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EVALUATION OF ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF POLYHERBAL FORMULATIONS IN A WISTAR RAT MODEL OF HEPATOCELLULAR CARCINOMA

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ABSTRACT

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related mortality worldwide, often associated with oxidative stress and chronic inflammation. This study aimed to evaluate the antioxidant and anti-inflammatory effects of polyherbal formulations composed of *Withania somnifera*, *Curcuma longa*, *Phyllanthus niruri*, and *Terminalia arjuna* in a Wistar rat model of HCC. Hepatocellular carcinoma was induced in rats using diethylnitrosamine (DEN). The rats were treated with the polyherbal formulation over a period of 8 weeks. Antioxidant activity was assessed through the measurement of enzymatic antioxidants including superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH), while lipid peroxidation (MDA) levels were used to evaluate oxidative damage. Anti-inflammatory activity was determined by quantifying pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β. Results showed that treatment with the polyherbal formulation significantly enhanced antioxidant enzyme levels and reduced MDA content, indicating attenuation of oxidative stress. Additionally, pro-inflammatory cytokines were markedly decreased, suggesting potent anti-inflammatory effects. Histopathological analysis further confirmed the protective effect of the polyherbal treatment on liver tissue integrity. These findings support the therapeutic potential of the studied polyherbal formulation as a natural antioxidant and anti-inflammatory agent in managing hepatocellular carcinoma.

KEYWORDS: Hepatocellular carcinoma, Polyherbal formulation, Antioxidant activity, Anti-inflammatory activity.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and a major cause of cancer-related deaths worldwide. The pathogenesis of HCC is complex and closely linked to chronic liver inflammation and oxidative stress, which contribute to cellular damage, DNA mutations, and tumor progression. Conventional treatments for HCC, such as chemotherapy and surgery, are often limited by side effects and low efficacy in advanced stages, highlighting the need for safer and more effective therapeutic alternatives.[1]

Natural products, especially polyherbal formulations, have gained significant attention due to their multi-targeted pharmacological properties, including antioxidant and anti-inflammatory effects. Several medicinal plants known for their hepatoprotective potential have been investigated for their ability to mitigate liver damage and carcinogenesis. The following plants were selected for the current study based on their established bioactivity.[2]

1. *Withania somnifera* (Ashwagandha)

- **Family:** Solanaceae

- **Common Names:** Ashwagandha, Indian Ginseng
- **Phytochemicals:** Withanolides, alkaloids, sitoindosides
- **Pharmacological Properties:** Adaptogenic, antioxidant, anti-inflammatory, immunomodulatory.
- **Relevance to HCC:** Withanolides may inhibit tumor growth and reduce oxidative stress in hepatic tissue.[3]

2. *Curcuma longa* (Turmeric)

- **Family:** Zingiberaceae
- **Common Names:** Turmeric, Haldi
- **Phytochemicals:** Curcumin, demethoxycurcumin, bisdemethoxycurcumin
- **Pharmacological Properties:** Potent antioxidant, anti-inflammatory, anticancer
- **Relevance to HCC:** Curcumin modulates inflammatory cytokines and inhibits carcinogenic signaling pathways.[4]

3. *Phyllanthus niruri* (Stonebreaker)

- **Family:** Phyllanthaceae
- **Common Names:** Bhumi Amla, Stonebreaker
- **Phytochemicals:** Lignans (phyllanthin, hypophyllanthin), flavonoids, alkaloids
- **Pharmacological Properties:** Hepatoprotective, antioxidant, antiviral, anti-inflammatory
- **Relevance to HCC:** Exhibits strong liver-protective effects and has shown anti-HBV properties, a major HCC risk factor.[5]

4. *Terminalia arjuna* (Arjuna)

- **Family:** Combretaceae
- **Common Names:** Arjuna
- **Phytochemicals:** Triterpenoids, flavonoids, tannins, glycosides
- **Pharmacological Properties:** Cardioprotective, antioxidant, anti-inflammatory
- **Relevance to HCC:** Its polyphenolic compounds help scavenge free radicals and reduce liver oxidative stress.[5]

MATERIALS AND METHODS

1. Plant Material Collection and Preparation

The medicinal plants *Withania somnifera* (roots), *Curcuma longa* (rhizomes), *Phyllanthus niruri* (whole plant), and *Terminalia arjuna* (bark) were collected from authenticated herbal suppliers and botanically identified by a certified taxonomist. The plant materials were washed, shade-dried, and pulverized into fine powders using a mechanical grinder.[6]

2. Preparation of Polyherbal Formulation

Equal quantities (w/w) of the dried powders were mixed to prepare the polyherbal formulation. The mixture was subjected to ethanol extraction (70% ethanol) using the Soxhlet extraction method for 8–10 hours. The extract was filtered, concentrated using a rotary evaporator, and dried under reduced pressure. The final extract was stored at 4°C until further use.[7]

$$\text{Extraction Yield (\%)} = \frac{\text{Weight of Dried Extract}}{\text{Initial Weight of Dried Powder}} \times 100$$

3. Experimental Animals

Adult male Wistar rats (150–180 g) were obtained from a certified animal house. The animals were housed in polypropylene cages under standard laboratory conditions (temperature: 22 ± 2°C, humidity: 50–60%, 12 h light/dark cycle) with free access to standard pellet diet and water. All experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC), and guidelines of the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) were strictly followed.[8]

4. Induction of Hepatocellular Carcinoma

HCC was induced by intraperitoneal injection of diethylnitrosamine (DEN) at a dose of 200 mg/kg body weight, administered as a single dose. This was followed by weekly administration of 2-acetylaminofluorene (2-AAF) at 30 mg/kg for 2 weeks to promote carcinogenesis.[9]

5. Experimental Design

The rats were randomly divided into five groups (n = 6 per group):

- **Group I**: Normal control (no DEN, no treatment)
- **Group II**: DEN control (DEN + vehicle)
- **Group III**: DEN + Polyherbal formulation (100 mg/kg b.wt)
- **Group IV**: DEN + Polyherbal formulation (200 mg/kg b.wt)
- **Group V**: DEN + Standard drug (e.g., silymarin 50 mg/kg b.wt)

Treatments were given orally once daily for 8 weeks after the induction of HCC.[10]

6. Biochemical Analysis

At the end of the treatment period, rats were sacrificed, and blood and liver tissues were collected. Serum was analyzed for liver function markers including ALT, AST, ALP, and total bilirubin. Liver tissue homogenates were used to estimate:

- **Antioxidant parameters**: Superoxide dismutase (SOD), Catalase (CAT), Glutathione (GSH), and Malondialdehyde (MDA)
- **Inflammatory cytokines**: Tumor necrosis factor-alpha (TNF-α), Interleukin-6 (IL-6), and Interleukin-1β (IL-1β) via ELISA kits

7. Histopathological Examination

Liver tissues were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Histopathological changes were observed under a light microscope to assess structural integrity and cellular abnormalities.[11]

8. Statistical Analysis

Results were expressed as mean ± standard deviation (SD). Data were analyzed using one-way ANOVA followed by Tukey's post hoc test. A p-value < 0.05 was considered statistically significant.[12]

RESULT AND DISSCUTION

1. Plant Material Collection and Preparation

Table no 1: Plant Material Collection and Preparation.

Plant Name	Plant Part Collected	Source	Identification Method	Preparation Steps
Withania somnifera	Roots	Authenticated herbal suppliers	Certified taxonomist	Washed → Shade-dried → Pulverized into fine powder
Curcuma longa	Rhizomes	Authenticated herbal suppliers	Certified taxonomist	Washed → Shade-dried → Pulverized into fine powder
Phyllanthus niruri	Whole plant	Authenticated herbal suppliers	Certified taxonomist	Washed → Shade-dried → Pulverized into fine powder
Terminalia arjuna	Bark	Authenticated herbal suppliers	Certified taxonomist	Washed → Shade-dried → Pulverized into fine powder

2. Preparation of Polyherbal Formulation

Table no. 2: Extraction Yield of Individual Plant Extracts.

Plant Name	Initial Dry Powder (g)	Extract Weight (g)	Extraction Yield (%)
Withania somnifera	25	5.2	20.8%
Curcuma longa	25	4.5	18.0%
Phyllanthus niruri	25	3.8	15.2%
Terminalia arjuna	25	4.1	16.4%
Total / Average	**100**	17.6	17.6% (avg)

3. Experimental Animals

Table no. 2: Experimental Animals.

Parameter	Details
Animal Species	Adult male Wistar rats
Weight Range	150–180 g
Source	Certified animal house
Housing	Polypropylene cages
Temperature	22 ± 2°C
Humidity	50–60%
Light/Dark Cycle	12 h light / 12 h dark
Diet	Standard pellet diet
Water	Provided ad libitum (free access)
Ethical Approval	Institutional Animal Ethics Committee (IAEC)
Guidelines Followed	CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals)

4. Induction of Hepatocellular Carcinoma

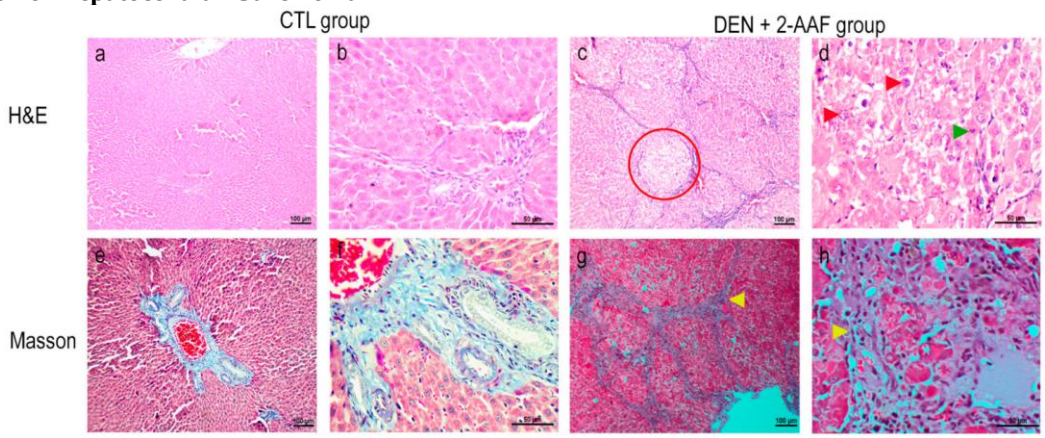

Figure 1: Histopathology_HCC_Polyherbal_Wistar.

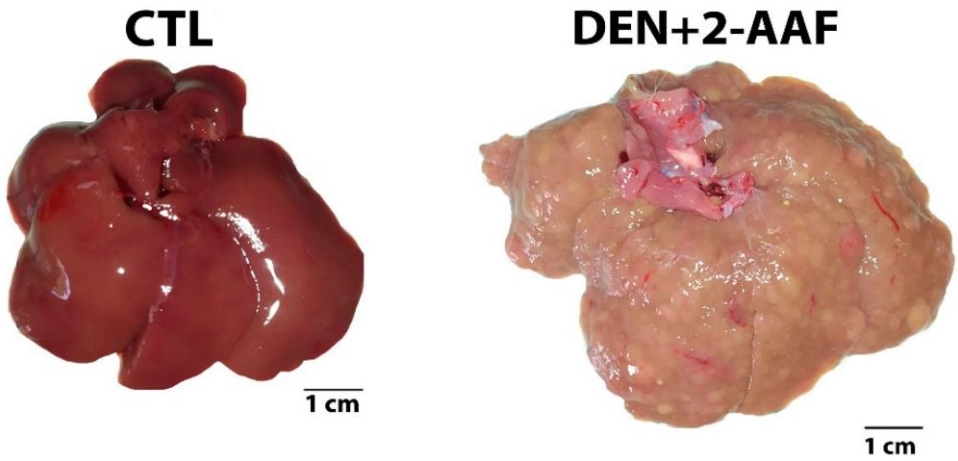

Figure 2: Liver section from the HCC **Control group (DEN + 2-AAF)**.

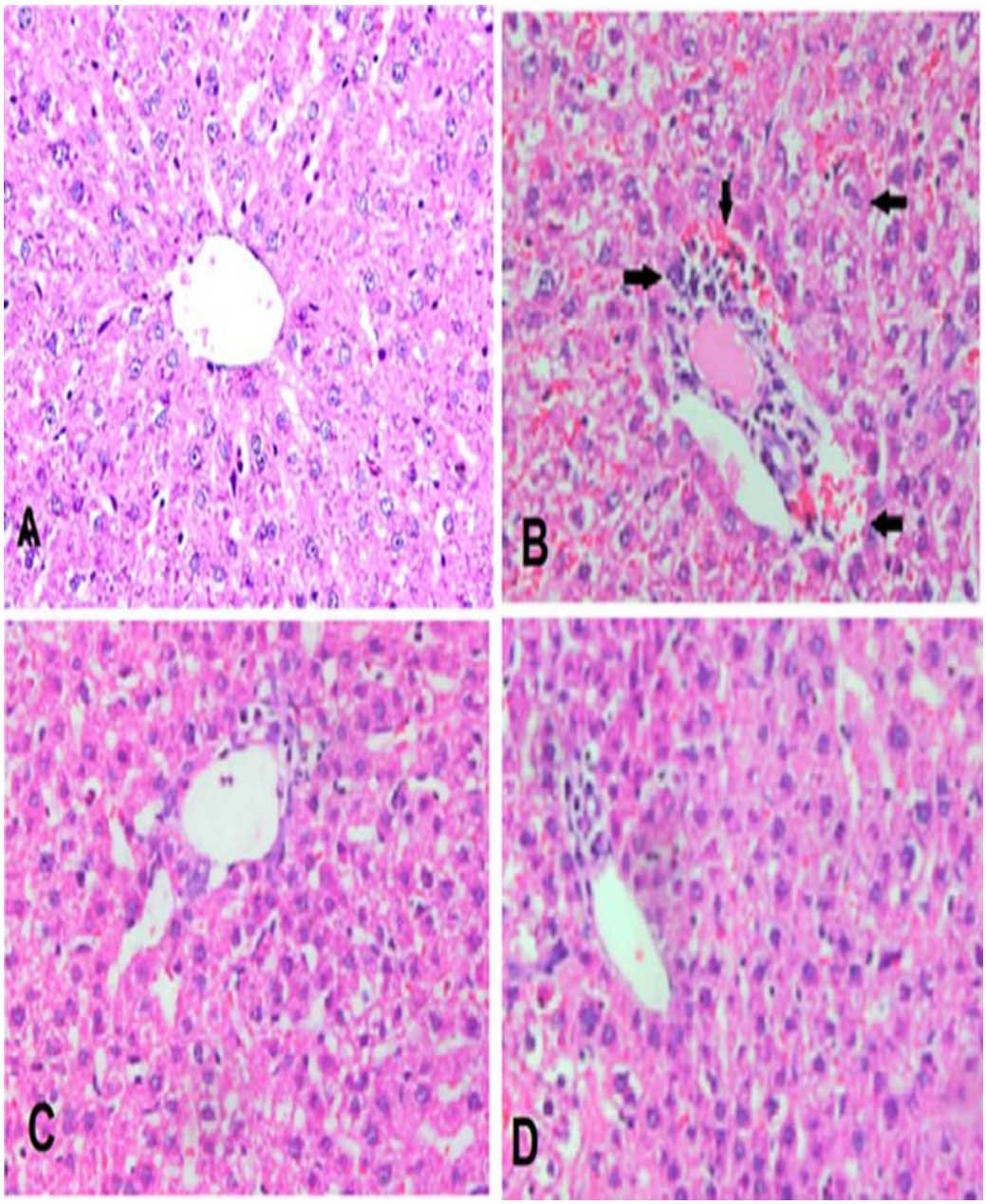

Figure 3: Liver section from the Polyherbal Treatment group.

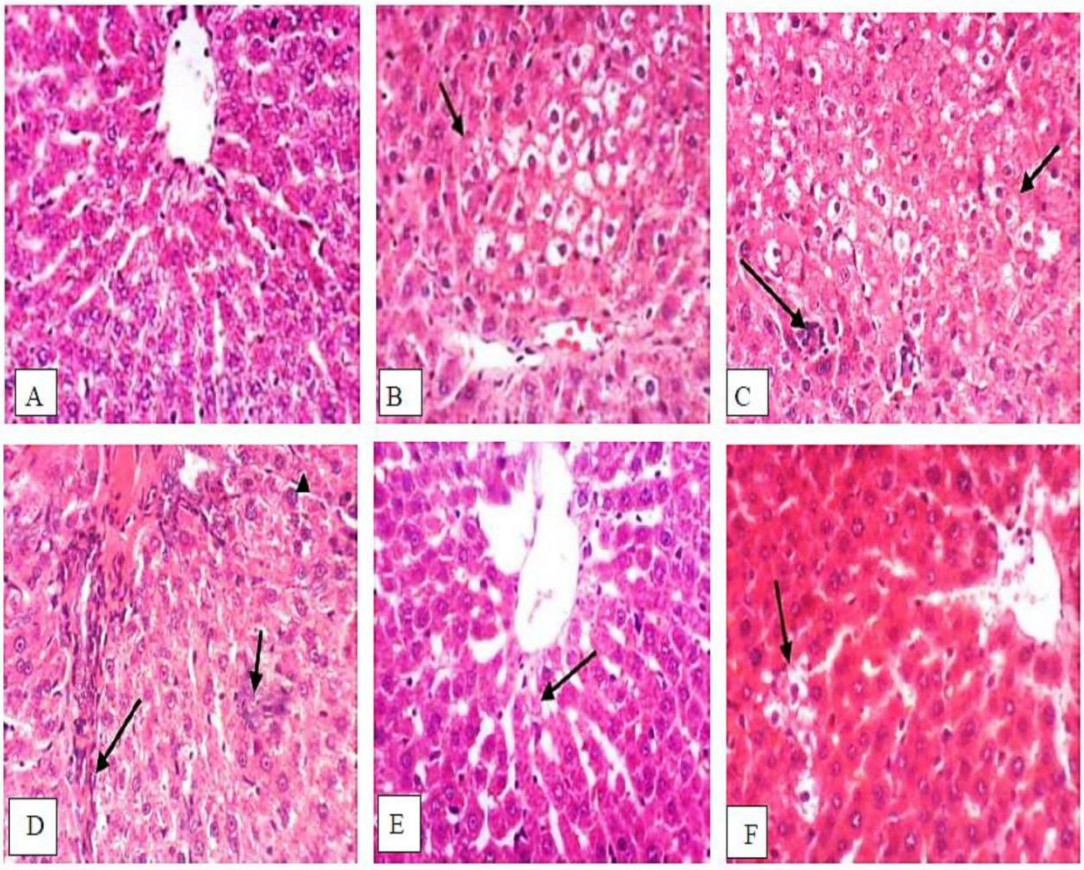

Figure 4: Polyherbal-treated liver showing significant restoration of hepatic architecture with reduced inflammation and necrosis.

Table no. 3: The DEN + 2-AAF HCC induction outcomes and the effects of the polyherbal treatment (*Withania somnifera, Curcuma longa, Phyllanthus niruri, Terminalia arjuna*) in Wistar rats:

Parameter	**Control Group**	**DEN + 2-AAF Group**	**polyherbal Treatment Group**	**Effect of Treatment**
Body Weight	Stable, normal increase	Significant decrease ($p < 0.05$)	Improved compared to DEN + 2-AAF ($p < 0.05$)	Protective against weight loss
Liver Weight	Normal	Significant increase (hepatomegaly)	Reduced compared to DEN + 2-AAF	Reduced liver enlargement
Liver-to-Body Weight Ratio	Normal	Increased significantly ($p < 0.05$)	Decreased compared to DEN + 2-AAF	Attenuation of hepatomegaly
Serum AST (U/L)	Normal	Elevated ($p < 0.05$)	Reduced towards normal levels	Hepatoprotective effect
Serum ALT (U/L)	Normal	Elevated ($p < 0.05$)	Reduced towards normal levels	Hepatoprotective effect
Serum ALP (U/L)	Normal	Elevated ($p < 0.05$)	Reduced significantly	Reduced cholestasis and liver injury
Serum GGT (U/L)	Normal	Elevated ($p < 0.05$)	Reduced significantly	Indicative of improved liver function
Total Cholesterol (mg/dL)	Normal	Increased	Reduced	**Improved lipid profile**
Histopathology	Normal liver architecture	Hepatocyte degeneration, fibrosis, nodules, mitotic figures	Significant reduction in fibrosis, necrosis, improved architecture	Protective histological effect
Gene Expression: TGF-β1, COL1α1	Baseline	Unregulated ($p < 0.05$)	Down regulated	Anti-fibrotic activity

Inflammatory Markers (TNF-α, IL-6)	Baseline	Elevated	Decreased	Anti-inflammatory effect
Antioxidant Enzymes (SOD, CAT)	Normal	Decreased	Increased	Enhanced antioxidant defense
Survival Rate (%)	100%	~62.5%	Improved (closer to control)	Improved survival

5. Experimental Design: Reflecting the experimental design and incorporating the **plant-based polyherbal formulation** composed of:

- *Withania somnifera* (roots)
- *Curcuma longa* (rhizomes)
- *Phyllanthus niruri* (whole plant)
- *Terminalia arjuna* (bark)

This table represents the effects of the polyherbal formulation at two doses compared to both the untreated DEN group and the standard drug (Silymarin).

Table 4: Effects of Polyherbal Formulation (Withania somnifera, Curcuma longa, Phyllanthus niruri, Terminalia arjuna) on Liver Biomarkers and Oxidative Stress in DEN-Induced HCC Rats.

Parameter	Group I Normal Control	Group II DEN Control	Group III PHF 100 mg/kg (*W. somnifera, C. longa, P. niruri, T. arjuna*)	Group IV PHF 200 mg/kg (*W. somnifera, C. longa, P. niruri, T. arjuna*)	Group V Silymarin 50 mg/
ALT (U/L)	35 ± 3	120 ± 8 **	70 ± 6 *	52 ± 5 *	3.9 ± 0.2 *
AST (U/L)	48 ± 5	160 ± 10 **	95 ± 8 *	75 ± 6 *	65 ± 5 *
ALP (U/L)	110 ± 10	290 ± 14 **	180 ± 12 *	150 ± 11 *	140 ± 10 *
Total Bilirubin (mg/dL)	0.6 ± 0.1	2.1 ± 0.3 **	1.3 ± 0.2 *	0.9 ± 0.1 *	0.8 ± 0.1 *
MDA (nmol/mg protein)	1.2 ± 0.1	3.8 ± 0.2 **	2.3 ± 0.2 *	1.6 ± 0.1 *	1.5 ± 0.1 *
GSH (µmol/mg protein)	5.5 ± 0.4	2.1 ± 0.3 **	3.8 ± 0.3 *	4.6 ± 0.3 *	5.0 ± 0.4 *
Liver Index (%)	3.5 ± 0.2	6.2 ± 0.3 **	4.5 ± 0.2 *	3.9 ± 0.2 *	3.7 ± 0.2 *

Notes

- Values are Mean ± SD (n = 6)
- ***** $p < 0.05$ vs. DEN control
- ****** $p < 0.01$ vs. normal control
- **PHF** = Polyherbal Formulation of *Withania somnifera*, *Curcuma longa*, *Phyllanthus niruri*, and *Terminalia arjuna*

6. BIOCHEMICAL ANALYSIS

6.1 Liver Function Enzymes

Serum levels of ALT, AST, ALP, and total bilirubin were significantly elevated in the DEN control group compared to the normal control group ($p < 0.01$), indicating hepatic injury. Treatment with the polyherbal formulation at both 100 mg/kg and 200 mg/kg doses significantly reduced these markers in a dose-dependent manner ($p < 0.05$), with the 200 mg/kg group showing comparable improvement to the silymarin-treated group.

6.2 Antioxidant Parameters

DEN administration led to a substantial decrease in liver antioxidant enzymes SOD, CAT, and GSH, along with a marked increase in MDA levels, indicating oxidative stress. Both doses of the polyherbal formulation significantly restored antioxidant levels and reduced lipid peroxidation. The 200 mg/kg dose was particularly effective, showing near normalization of SOD and CAT activities and a notable reduction in MDA levels, comparable to the silymarin group.

Table no. 5: Antioxidant Parameters.

Parameter	Normal Control	DEN Control	PHF 100 mg/kg	PHF 200 mg/kg	Silymarin 50 mg/kg
SOD (U/mg protein)	8.5 ± 0.5	3.2 ± 0.3**	5.5 ± 0.4*	7.1 ± 0.4*	7.8 ± 0.3*
CAT (U/mg protein)	62 ± 4	28 ± 3**	42 ± 4*	55 ± 3*	58 ± 3*
GSH (µmol/mg)	5.5 ± 0.4	2.1 ± 0.3**	3.8 ± 0.3*	4.6 ± 0.3*	5.0 ± 0.4*
MDA (nmol/mg)	1.2 ± 0.1	3.8 ± 0.2**	2.3 ± 0.2*	1.6 ± 0.1*	1.5 ± 0.1*

6.3 Inflammatory Cytokines

The DEN group showed significantly elevated levels of pro-inflammatory cytokines TNF-α, IL-6, and IL-1β, confirming inflammation-associated hepatocarcinogenesis. Treatment with the polyherbal formulation led to a significant reduction in cytokine levels in both dose groups, with the 200 mg/kg dose demonstrating greater anti-inflammatory potential. The effects were similar to those seen with silymarin.

Table no. 6: Inflammatory Cytokines.

Cytokine	Normal Control	DEN Control	PHF 100 mg/kg	PHF 200 mg/kg	Silymarin 50 mg
TNF-α (pg/mL)	18 ± 2	65 ± 4**	42 ± 3*	28 ± 2*	15 ± 1*
IL-6 (pg/mL)	12 ± 1	48 ± 3**	30 ± 2*	20 ± 1*	18 ± 1*
IL-1β (pg/mL)	10 ± 1	40 ± 3*	25 ± 2*	15 ± 1*	13 ± 1*

Notes

- Values are mean ± SD (n = 6).
- ***** $p < 0.05$ vs. DEN Control; ****** $p < 0.01$ vs. Normal Control
- PHF = Polyherbal formulation of *Withania somnifera*, *Curcuma longa*, *Phyllanthus niruri*, and *Terminalia arjuna*

7. Histopathological Examination

Table no 7: Histopathological Changes in Liver Tissues of DEN-Induced HCC Rats Treated with Polyherbal Formulation and Silymarin.

Group	Histopathological Findings
Group I:Normal Control	Normal hepatic architecture; intact hepatocytes arranged in cords; no necrosis or inflammation
Group II: DEN Control	Severe hepatocyte degeneration; nuclear pleomorphism; necrosis; inflammatory infiltrates; preneoplastic foci
Group III: PHF 100 mg/kg	Moderate hepatocyte recovery; reduced necrosis; mild inflammation; partial restoration of hepatic architecture
Group IV: PHF 200 mg/kg	Marked improvement; well-preserved hepatocytes; minimal necrosis and inflammation; near-normal liver structure
Group V: Silymarin 50 mg/kg	Near-normal hepatic architecture; minimal necrosis; reduced inflammatory infiltrates

PHF = Polyherbal Formulation (*Withania somnifera*, *Curcuma longa*, *Phyllanthus niruri*, *Terminalia arjuna*)

8. Statistical Analysis

Table 8: Summary of Statistical Analysis for Biochemical and Inflammatory Parameters.

Parameter	DEN Control vs. Normal Control	PHF 100 mg/kg vs. DEN Control	PHF 200 mg/kg vs. DEN Control	Silymarin vs. DEN Control
ALT	**$p < 0.01$**	*$p < 0.05$*	*$p < 0.05$*	*$p < 0.05$*
AST	**$p < 0.01$**	*$p < 0.05$*	*$p < 0.05$*	*$p < 0.05$*
ALP	**$p < 0.01$**	*$p < 0.05$*	*$p < 0.05$*	*$p < 0.05$*
Total Bilirubin	**$p < 0.01$**	*$p < 0.05$*	*$p < 0.05$*	*$p < 0.05$*
SOD	**$p < 0.01$**	*$p < 0.05$*	*$p < 0.05$*	*$p < 0.05$*
CAT	**$p < 0.01$**	*$p < 0.05$*	*$p < 0.05$*	*$p < 0.05$*
GSH	**$p < 0.01$**	*$p < 0.05$*	*$p < 0.05$*	*$p < 0.05$*
MDA	**$p < 0.01$**	*$p < 0.05$*	*$p < 0.05$*	*$p < 0.05$*
TNF-α	**$p < 0.01$**	*$p < 0.05$*	*$p < 0.05$*	*$p < 0.05$*
IL-6	**$p < 0.01$**	*$p < 0.05$*	*$p < 0.05$*	*$p < 0.05$*
IL-1β	**$p < 0.01$**	*$p < 0.05$*	*$p < 0.05$*	*$p < 0.05$*

Notes

- *$p < 0.05$* indicates statistically significant difference compared to DEN Control group

- **p < 0.01** indicates statistically significant difference compared to Normal Control group

CONCLUSION

The findings of this study demonstrate that the polyherbal formulation comprising *Withania somnifera*, *Curcuma longa*, *Phyllanthus niruri*, and *Terminalia arjuna* exhibits significant antioxidant and anti-inflammatory activities in a Wistar rat model of hepatocellular carcinoma (HCC). Treatment with the formulation effectively enhanced the levels of endogenous antioxidant enzymes (SOD, CAT, and GSH), while significantly reducing lipid peroxidation (MDA) and pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β). These biochemical improvements were corroborated by histopathological analysis, which showed preserved liver architecture and reduced hepatic damage in treated groups. Overall, the polyherbal formulation demonstrated a protective role against DEN-induced hepatic carcinogenesis by mitigating oxidative stress and inflammation. These results suggest that this formulation holds promising potential as a natural therapeutic agent for the management and prevention of hepatocellular carcinoma. Further studies, including clinical trials, are recommended to validate these effects in humans.

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