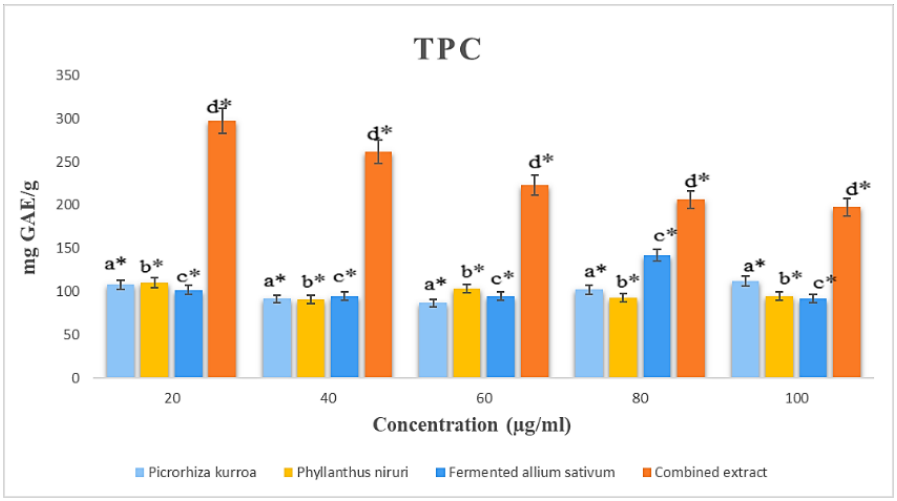
	Fermented Allium sativum	105.00 ± 7.01 mg GAE/g
	Combined Extract	237.29 ± 10.42 mg GAE/g
2. **Total Flavonoid Content (TFC):**	Picrorhiza kurroa	38.72 ± 5.12 mg QE/g
	Phyllanthus niruri	51.97 ± 5.24 mg QE/g
	Fermented Allium sativum	46.48 ± 3.71 mg QE/g
	Combined Extract	120.38 ± 9.91 mg QE/g





760nm				
Concentration (µg/ml)	**Picrorhiza kurroa**	**Phyllanthus niruri**	**Fermented Allium sativum**	**Combined extract**
20	107.92	110.00	101.67	297.5
40	91.46	90.94	94.58	261.77
60	86.67	103.33	94.65	223.13
80	101.98	92.86	142.08	206.41
100	111.79	94.29	92	197.63



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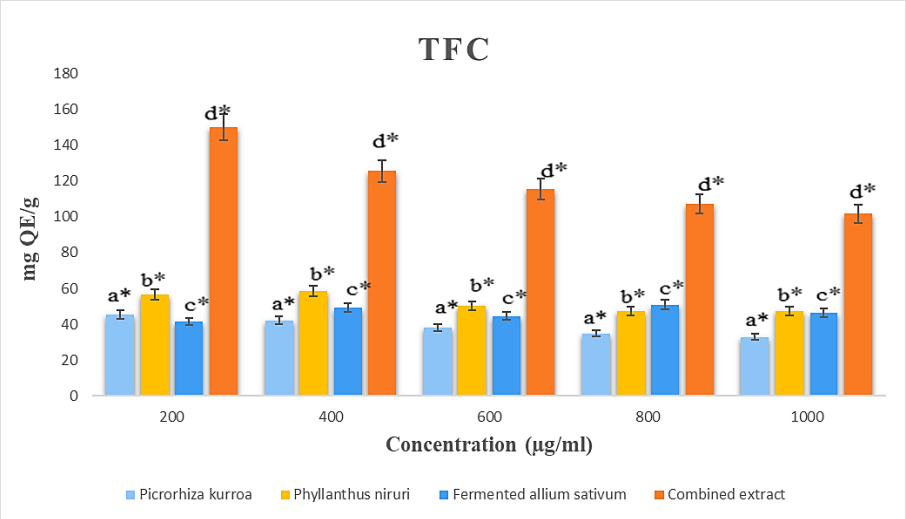
Total Phenolic Content (TPC) of the plant extracts Picrorhiza kurroa, Phyllanthus niruri, Fermented Allium sativum and Combined extract, expressed as milligrams of gallic acid equivalents (mg GAE) per gram of dry weight (mg/g). Bar graphs labeled with different letters (a*, b*, c*, d*) indicate significant differences between groups (P = 0.0068), while bars sharing the same letter marked with an asterisk (*) represent highly significant differences within groups (P < 0.0002).

510nm				
Concentration (µg/ml)	**Picrorhiza kurroa**	**Phyllanthus niruri**	**Fermented Allium sativum**	**Combined extract**
200	45.39	56.50	41.5	150.06
400	42.14	58.53	49.08	125.47
600	38.28	50.13	44.57	115.31
800	34.82	47.18	50.93	107.32
1000	32.97	47.52	46.3	101.74



Total Flavonoid Content (TPC) of the plant extracts Picrorhiza kurroa, Phyllanthus niruri, Fermented Allium sativum and Combined extract, expressed as milligrams of quercetin equivalents (mg QE) per gram of dry weight (mg/g). Bar graphs labeled with different letters (a*, b*, c*) indicate significant differences between groups (P = 0.0056), while bars sharing the same letter marked with an asterisk (*) represent highly significant differences within groups (P < 0.0003).

5.1.3 Radical Scavenging Activity Assays:

Table 4: The concentration of plant extracts (Picrorhiza kurroa, Phyllanthus niruri, Fermented Allium sativum) and Combined Extract required to inhibit 50% of free radical formation (IC_{50}).

Nature of Radical Species Generated	Test Sample	Half-Maximal Inhibitory Concentration (IC_{50}) of the Sample
1. DPPH radical species	Picrorhiza kurroa	39.85 ± 0.86 µg/ml

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	Phyllanthus niruri	30.74 ± 0.12 µg/ml
	Fermented Allium sativum	33.80 ± 0.17 µg/ml
	Ascorbic acid (Standard)	22.04 ± 0.11 µg/ml
	Combined Extract	14.15 ± 0.45 µg/ml
2. Nitric oxide radical species	Picrorhiza kurroa	30.65 ± 0.72 µg/ml
	Phyllanthus niruri	39.12 ± 0.72 µg/ml
	Fermented Allium sativum	31.94 ± 1.40 µg/ml
	Ascorbic acid (Standard)	50.00 ± 0.32 µg/ml
	Combined Extract	25.70 ± 0.76 µg/ml
3. ABTS radical species	Picrorhiza kurroa	22.73 ± 0.02 µg/ml
	Phyllanthus niruri	19.06 ± 0.01 µg/ml
	Fermented Allium sativum	21.02 ± 0.06 µg/ml
	Ascorbic acid (Standard)	30.45 ± 0.16 µg/ml
	Combined Extract	13.69 ± 0.03 µg/ml
4. Superoxide radical species	Picrorhiza kurroa	37.32 ± 0.16 µg/ml
	Phyllanthus niruri	30.97 ± 0.44 µg/ml
	Fermented Allium sativum	28.61 ± 0.16 µg/ml
	Ascorbic acid (Standard)	28.37 ± 0.10 µg/ml
	Combined Extract	15.89 ± 0.26 µg/ml
5. MDA species	Picrorhiza kurroa	27.88 ± 0.34 µg/ml
	Phyllanthus niruri	26.82 ± 0.18 µg/ml
	Fermented Allium sativum	28.73 ± 0.27 µg/ml
	Ascorbic acid (Standard)	30.59 ± 0.46 µg/ml
	Combined Extract	17.71 ± 0.24 µg/ml
6. Hydrogen peroxide radical species	Picrorhiza kurroa	19.03 ± 0.03 µg/ml
	Phyllanthus niruri	19.38 ± 0.11 µg/ml
	Fermented Allium sativum	24.05 ± 0.09 µg/ml
	Ascorbic acid (Standard)	25.41 ± 0.09 µg/ml
	Combined Extract	13.52 ± 0.13 µg/ml
7. Iron (II)-ferrozine complex species	Picrorhiza kurroa	28.64 ± 0.35 µg/ml
	Phyllanthus niruri	33.48 ± 0.49 µg/ml
	Fermented Allium sativum	35.16 ± 0.85 µg/ml
	EDTA (Standard)	34.24 ± 0.82 µg/ml
	Combined Extract	16.86 ± 0.16 µg/ml

Abbreviations: DPPH – 2,2-diphenyl-1-picrylhydrazyl; MDA – malondialdehyde; EDTA – ethylenediaminetetraacetic acid; ABTS – 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid).
Results are expressed as mean ± standard deviation (SD) for triplicate measurements (n = 3).

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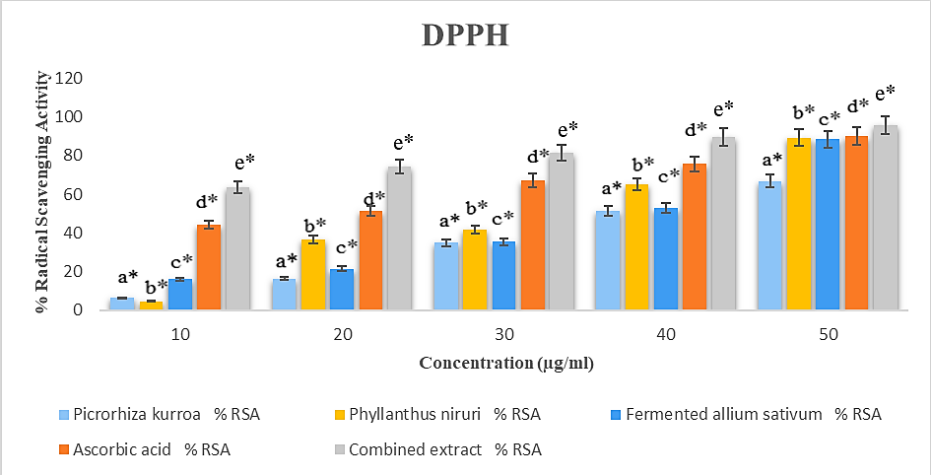
a. DPPH (2,2-Diphenyl-1-picrylhydrazyl) Assay:

517nm					
Concentration (µg/ml)	**Picrorhiza kurroa**	**Phyllanthus niruri**	**Fermented Allium sativum**	**Ascorbic acid**	**Combined extract**
	% RSA	**% RSA**	**% RSA**	**% RSA**	**% RSA**
10	6.38	4.66	15.98	44.31	63.71
20	16.28	36.57	21.49	51.21	74.37
30	34.84	41.87	35.35	67.22	81.38
40	51.57	65.13	52.97	75.78	89.69
50	66.89	89.4	88.54	90.07	95.8



In vitro 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was evaluated for Picrorhiza kurroa, Phyllanthus niruri, Fermented Allium sativum plant extract, Combined extract, and Ascorbic acid (Standard). Bar graphs labeled with different letters (a*, b*, c*, d*, e*) indicate significant differences between groups ($P = 0.0069$), while bars sharing the same letter marked with an asterisk (*) represent highly significant differences within groups ($P<0.0001$).

b. ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) Assay:

734nm					
Concentration (µg/ml)	**Picrorhiza kurroa**	**Phyllanthus niruri**	**Fermented Allium sativum**	**Ascorbic acid**	**Combined extract**
	%RSA	**%RSA**	**%RSA**	**%RSA**	**%RSA**
10	38.05	40.91	33.48	18.78	65.54

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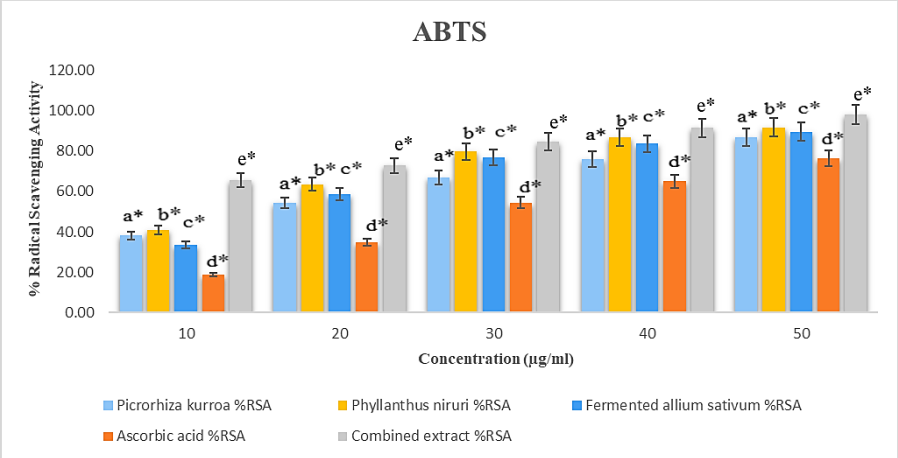
20	54.25	63.45	58.69	34.69	72.81
30	66.84	79.67	76.71	54.49	84.56
40	75.86	86.58	83.62	65.03	91.35
50	86.84	91.59	89.53	76.46	98.08



In vitro 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activity was evaluated for Picrorhiza kurroa, Phyllanthus niruri, Fermented Allium sativum plant extract, Combined extract, and Ascorbic acid (Standard). Bar graphs labeled with different letters (a*, b*, c*, d*, e*) indicate significant differences between groups (P = 0.0040), while bars sharing the same letter marked with an asterisk (*) represent highly significant differences within groups (P<0.0001).

c. SOD (Superoxide Dismutase) Mimetic Activity Assay:

562nm					
Concentration (µg/ml)	**Picrorhiza kurroa**	**Phyllanthus niruri**	**Fermented Allium sativum**	**Ascorbic acid**	**Combined extract**
	%RSA	**%RSA**	**%RSA**	**%RSA**	**%RSA**
10	13.58	19.03	17.34	15.31	61.91
20	25.4	33.77	35.48	35.3	67.87
30	43.1	50.24	51.86	51.65	76.73
40	54.75	66.14	71.81	70.54	85.27
50	64.87	75.85	85.76	90.58	93.58

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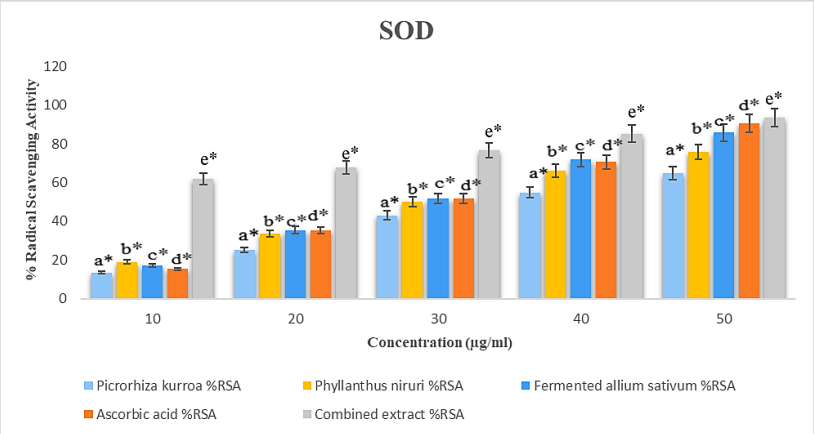
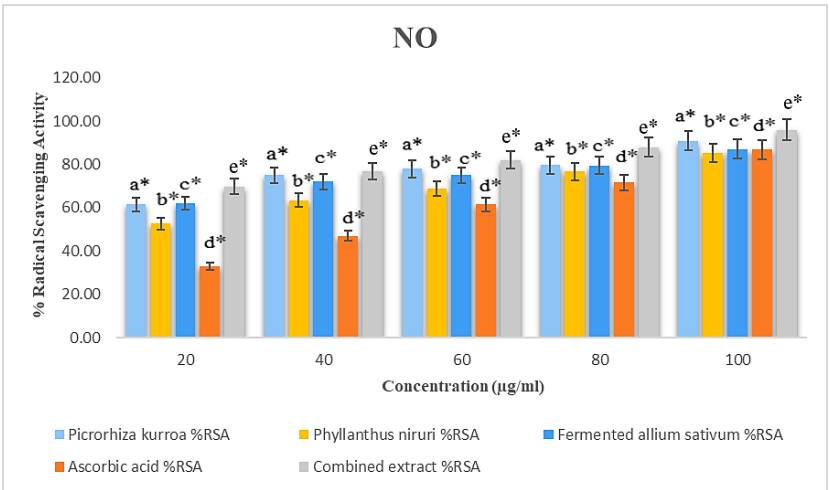


In vitro Superoxide Dismutase (SOD) radical scavenging activity was evaluated for Picrorhiza kurroa, Phyllanthus niruri, Fermented Allium sativum plant extract, Combined extract, and Ascorbic acid (Standard). Bar graphs labeled with different letters (a*, b*, c*, d*, e*) indicate significant differences between groups (P = 0.0069), while bars sharing the same letter marked with an asterisk (*) represent highly significant differences within groups (P<0.0001).

d. NO (Nitric Oxide) Scavenging Assay:

540nm					
Concentration (µg/ml)	**Picrorhiza kurroa**	**Phyllanthus niruri**	**Fermented Allium sativum**	**Ascorbic acid**	**Combined extract**
	%RSA	**%RSA**	**%RSA**	**%RSA**	**%RSA**
20	61.44	52.56	62.10	33.02	69.76
40	74.98	63.42	72.05	46.94	76.78
60	77.90	68.86	74.97	61.52	82.06
80	79.67	76.64	79.54	71.72	87.99
100	90.94	85.43	87.07	86.79	95.97



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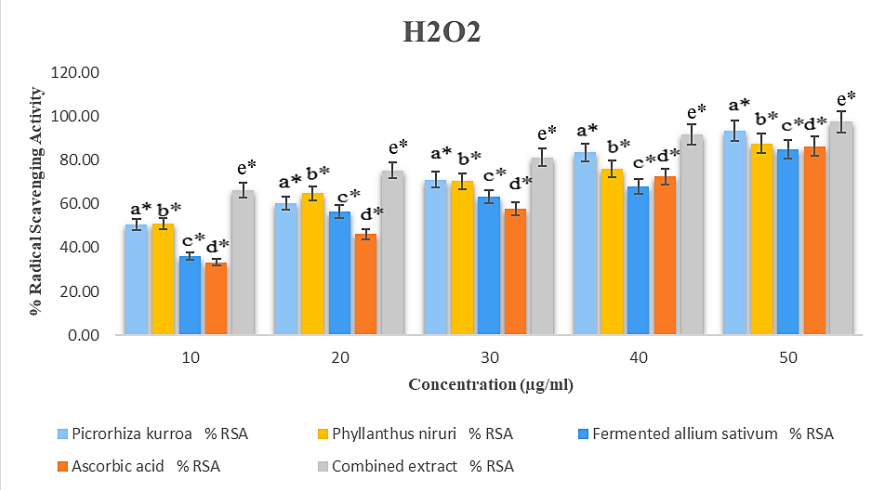
In vitro Nitric oxide (NO) radical scavenging activity was evaluated for Picrorhiza kurroa, Phyllanthus niruri, Fermented Allium sativum plant extract, Combined extract, and Ascorbic acid (Standard). Bar graphs labeled with different letters (a*, b*, c*, d*, e*) indicate significant differences between groups (P = 0.0207), while bars sharing the same letter marked with an asterisk (*) represent highly significant differences within groups (P<0.0001).

e. H_2O_2 (Hydrogen Peroxide) Scavenging Assay:

230nm					
Concentration (µg/ml)	**Picrorhiza kurroa**	**Phyllanthus niruri**	**Fermented Allium sativum**	**Ascorbic acid**	**Combined extract**
	% RSA	**% RSA**	**% RSA**	**% RSA**	**% RSA**
10	50.72	51.12	36.10	33.33	66.23
20	60.38	64.79	56.51	46.10	75.35
30	71.02	70.28	63.28	57.82	81.3
40	83.47	76.18	67.79	72.50	91.8
50	93.22	87.56	84.94	86.33	97.5



In vitro Hydrogen Peroxide (H_2O_2) radical scavenging activity was evaluated for Picrorhiza kurroa, Phyllanthus niruri, Fermented Allium sativum plant extract, Combined extract, and Ascorbic acid (Standard). Bar graphs labeled with different letters (a*, b*, c*, d*, e*) indicate significant differences between groups (P = 0.0054), while bars sharing the same letter marked with an asterisk (*) represent highly significant differences within groups (P<0.0001).

f. LPO (Lipid Peroxidation Inhibition) Assay:

532nm					
Concentration (µg/ml)	**Picrorhiza kurroa**	**Phyllanthus niruri**	**Fermented Allium sativum**	**Ascorbic acid**	**Combined extract**
	% LPO	**% LPO**	**% LPO**	**% LPO**	**% LPO**

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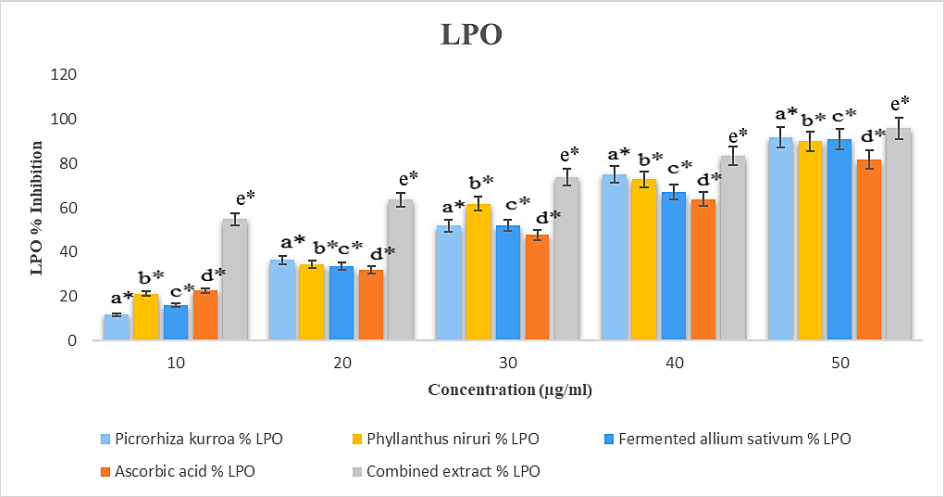
10	11.7	21.09	16.1	22.6	54.98
20	36.42	34.39	33.71	32.06	63.7
30	51.95	61.86	52.01	47.68	73.77
40	75.09	72.82	66.98	63.87	83.56
50	91.95	90.04	91.17	81.73	95.92



In vitro Lipid Peroxidation Inhibition (LPO) radical scavenging activity was evaluated for Picrorhiza kurroa, Phyllanthus niruri, Fermented Allium sativum plant extract, Combined extract, and Ascorbic acid (Standard). Bar graphs labeled with different letters (a*, b*, c*, d*, e*) indicate significant differences between groups (P = 0.0179), while bars sharing the same letter marked with an asterisk (*) represent highly significant differences within groups (P<0.0001).

2. Metal Chelating Activity:

a. Iron Chelating Assay:

562nm					
Concentration (µg/ml)	**Picrorhiza kurroa**	**Phyllanthus niruri**	**Fermented Allium sativum**	**Edta**	**Combined extract**
	% IRON	**% IRON**	**% IRON**	**% IRON**	**% IRON**
10	32.51	35.43	33.32	19.34	59.3
20	49.53	44.54	45.34	30.58	66.75
30	59.91	62.84	59.61	47.41	74.33
40	77.06	72.52	70.48	56.71	81.24
50	90.48	87.65	86.36	70.16	94.45

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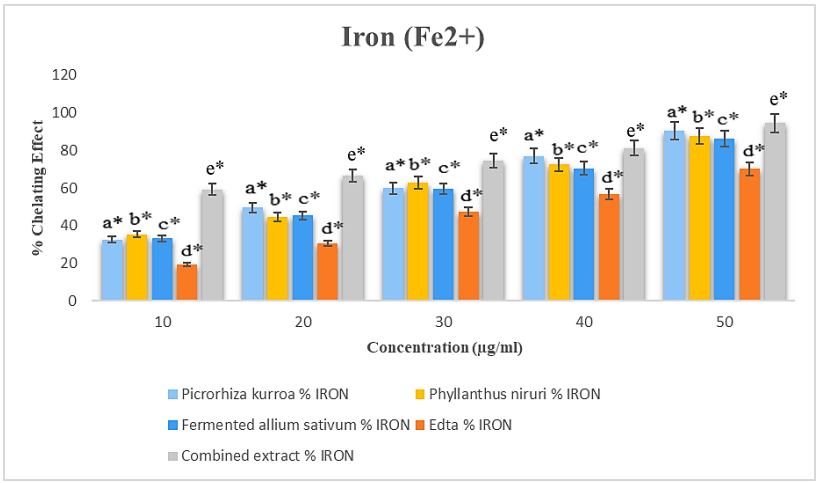

In vitro Iron (Fe2+) Chelating Assay was evaluated for Picrorhiza kurroa, Phyllanthus niruri, Fermented Allium sativum plant extract, Combined extract, and EDTA (Standard). Bar graphs labeled with different letters (a*, b*, c*, d*, e*) indicate significant differences between groups (P = 0.0033), while bars sharing the same letter marked with an asterisk (*) represent highly significant differences within groups (P<0.0001).

3. Total Antioxidant Capacity (TAC) Assays:

Table 5: The IC_{50} values for Total Antioxidant Capacity (TAC) represent the concentration of plant extracts (Picrorhiza kurroa, Phyllanthus niruri, Fermented Allium sativum) and Combined Extract required to achieve 50% inhibition of TAC activity.

Total Antioxidant Capacity (TAC) Activity	Test Sample	Half-Maximal Inhibitory Concentration (IC_{50}) of the Sample
1. FRAP Activity	Picrorhiza kurroa	212.42 ± 1.91 µg/ml
	Phyllanthus niruri	241.56 ± 0.94 µg/ml
	Fermented Allium sativum	277.84 ± 2.84 µg/ml
	Ascorbic acid (Standard)	324.59 ± 3.69 µg/ml
	Combined Extract	151.00 ± 0.61 µg/ml
2. Phosphomolybdenum Activity	Picrorhiza kurroa	59.44 ± 0.80 µg/ml
	Phyllanthus niruri	48.98 ± 1.21 µg/ml
	Fermented Allium sativum	54.25 ± 0.73 µg/ml
	Ascorbic acid (Standard)	57.10 ± 0.41 µg/ml
	Combined Extract	21.38 ± 0.34 µg/ml

Abbreviations: FRAP – Ferric reducing antioxidant power.
Results are expressed as mean ± standard deviation (SD) for triplicate measurements (n = 3).

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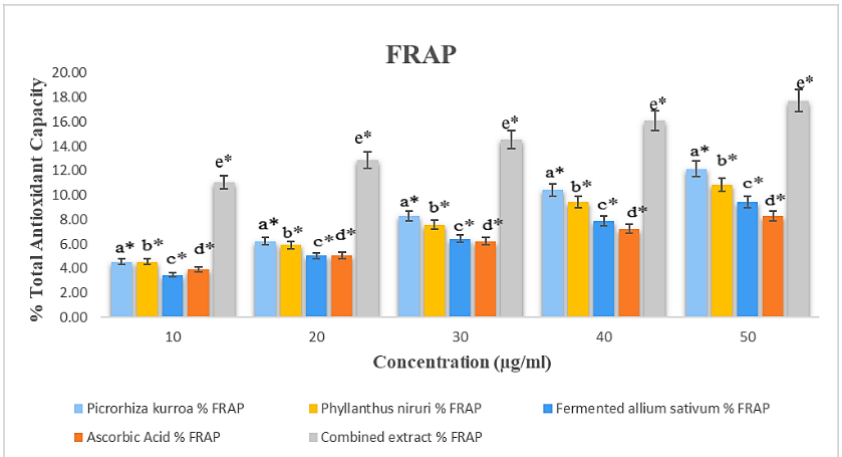
a. FRAP (Ferric Reducing Antioxidant Power) Assay:

593nm					
Concentration (µg/ml)	**Picrorhiza kurroa**	**Phyllanthus niruri**	**Fermented Allium sativum**	**Ascorbic Acid**	**Combined extract**
	% FRAP	**% FRAP**	**% FRAP**	**% FRAP**	**% FRAP**
10	4.54	4.56	3.47	3.95	11.06
20	6.22	5.93	5.04	5.05	12.86
30	8.28	7.57	6.44	6.23	14.53
40	10.42	9.40	7.87	7.23	16.1
50	12.15	10.86	9.44	8.28	17.73



In vitro Ferric Reducing Antioxidant Power (FRAP) Assay was evaluated for Picrorhiza kurroa, Phyllanthus niruri, Fermented Allium sativum plant extract, Combined extract, and Ascorbic acid (Standard). Bar graphs labeled with different letters (a*, b*, c*, d*, e*) indicate significant differences between groups (P = 0.0029), while bars sharing the same letter marked with an asterisk (*) represent highly significant differences within groups (P<0.0001).

b. Phosphomolybdenum Assay:

695nm					
Concentration (µg/ml)	**Picrorhiza kurroa**	**Phyllanthus niruri**	**Fermented Allium sativum**	**Ascorbic Acid**	**Combined extract**
	% TAC	**% TAC**	**% TAC**	**% TAC**	**% TAC**
20	20.51	36.50	26.89	26.23	61.09
40	41.14	54.21	46.30	46.11	70.89
60	52.79	64.78	61.33	57.94	76.77
80	66.06	71.48	68.36	66.04	80.73
100	76.69	77.69	77.92	73.61	85.56

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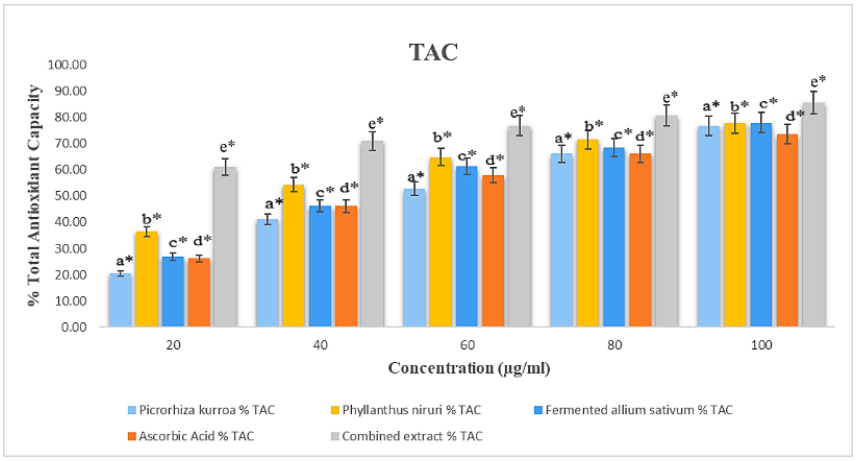

In vitro Phosphomolybdenum (TAC) Assay was evaluated for Picrorhiza kurroa, Phyllanthus niruri, Fermented Allium sativum plant extract, Combined extract, and Ascorbic acid (Standard). Bar graphs labeled with different letters (a*, b*, c*, d*, e*) indicate significant differences between groups (P = 0.0192), while bars sharing the same letter marked with an asterisk (*) represent highly significant differences within groups (P<0.0001).

4. Enzymatic Antioxidant Activity Assays:

Table 6: The % Units/mg of antioxidant enzyme activities reflect the concentration of plant extracts (Picrorhiza kurroa, Phyllanthus niruri, Fermented Allium sativum) and Combined Extract required to express enzymatic activity as a percentage of antioxidant units per milligram.

Enzymatic Antioxidant Activity	Test Sample	% Units/mg
1. Catalase Assay	Picrorhiza kurroa	40.58 ± 0.53 Units/mg
	Phyllanthus niruri	34.51 ± 0.51 Units/mg
	Fermented Allium sativum	38.68 ± 0.77 Units/mg
	Catalase	34.17 ± 2.29 Units/mg
	Combined Extract	74.63 ± 6.78 Units/mg
2. Peroxidase Assay	Picrorhiza kurroa	38.37 ± 0.91 Units/mg
	Phyllanthus niruri	35.06 ± 0.22 Units/mg
	Fermented Allium sativum	33.92 ± 0.43 Units/mg
	Peroxidase	33.26 ± 0.37 Units/mg
	Combined Extract	77.19 ± 1.47 Units/mg

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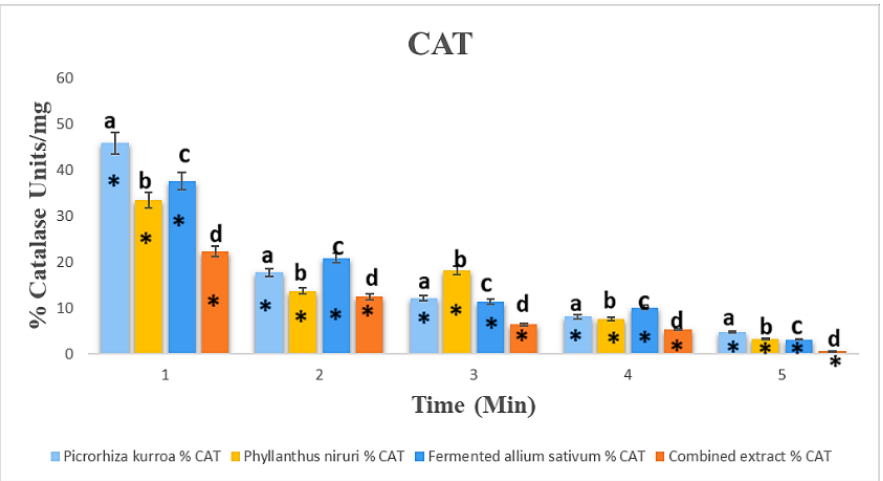
Enzyme activity units were defined as follows: POD (Peroxidase): One unit of enzyme activity corresponds to a 0.001 change in absorbance per minute under standard assay conditions. CAT (Catalase): One unit of catalase activity is defined as the amount of enzyme that induces (Units/mg).

a 0.001 absorbance change per minute under assay conditions. Results are expressed as mean ± standard error of the mean (SEM) in % enzymatic antioxidant activity

a. CAT (Catalase) Activity Assay:

240nm				
Time (in Min)	**Picrorhiza kurroa**	**Phyllanthus niruri**	**Fermented Allium sativum**	**Combined extract**
	% CAT	**% CAT**	**% CAT**	**% CAT**
1	45.91	33.44	37.68	22.38
2	17.75	13.7	20.9	12.47
3	12.09	18.13	11.45	6.39
4	8.07	7.68	10.1	5.34
5	4.73	3.29	3.14	0.59



In vitro Catalase (CAT) Activity Assay was evaluated for Picrorhiza kurroa, Phyllanthus niruri, Fermented Allium sativum plant extract, and Combined extract. Bar graphs labeled with different letters (a, b, c, d) indicate not significant differences between groups (P = 0.0870), while bars sharing the same letter marked with an asterisk (*) represent highly significant differences within groups (P<0.0001).

b. Peroxidase (POD) Assay:

420nm				
Time (in Min)	**Picrorhiza kurroa**	**Phyllanthus niruri**	**Fermented Allium sativum**	**Combined extract**
	% POD	**% POD**	**% POD**	**% POD**
1	1.04	0.98	1.82	0.18
2	2.8	2.28	3.37	0.79

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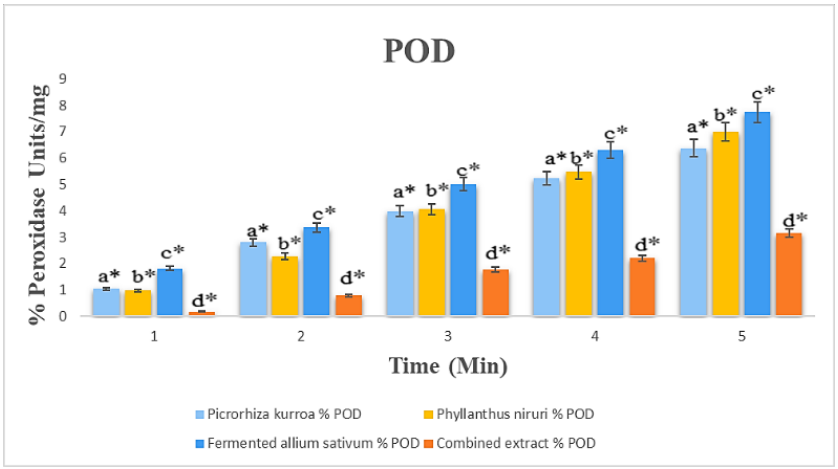
3	3.99	4.07	5.01	1.78
4	5.24	5.48	6.3	2.21
5	6.38	6.99	7.74	3.15



In vitro Peroxidase (POD) Activity Assay was evaluated for Picrorhiza kurroa, Phyllanthus niruri, Fermented Allium sativum plant extract, and Combined extract. Bar graphs labeled with different letters (a*, b*, c*, d*) indicate significant differences between groups (P = 0.0119), while bars sharing the same letter marked with an asterisk (*) represent highly significant differences within groups (P<0.0001).

c. GSH (Reduced Glutathione) Content Assay:

Table 7: The % Mean Reduced Glutathione content (µg/mL) represents the antioxidant activity of plant extracts (Picrorhiza kurroa, Phyllanthus niruri, Fermented Allium sativum) and Combined Extract, indicating the concentration required to achieve the observed GSH levels.

Reduced Glutathione Antioxidant Activity	Test Sample	% Mean Reduced Glutathione content (µg/ml)
Glutathione Assay	Picrorhiza kurroa	24.91 ± 0.05 µg/ml
	Phyllanthus niruri	25.14 ± 0.11 µg/ml
	Fermented Allium sativum	24.97 ± 0.06 µg/ml
	Oxidized Glutathione (GSSG)	25.70 ± 0.08 µg/ml
	Combined Extract	58.82 ± 0.47 µg/ml

Results are expressed as mean ± standard deviation (SD) for triplicate measurements (n = 3).

412nm				
Concentration (µg/ml)	**Picrorhiza kurroa**	**Phyllanthus niruri**	**Fermented Allium sativum**	**Combined extract**
	% GSH	**% GSH**	**% GSH**	**% GSH**
10	0.07	0.12	0.1	0.45
20	0.23	0.21	0.19	0.55
30	0.31	0.28	0.28	0.66
40	0.41	0.39	0.36	0.74

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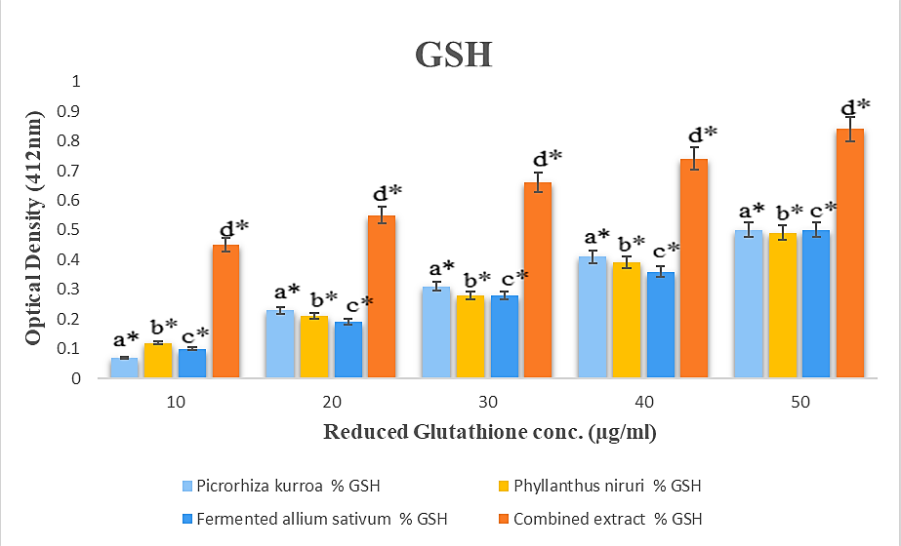
50	0.5	0.49	0.5	0.84



In vitro Reduced Glutathione (GSH) Content Assay was evaluated for Picrorhiza kurroa, Phyllanthus niruri, Fermented Allium sativum plant extract, and Combined extract. Bar graphs labeled with different letters (a*, b*, c*, d*) indicate significant differences between groups (P = 0.0034), while bars sharing the same letter marked with an asterisk (*) represent highly significant differences within groups (P<0.0001).

5.2 Evaluation of Polyherbal Solution (PHS)

5.2.1 Organoleptic Characteristics of Polyherbal Solutions (PHS)

The analysis revealed that the three formulations—PHF-A, PHF-B, and PHF-C—exhibited comparable morphological characteristics, including a reddish-black color, a mildly bitter taste with a distinct aroma, and a liquid consistency. These formulations correspond to different dose levels of the Polyherbal Solution (PHS): indicate that the formulation remained physically and chemically stable, confirming its suitability for long-term storage.

PHF-A represents the low dose (100 mg/kg), PHF-B the medium dose (200 mg/kg), and PHF-C the high dose (400 mg/kg), all administered orally.

5.2.2 Accelerated Stability studies

The polyherbal formulation demonstrated excellent stability over the three-month period under accelerated conditions. No significant changes were observed in viscosity, pH, Particle Size and Density. These findings

5.2.3 Physicochemical Characteristics of Polyherbal Solutions (PHS)

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Polyherbal Solutions (PHS)s (PHS) were formulated and assessed for various attributes, such as, pH, viscosity, and density as indicated.

Table 8: Physicochemical Evaluation of Polyherbal Solutions (PHS)

Formulation	pH	Viscosity (cP)	Flow rate (mL/sec)	Particle Size (µm)	Density
PHS-A	6.70	24	5/16	520.1	1.032
PHS-B	6.83	48	5/32	408.7	1.092
PHS-C	6.91	72	5/49	468.9	1.037

5.3 In vivo findings

5.3.1 Acute toxicity study

Acute toxicity was assessed at doses of 50 mg/kg, 300 mg/kg, 2000 mg/kg, and the limit dose of 5000 mg/kg, in accordance with OECD guidelines. No observable changes were noted in the skin, fur, eyes, or mucous membranes at any of the tested doses. Additionally, no signs of tremors, convulsions, salivation, diarrhea, lethargy, sleep disturbances, or coma were detected. Body weight remained unaffected throughout the study, and no mortality was recorded at any dose level.

5.3.2 Physiological Parameter:

1. CCl4 method

Table 9: Effect of Treatments on Body and Liver Weights in Normal, Disease (CCl_4-Induced), Standard, and Test Dose Groups

Treatment Groups and Dose	IBW (g)	FBW (g)	LW (g)	RLW (Liverwt./100 gb.wt.)
I: Normal	198.17 ± 3.35	200.83 ± 3.16	8.97 ± 0.13	4.47 ± 0.04
II: Disease (Negative Control)	203.33 ± 4.98	205 ± 4.46	9.16 ± 0.08	4.48 ± 0.06
III: Standard (Positive Control)	213.17 ± 8.32	214.50 ± 7.58	9.24 ± 0.08	4.33 ± 0.10
IV: Test Low Dose (100mg/kg)	212.33 ± 4.41	213.33 ± 4.87	9.17 ± 0.07	4.31 ± 0.06
V: Test Medium Dose (200mg/kg)	22.67 ± 6.48	224.67 ± 5.73	9.33 ± 0.06	4.16 ± 0.08
VI: Test High Dose (400mg/kg)	213.33 ± 3.63	214.83 ± 3.44	9.29 ± 0.04	4.33 ± 0.05

IBW – Initial Body Weight; FBW – Final Body Weight; LW – Liver Weight; RLW – Relative Liver Weight (Liver weight per 100 g of body weight)

2. PCM method:

Table 10: Effect of Treatments on Body and Liver Weights in Normal, Disease (PCM-Induced), Standard, and Test Dose Groups

Treatment groups and dose	IBW (g)	FBW (g)	LW (g)	RLW (Liverwt./100gb.wt.)
I: Normal	198.17 ± 3.35	200.83 ± 3.16	8.97 ± 0.13	4.47 ± 0.04
II: Disease (Negative Control)	212.17 ± 8.74	213.17 ± 8.20	9.23 ± 0.09	4.36 ± 0.12

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III: Standard (Positive Control)	228.50 ± 11.15	229.67 ± 10.95	9.27 ± 0.21	4.07 ± 0.11
IV: Test Low Dose (100mg/kg)	232.67 ± 12.92	233.83 ± 12.79	9.37 ± 0.12	4.06 ± 0.18
V: Test Medium Dose (200mg/kg)	250.67 ± 14.67	251.00 ± 13.87	9.51 ± 0.11	3.85 ± 0.18
VI: Test High Dose (400mg/kg)	240.33 ± 10.50	241.33 ± 10.70	9.49 ± 0.09	3.97 ± 0.14

IBW – Initial Body Weight; FBW – Final Body Weight; LW – Liver Weight; RLW – Relative Liver Weight (Liver weight per 100 g of body weight)

5.3.3 Biochemical Parameter:

1. **CCL4 method:**

Table 11: Effect of Normal, CCl_4-Induced Disease, Standard, and Polyherbal Test (100, 200, 400 mg/kg) Groups on Liver Function Biomarkers (ALP, PT, OT, TP, TBil, GGT, ALB)

Treatment and dose	ALP	PT	OT	TP	TBil	GGT	ALB
I: Normal	43.92 ± 5.74	71.62 ± 3.29	73.33 ± 2.49	6.12 ± 0.09	0.19 ± 0.03	2.49 ± 0.44	4.25 ± 0.22
II: Disease (Negative Control)	1129.93 ± 15.16	359.87 ± 4.44	547.56 ± 8.55	5.51 ± 0.14	0.39 ± 0.05	5.09 ± 0.47	3.05 ± 0.19
III: Standard (Positive Control)	502.72 ± 9.56	173.44 ± 3.74	325.21 ± 3.24	6.31 ± 0.11	0.24 ± 0.04	2.92 ± 0.74	3.96 ± 0.22
IV: Test Low Dose (100mg/kg)	635.84 ± 6.64	318.84 ± 4.06	432.08 ± 4.29	7.20 ± 0.09	0.32 ± 0.03	3.02 ± 0.44	3.85 ± 0.16
V: Test Medium Dose (200mg/kg)	514.25 ± 3.29	291.05 ± 6.62	331.53 ± 5.50	6.33 ± 0.07	0.24 ± 0.02	2.81 ± 0.47	3.94 ± 0.25
VI: Test High Dose (400mg/kg)	347.00 ± 13.68	230.05 ± 9.43	260.17 ± 4.37	6.22 ± 0.09	0.20 ± 0.03	2.44 ± 0.68	4.09± 0.25

2. **PCM Method:**

Table 12: Effect of Normal, PCM-Induced Disease, Standard, and Polyherbal Test (100, 200, 400 mg/kg) Groups on Liver Function Biomarkers (ALP, PT, OT, TP, TBil, GGT, ALB)

Treatment and dose	ALP	PT	OT	TP	TBil	GGT	ALB
I: Normal	43.92 ± 5.74	71.62 ± 3.29	73.33 ± 2.49	6.12 ± 0.09	0.19 ± 0.03	2.49 ± 0.44	4.25 ± 0.22
II: Disease (Negative Control)	953.76 ± 13.24	265.48 ± 14.97	463.04 ± 18.52	5.57 ± 0.16	0.44 ± 0.02	5.74 ± 0.09	3.11 ± 0.13
III: Standard (Positive Control)	476.50 ± 24.49	172.7 ± 19.75	275.16 ± 18.41	6.57 ± 0.12	0.26 ± 0.04	2.14 ± 0.75	4 ± 0.22
IV: Test Low Dose (100mg/kg)	740.53 ± 13.59	144.39 ± 14.19	338.12 ± 10.03	6.67 ± 0.05	0.28 ± 0.03	3.97 ± 0.3	3.66 ± 0.25

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V: Test Medium Dose (200mg/kg)	494.87 ± 5.07	141.64 ± 10.99	156.54 ± 7.41	6.59 ± 0.05	0.26 ± 0.03	3.29 ± 0.58	3.88 ± 0.18
VI: Test High Dose (400mg/kg)	430.98 ± 6.83	97.49 ± 4.41	85.08 ± 8.42	6.41 ± 0.08	0.22 ± 0.03	2.28 ± 0.31	4.07 ± 0.16

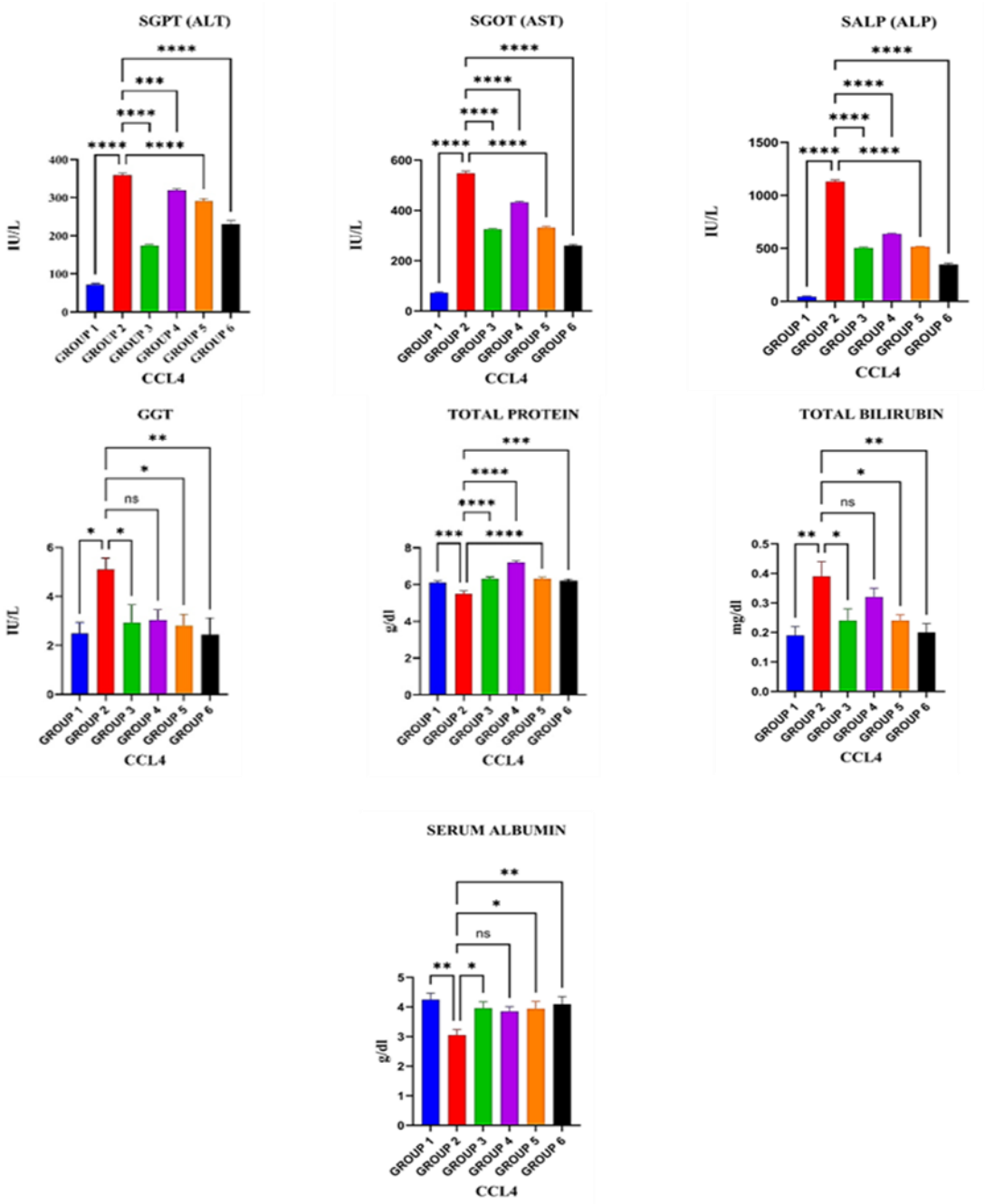

Figure 1. The hepatoprotective effect of a polyherbal solution (containing Picrorhiza kurroa, Phyllanthus niruri, and Fermented Allium sativum) was evaluated on liver biochemical markers in control and experimental rats. Values are expressed as Mean ± SEM. Statistical significance was determined by comparing all groups to the disease-induced

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(Negative Control) group, with results indicated as follows: *p < 0.05, **p < 0.01, ***p<0.001, ****p<0.0001. The experimental groups were: GROUP 1 – Normal Control; GROUP 2 – Negative Control (Carbon tetrachloride 1 mL/kg, disease-induced); GROUP 3 – Positive Control (Silymarin 50 mg/kg, standard); GROUP 4 – Test Low Dose (100 mg/kg polyherbal solution); GROUP 5 – Test Medium Dose (200 mg/kg polyherbal solution); GROUP 6 – Test High Dose (400 mg/kg polyherbal solution).

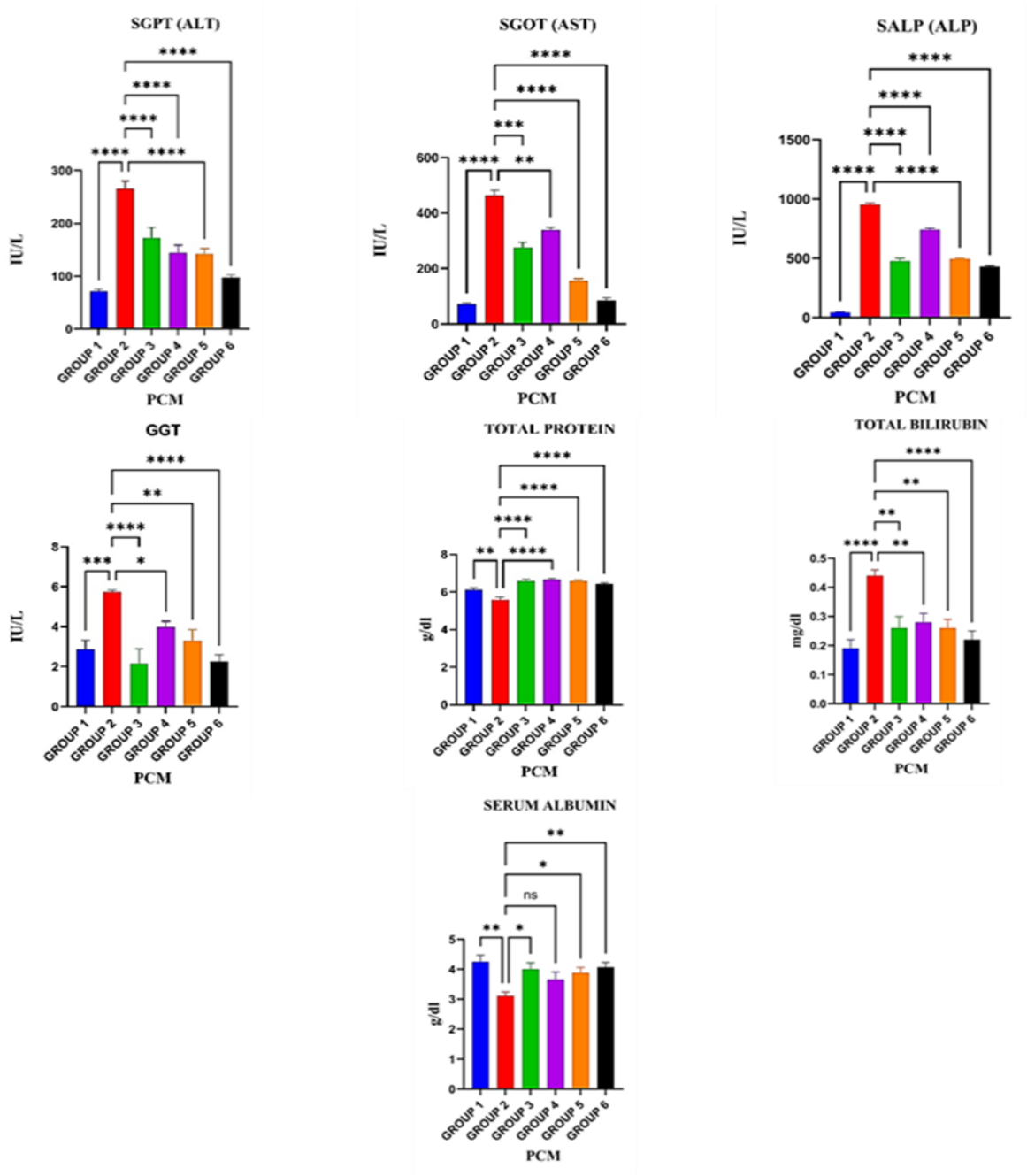

Figure 2. The hepatoprotective effect of a polyherbal solution (containing Picrorhiza kurroa, Phyllanthus niruri, and Fermented Allium sativum) was evaluated on liver biochemical markers in control and experimental rats. Values are expressed as Mean ± SEM. Statistical significance was determined by comparing all groups to the disease-induced (Negative Control) group, with results indicated as follows: *p < 0.05, **p < 0.01, ***p<0.001, ****p<0.0001. The

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experimental groups were: GROUP 1 – Normal Control; GROUP 2 – Negative Control (Carbon tetrachloride 1 mL/kg, disease-induced); GROUP 3 – Positive Control (Silymarin 50 mg/kg, standard); GROUP 4 – Test Low Dose (100 mg/kg polyherbal solution); GROUP 5 – Test Medium Dose (200 mg/kg polyherbal solution); GROUP 6 – Test High Dose (400 mg/kg polyherbal solution).

5.3.3 Histopathological Evaluation of Hepatic Tissues in CCl_4-Induced Hepatotoxicity Model

The histopathological examination of liver tissues from the normal control group revealed well-preserved hepatic architecture characterized by narrow sinusoids, radially arranged hepatic cords surrounding the central vein, and distinct, prominent nuclei. In contrast, the liver sections of rats administered carbon tetrachloride (1 mL/kg) exhibited extensive hepatic damage, including hepatocyte ballooning, macrovesicular fatty infiltration, and significant architectural distortion of the hepatic lobules. Rats treated with the standard hepatoprotective agent, silymarin (50 mg/kg), demonstrated marked histological improvement with largely preserved lobular architecture, reduced macrovesicular fatty infiltration, and minimal hepatocellular degeneration, indicating effective protection against CCl_4-induced hepatotoxicity. Administration of the polyherbal solution (PHS) at doses of 100 and 200 mg/kg resulted in mild amelioration of hepatic damage; however, the histopathological features were not substantially different from those observed in the CCl_4-treated group. Notably, rats treated with the high dose of PHS (400 mg/kg) exhibited significant histological improvements, including reduced cellular degeneration, minimal macrovesicular fatty infiltration, and near-normal hepatic architecture, suggesting a dose-dependent hepatoprotective effect.

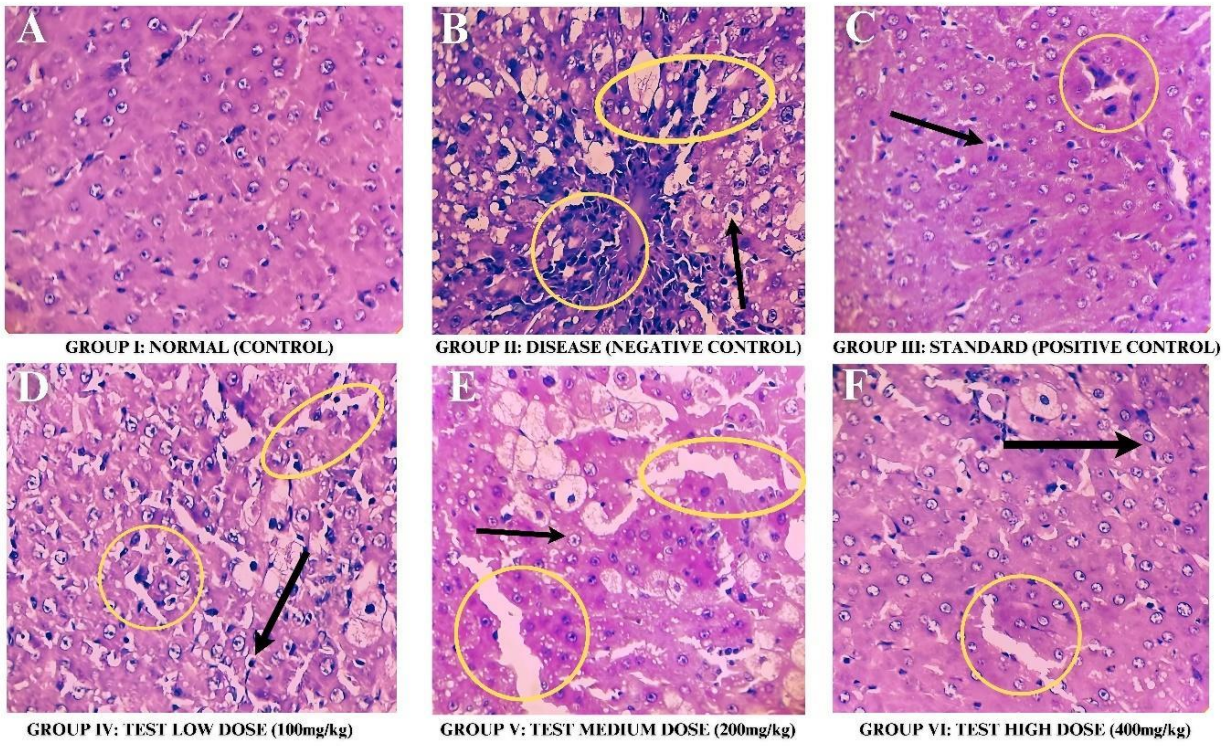

Figure 3: Histopathological analysis of liver tissues in control and experimental rat groups: (A) Normal Control Group: Exhibited well-defined hepatic architecture with vesicular nuclei; (B) Hepatotoxic Group: Displayed marked degenerative changes in hepatocytes along with necrosis and macrovesicular fatty infiltration; (C) Silymarin-Treated Group (50 mg/kg): Showed restoration of normal hepatic architecture with well-defined nuclei; (D) PHS-Treated Group (100 mg/kg): Revealed proliferative hepatocytes accompanied by degenerative changes and widened intercellular spaces; (E) PHS-

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Treated Group (200 mg/kg): Demonstrated proliferative hepatocytes with mild degenerative changes and increased intercellular space; (F) PHS-Treated Group (400 mg/kg): Presented normal hepatic architecture and well-defined nuclei, comparable to both the Normal Control (A) and Silymarin-Treated (C) groups

5.6 Histopathological Assessment of Liver Tissues in Paracetamol-Induced Hepatotoxicity Models

Histological examination of liver sections from the normal control group displayed intact hepatic architecture, with well-organized hepatic cords, narrow sinusoids, and centrally located, prominent nuclei. In contrast, the liver tissues of rats administered paracetamol (500 mg/kg) exhibited significant pathological changes, including widespread hepatocellular necrosis, vacuolar degeneration, and macrovesicular fatty infiltration, indicative of acute liver injury. Treatment with the standard reference compound, silymarin (50 mg/kg), resulted in notable histological improvement, as evidenced by diminished cellular damage and near-normal hepatic structure. Administration of the polyherbal solution (PHS) at 100 and 200 mg/kg led to only slight improvements in liver histology, which were not markedly different from the pathological features observed in the paracetamol-induced group. However, a substantial histological recovery was observed in rats treated with the high dose of PHS (400 mg/kg), characterized by reduced necrosis, minimal macrovesicular fatty infiltration, and restoration of normal hepatic architecture, highlighting a dose-dependent hepatoprotective effect.

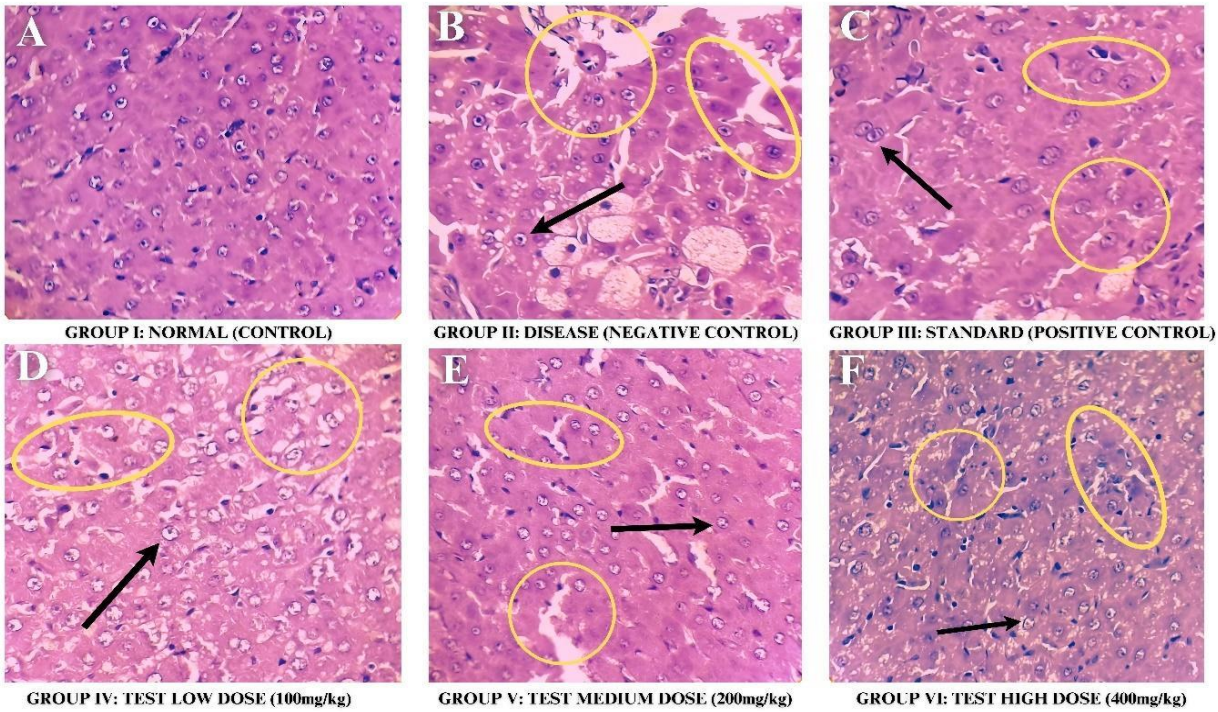

Figure 4: Histopathological analysis of liver tissues in control and experimental rat groups: (A) Normal Control Group: Exhibited well-defined hepatic architecture with vesicular nuclei; (B) Hepatotoxic Group: Displayed marked degenerative changes in hepatocytes along with necrosis and macrovesicular fatty infiltration; (C) Silymarin-Treated Group (50 mg/kg): Showed restoration of normal hepatic architecture with well-defined nuclei; (D) PHS-Treated Group (100 mg/kg): Revealed proliferative hepatocytes accompanied by degenerative changes and widened intercellular spaces; (E) PHS-Treated Group (200 mg/kg): Demonstrated proliferative hepatocytes with mild degenerative changes and increased intercellular space; (F) PHS-Treated Group (400 mg/kg): Presented normal hepatic architecture and well-defined nuclei, comparable to both the Normal Control (A) and Silymarin-Treated (C) groups.

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6. Discussion

Phytochemical screening of the extracts revealed the presence of several active constituents. Picrorhiza kurroa, Phyllanthus niruri, and Allium sativum all showed the presence of tannins, flavonoids, and phenols, indicating potential antioxidant activity. P. kurroa contained cardiac glycosides, terpenoids, glycosides, steroids, and coumarins. P. niruri tested positive for alkaloids, saponins, terpenoids, coumarins, and sterols. A. sativum exhibited alkaloids, carbohydrates, proteins, glycosides, and shared flavonoids and phenols with the others. Carbohydrates and proteins were uniquely present in A. sativum, while absent in the other extracts. The polyherbal formulation exhibited significant antioxidant potential across various in vitro assays. The Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) were highest in the combined extract of Picrorhiza kurroa, Phyllanthus niruri, and fermented Allium sativum, showing statistically significant differences between groups ($P = 0.0068$ for TPC and $P = 0.0056$ for TFC). Radical scavenging assays, including DPPH ($P = 0.0069$), ABTS ($P = 0.0040$), superoxide dismutase (SOD) ($P = 0.0069$), nitric oxide (NO) ($P = 0.0207$), hydrogen peroxide (H_2O_2) ($P = 0.0054$), and lipid peroxidation (LPO) inhibition ($P = 0.0179$), demonstrated that the combined extract exhibited significantly stronger scavenging activity compared to individual plant extracts, often approaching the efficacy of standards like ascorbic acid.

The iron chelation assay ($P = 0.0033$) and FRAP assay ($P = 0.0029$) confirmed the superior ferric reducing and metal chelating abilities of the combined extract. Total antioxidant capacity (TAC), evaluated by the phosphomolybdenum assay, also showed significant enhancement ($P = 0.0192$). Enzymatic antioxidant assays revealed significant differences in peroxidase (POD) ($P = 0.0119$) and reduced glutathione (GSH) content ($P = 0.0034$), while catalase (CAT) activity, though improved, was not statistically significant across groups ($P = 0.0870$).

Overall, the polyherbal combination displayed highly significant antioxidant and free radical scavenging properties ($P < 0.0001$ within groups), supporting a synergistic interaction among the constituents. These results suggest the combined extract's strong potential for therapeutic application, particularly in conditions involving oxidative stress and liver injury.

The polyherbal solution (PHS) exhibited uniform and acceptable organoleptic characteristics across all tested formulations (PHF-A, PHF-B, and PHF-C), including a reddish-black appearance, liquid consistency, a mildly bitter taste, and a distinct herbal aroma, indicating good sensory acceptance for oral use. Physicochemical evaluation revealed formulation-specific variations with pH values ranging from 6.70 to 6.91, viscosity from 24 to 72 cP, consistent flow rates and particle sizes, suggesting dose-dependent differences while maintaining overall formulation integrity. Accelerated stability studies conducted over three months showed no significant changes in pH, viscosity, particle size, or density, indicating excellent physical and chemical stability under stressed conditions. Furthermore, acute oral toxicity studies conducted as per OECD guidelines demonstrated no clinical signs of toxicity, behavioral abnormalities, or mortality at doses up to 5000 mg/kg, affirming the safety profile of the formulation for further pharmacological investigation.

The present study investigated the hepatoprotective activity of a polyherbal solution composed of Picrorhiza kurroa, Phyllanthus niruri, and Fermented Allium sativum against carbon tetrachloride (CCl_4)-induced hepatotoxicity in rats. In the disease-induced group (Negative Control), there was a significant elevation in liver biochemical markers, including SGOT, SGPT, ALP, and total bilirubin, compared to the Normal Control group ($p < 0.0001$), confirming severe hepatic damage. Treatment with the polyherbal solution at low (100 mg/kg), medium (200 mg/kg), and high (400 mg/kg) doses led to a significant, dose-dependent reduction in these elevated liver enzymes. At the highest dose (400 mg/kg), the polyherbal solution significantly normalized liver function markers compared to the Negative Control group ($p < 0.001$ to $p < 0.0001$), indicating a strong hepatoprotective effect. The medium dose (200 mg/kg) also showed a highly significant improvement ($p < 0.01$ to $p < 0.001$), while the low dose (100 mg/kg) showed a moderate but significant reduction ($p < 0.05$ to $p < 0.01$) in liver marker levels. The effects observed with the polyherbal solution were comparable to those seen with the standard hepatoprotective agent, silymarin (50 mg/kg), further suggesting the therapeutic

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potential of the formulation. The hepatoprotective action may be attributed to the combined antioxidant, anti-inflammatory, and membrane-stabilizing properties of the constituent herbs, particularly the fermented Allium sativum, which is known to enhance bioavailability and antioxidant potency. Overall, the polyherbal formulation demonstrated significant protection against CCl_4-induced hepatic injury, emphasizing its potential as a natural therapeutic alternative for managing liver disorders. Further mechanistic studies and clinical trials are warranted to substantiate and explore its efficacy in human populations.

7. Conclusion

The present investigation conclusively demonstrates the hepatoprotective efficacy of a carefully formulated polyherbal solution (PHS) comprising Picrorhiza kurroa, Phyllanthus niruri, and Fermented Allium sativum. Comprehensive preliminary phytochemical screening revealed a rich composition of bioactive phytoconstituents—including flavonoids, phenolics, saponins, and terpenoids—well known for their potent antioxidant and hepatoprotective properties. These compounds are believed to play a crucial role in neutralizing oxidative stress and restoring hepatic cellular integrity. The strong antioxidant potential of the PHS was confirmed through a series of twelve in vitro antioxidant assays, which collectively demonstrated remarkable free radical scavenging ability, metal ion chelation, and enhancement of endogenous enzymatic defenses. These findings highlight the robust antioxidative capacity of the formulation, which is essential for counteracting oxidative liver damage. Further validation through in vivo studies using rat models of carbon tetrachloride (CCl_4) and paracetamol-induced hepatotoxicity revealed statistically significant improvements in key biochemical indicators of liver function. Notably, reductions in serum ALT, AST, ALP, and total bilirubin levels, alongside increases in albumin concentrations, point to substantial hepatocellular protection. Histopathological observations further confirmed the biochemical outcomes, showing preserved liver architecture and reduced cellular necrosis, particularly at the highest tested dose (400 mg/kg body weight). The hepatoprotective efficacy observed at this high dose was comparable to that of silymarin, a well established reference drug, thus suggesting a dose-dependent therapeutic effect of the polyherbal formulation. The synergy between the selected herbal extracts appears to amplify their individual bioactivities, supporting the concept of polyherbal in traditional medicine. Collectively, the results not only validate the ethnomedicinal claims surrounding these herbs but also underscore the potential of their combined use as a natural, multi-target therapeutic approach for the prevention and management of liver disorders. Future studies involving molecular-level mechanisms and clinical validation may further establish the PHS as a promising hepatoprotective candidate.

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