IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

Evaluation Of Anti-Cancer Activity Of Rutin And Piperine In Experimentally Induced Hepatocellular Carcinoma

Author: Yadala Prapurna Chandra M.Pharma.,Ph.D.[*1], A.H.M. Viswanath swamy M.Pharma.,Ph.D.[2]

[1]Principal & Professor, Dept of Pharmacology, Ratnam Institute of Pharmacy, Nellore, A.P., India.

[2] Principal & Professor, K.L.E. University's college of Pharmacy, Vidhyanagar, Hubli, Karnataka, India.

Abstract

Chemical carcinogens like N-Nitrosodiethylamine (DEN) frequently cause hepatocellular carcinoma (HCC), a major global health problem. The chemopreventive potential of the natural bioactive compounds Rutin and piperine against DEN-induced and phenobarbital-induced HCC in Wistar rats is the focus of this investigation. Rutin (R), Piperine (P), or a combination of the two were administered to the animals, who were then divided into initiation and promotion models. DEN administration led to significant hepatic damage, evidenced by reduced body weight, increased liver weight, elevated hepatic enzymes (AST, ALT, ALP, ACP, LDH, γ-GT), tumor markers (AFP, CEA), DNA content, and disrupted electrolyte balance. The combination of R and P significantly restored biochemical parameters, normalized enzyme activities, and enhanced histopathological architecture during treatment. RP showed superior efficacy in reducing hepatomegaly, restoring membrane-bound enzymes (Na^+/K^+ ATPase, Ca^{2+} ATPase, Mg^{2+} ATPase), and correcting serum sodium, potassium, calcium, and magnesium levels. Histological analysis confirmed reduced necrosis and improved liver morphology in RP-treated groups. These results suggest that Rutin and Piperine have a synergistic effect on chemoprevention and hepatoprotection, which has potential as a treatment for chemically induced liver carcinogenesis.

Keywords

Hepatocellular carcinoma, Rutin, Piperine, N-Nitrosodiethylamine, Chemoprevention, Liver enzymes

Introduction:

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related mortality worldwide, often associated with chronic liver injury and exposure to chemical carcinogens like N-Nitrosodiethylamine (DEN) [1]. Natural compound chemoprevention is a promising approach to reducing hepatic carcinogenesis[2]. Both the black pepper alkaloid piperine and the flavonoid rutin have potent antioxidant and anti-inflammatory properties. This study looks at how they affect DEN-induced and Phenobarbital-promoted HCC in rats individually and together[3]. By

The research aims to clarify the synergistic hepatoprotective potential of Rutin and Piperine during the initiation and promotion phases of liver carcinogenesis by evaluating biochemical, histopathological, and enzymatic parameters [4].

Aim

Evaluation of Anti-cancer action of Rutin and Piperine on N-Nitrosodiethylamine-initiated and Phenobarbital-promoted experimental rat hepatocellular carcinoma.

Objective

To evaluate the effect of Rutin and Piperine in an experimentally induced hepatocellular carcinoma in the initiation and promotion model.

Material and Methods

Animal Housing and Acclimatization: Polypropylene cages were used to house male Wistar rats in controlled conditions (35 °C, 40-70% humidity, 12-hour light/dark cycle). Standard pellet diet and water were provided ad libitum. Animals were acclimatized for two weeks prior to experimentation [5].

Animals Housing & Treatment.

The Experimental animals were retained in polypropylene coops at room temperature (35 ±2 0C) and relative humidity (40-70 %) was maintained, also sustained 12 12-hour dark & light cycle. The rats were fed with standard pellets and water ad libitum. About two weeks prior to the start of the experiment, the animals were exposed to the conditions of the laboratory [6].

Animal Grouping & Treatment Protocol

Table 1: Experimental Protocol for Initiation and Promotion Models

Group	Treatment	Study Duration
I. Normal Control	0.9% w/v normal saline daily	45 days / 16 weeks
II. Disease Control	DEN (200 mg/kg, i.p.); in promotion model, followed by Phenobarbital 0.05% w/v (drinking water)	45 days / 16 weeks
III. DEN + Rutin (50 mg/kg)	DEN (200 mg/kg, i.p.); Rutin (50 mg/kg, p.o.) from 2nd week	45 days / 16 weeks
IV. DEN + Piperine (50 mg/kg)	DEN (200 mg/kg, i.p.); Piperine (50 mg/kg, p.o.) from 2nd week	45 days / 16 weeks

V. DEN + Rutin + Piperine (50 mg/kg each)	DEN (200 mg/kg, i.p.); Rutin + Piperine (50 mg/kg each, p.o.) from 2nd week	45 days / 16 weeks
VI. Rutin Only (50 mg/kg)	Rutin (50 mg/kg, p.o.) from 2nd week	45 days / 16 weeks
VII. Piperine Only (50 mg/kg)	Piperine (50 mg/kg, p.o.) from 2nd week	45 days / 16 weeks
VIII. Rutin + Piperine Only (50 mg/kg each)	Rutin + Piperine (50 mg/kg each, p.o.) from 2nd week	45 days / 16 weeks

Experimental Design: The study looked at how Rutin (R) and Piperine (P) prevented hepatocellular carcinoma (HCC) caused by phenobarbital (PB) and N-nitrosodiethylamine (DEN). For both the initiation (45 days) and promotion (16 weeks) phases, rat populations were divided into eight groups (n=6 per group) [7].

Treatment Protocol: Group I received normal saline. DEN (200 mg/kg, intraperitoneally) and PB (0.05% w/v in drinking water) were given to Group II. Groups III–V received DEN plus R (50 mg/kg), P (50 mg/kg), or both (RP, 50 mg/kg each) orally from week 2. Groups VI–VIII received only R, P, or RP without DEN [8].

Biochemical and Histological Assessments: The liver weight, relative liver weight, and body weight were all recorded. Serum was analyzed for hepatic enzymes (AST, ALT, ALP, ACP, LDH, γ-GT), bilirubin, tumor markers (AFP, CEA), DNA, total protein, and electrolytes. The Na+/K+ ATPase, Ca2+ ATPase, and Mg2+ ATPase enzymes that were bound to the membrane were measured. Liver morphology and histopathology were examined to assess tissue architecture and necrosis[9].

Results

In vivo evaluation of Rutin and Piperine in DEN induced hepatocellular carcinoma-Initiation & Promotion model.

General Observations and Liver Pathophysiological Markers

Food and water consumption, body weight, and liver indices were monitored to assess the systemic impact of DEN-induced hepatocarcinogenesis and the protective role of rutin (R), piperine (P), and their combination (RP) [10].

Food and water intake: Normal rats showed a gradual increase across weeks in both initiation and promotion phases. DEN-administered groups exhibited significant reductions in consumption from the first week ($P<0.001–0.05$) compared with controls. There was no significant dietary influence from R, P, or RP, as the treated groups did not differ significantly from DEN[11].

Body weight: Controls displayed steady weight gain, while DEN caused significant reductions in both models. The R, P, and RP groups performed no better than the DEN group, indicating that hepatotoxicity outweighed nutritional recovery[12].

Relative liver weight: During initiation and promotion, DEN's liver weight significantly increased (P 0.001), indicating hepatomegaly as a result of the tumor burden. R, P, and RP reduced relative liver weight significantly (P 0.001), with RP having the greatest impact[13].

Biochemical markers: Initial DEN levels of AST, ALT, ALP, ACP, LDH, -GT, and bilirubin were significantly elevated, indicating hepatic injury. These elevations were reduced by treatment, and RP brought levels back to normal. Similar trends were observed in promotion, where DEN produced stronger alterations in enzyme profiles and bilirubin. R, P, and especially RP reduced transaminases, phosphatases, LDH, and γ-GT, while restoring bilirubin toward normal values[14].

In general, DEN caused severe biochemical disturbances, hepatomegaly, growth retardation, and systemic toxicity. Supporting their synergistic hepatoprotective function, treatment with R and P, particularly when used in combination, significantly reduced alterations in liver weight and hepatic enzymes[15].

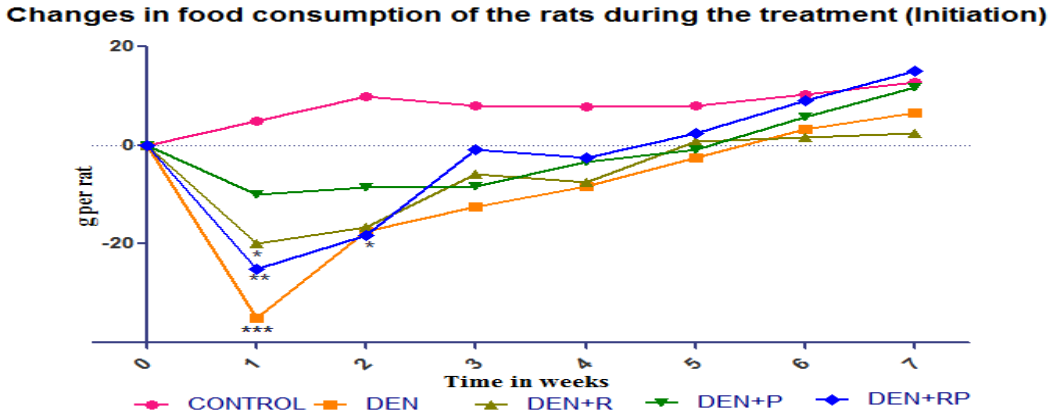

Figure1: Change in Food consumption of the Experimental animals (Initiation)

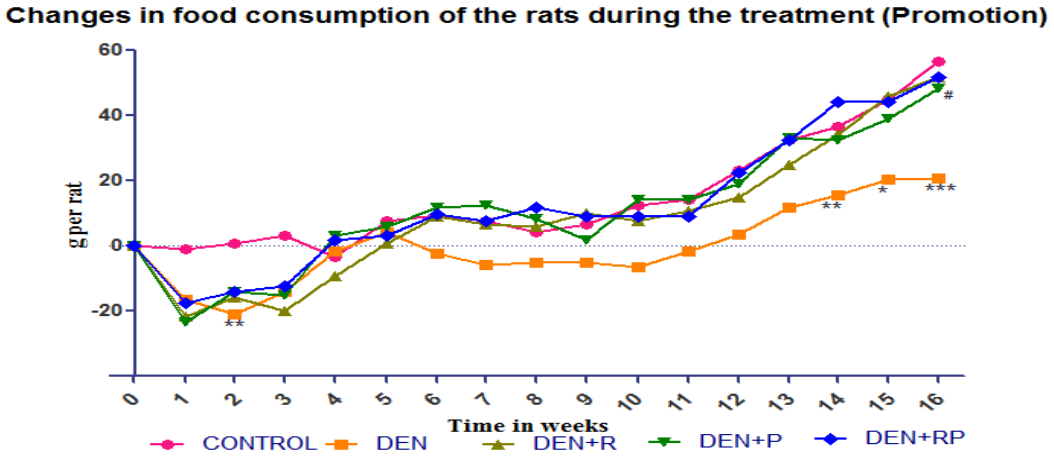

Figure 2: Change in Food consumption of the Experimental animals (Promotion)

Change in water consumption

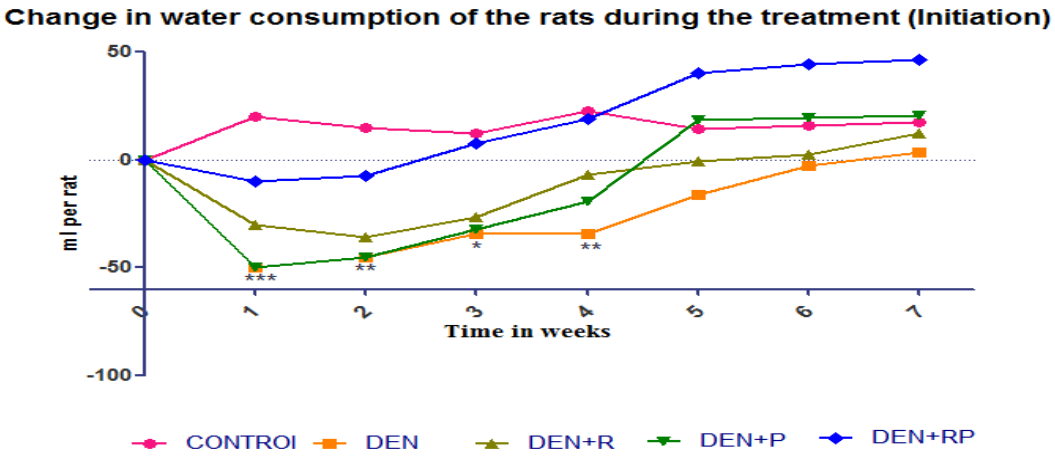

Figure 3: Change in water consumption of the Experimental animals (Initiation)

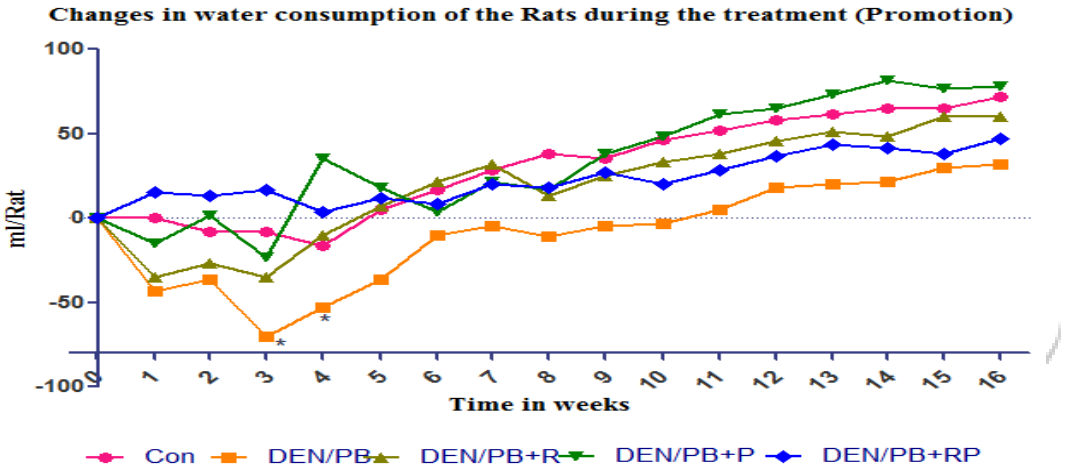

Figure 4: Change in water consumption of the Experimental animals (Promotion)

Change in body weight

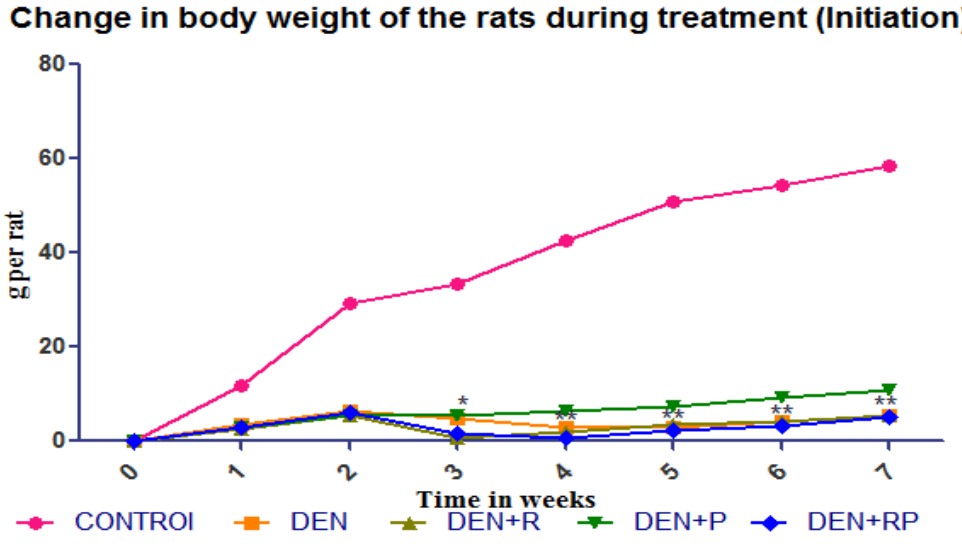

Figure 5: Change in body weight of the Experimental animals (Initiation)

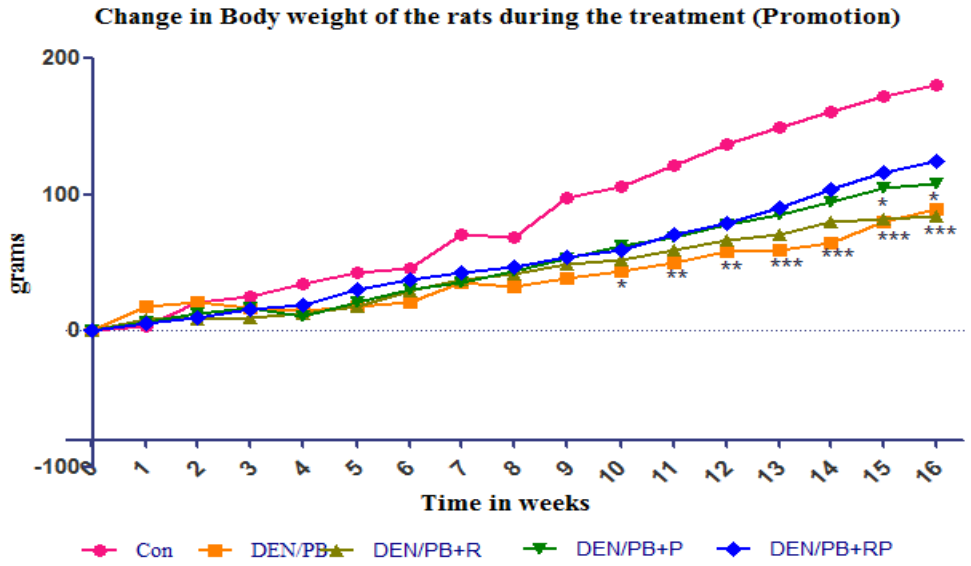

Figure 6: Change in body weight of the Experimental animals (Promotion)

Liver weight & Relative liver weight (Initiation)

Table 2: Liver weight & Relative weight (Initiation)

Group	Treatment	Initial Weight (Grams)	Final Weight (Grams)	Liver weight (Grams)	Relative liver weight (Grams)
I	Control	134.3±3.93	142.8±3.19	6.3±0.27	4.4 ± 0.19
II	DEN	136.8±3.31	134.5±3.33	6.8±0.27	5.1 ±0.31
III	DEN + R	135.7±5.89	134.7±5.47	6.7±0.15	5.0 ±0.12
IV	DEN+ P	134.5±2.26	135.7±1.03	6.6±0.13	4.9 ±0.08
V	DEN+ RP	135.8±1.94	132.8±2.79	6.5±0.11	4.9 ±0.10
VI	R Only	131.83 ± 1.58	144 ± 1.38	6.15±0.19	4.3 1.3
VII	P Only	135.0 ±2.24	146.7 ± 1.05	6.3±0.26	4.3 0.2
VIII	RP Only	133.3 ± 1.67	145.0 ± 1.83	6.35±0.23	4.4 0.18

Results are expressed as Mean ± SEM.

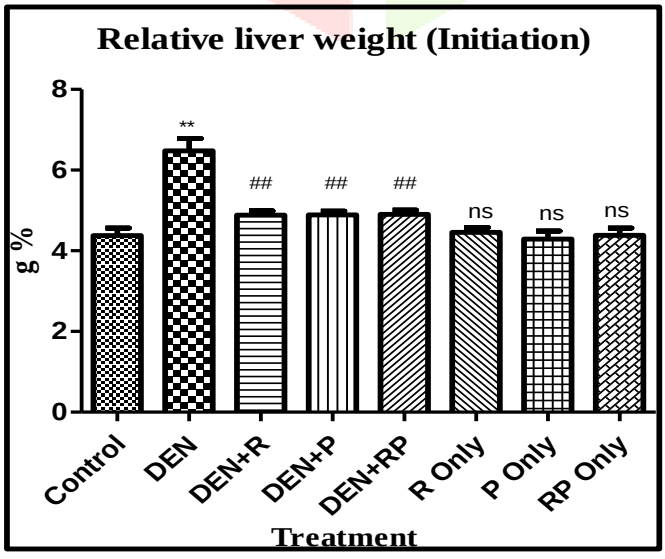

Figure 7: Relative Liver weight for Initiation

Table 3: Liver weight & Relative liver weight (Promotion)

Group	Treatment	Initial Weight (Grams)	Final Weight (Grams)	Liver weight (Grams)	Relative liver weight (Grams)
I	Control	140.83 ±3.52	317.5 ± 9.98	12 ± 0.51	3.8 ± 0.12
II	DEN/PB	171.67 ±4.94	251.67±11.45	13.7 ±0.36	5.5 ± 0.18
III	DEN/PB +R	170.0 ± 4.28	245.0 ± 6.58	10.8 ±0.20	4.4 ± 0.10
IV	DEN/PB+P	167.50 ± 7.39	268.33±10.22	10.7 ± 0.34	4.0 ± 0.15
V	DEN/PB+RP	166.67 ± 5.43	285.0 ± 12.85	11.2 ± 0.36	4.0 ± 0.15
VI	R Only	148.83 ± 5.29	301.67 ± 5.87	12 ± 0.47	4.0 ± 0.20
VII	P Only	145.0 ± 5.32	320.83 ± 8.31	12.4 ±0.36	3.9 ± 0.09
VIII	RP Only	159.17 ± 7.46	299.17 ± 5.83	11.2 ± 0.18	3.8 ± 0.07

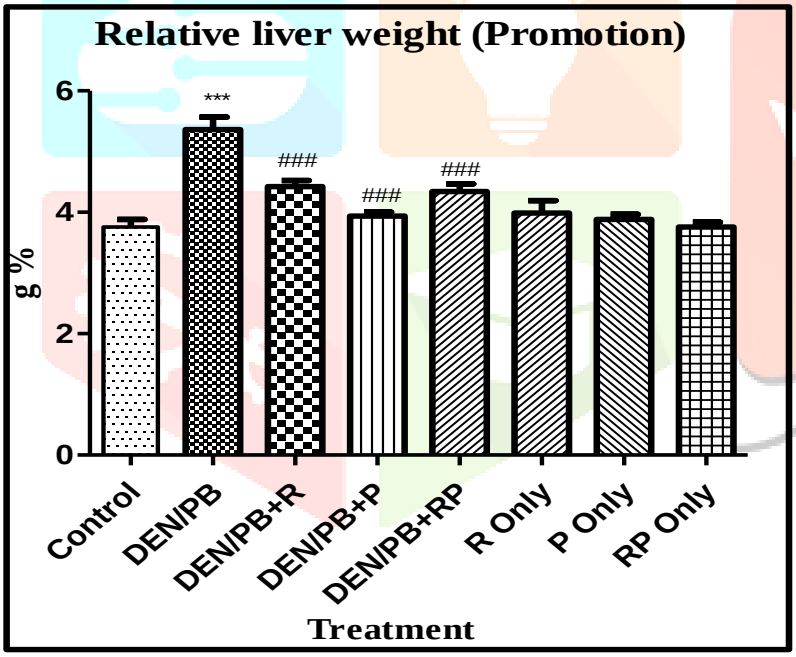

Figure 8: Relative Liver weight for Promotion

Liver Pathophysiological markers (Initiation)

Table 4: Liver pathophysiological markers for initiation

Particulars	Group I (Control)	Group II(DEN)	Group III (DEN + R)	Group IV(P)	Group IV(RP)	Group VI(R only)	Group VII(P only)	Group VIII(RP only)
AST	162.0 ± 16.36	328.9 ± 25.36	187.9 ± 16.21	207.7 ± 6.60	150.7 ± 18.26	160.1 ± 14.78	158.3 ± 14.33	174.3 ± 14.62
ALT	48.7 ± 5.35	120.4 ± 4.02	61.3 ± 1.25	63.3 ± 3.43	50.2 ± 3.95	48.8 ± 4.13	49.5 ± 4.78	50.2 ± 3.63
ALP	148.8 ± 7.34	344.5 ± 12.57	280.3 ± 6.47	244.1 ± 4.04	247.9 ± 4.40	145.3 ± 11.08	144.1 ± 8.92	160.3 ± 18.69
ACP	0.8 ± 0.15	3.3 ± 0.55	1.3 ± 0.33	1.2 ± 0.28	1.4 ± 0.57	1.0 ± 0.11	1.1 ± 0.09	0.9 ± 0.14
LDH	80.1 ± 7.11	152.2 ± 7.71	100.1 ± 6.76	106.0 ± 8.41	107.2 ± 7.16	93.9 ± 4.66	89.7 ± 9.95	86.4 ± 6.42
γ-GT	18.8 ± 0.40	29.1 ± 1.46	24.9 ± 1.87	26.5 ± 1.66	23.0 ± 1.59	19.4 ± 1.18	19.5 ± 1.09	22.5 ± 1.26
TBL(mg/100 ml)	0.4 ± 0.07	1.2 ± 0.10	0.8 ± 0.07	0.9 ± 0.06	0.7 ± 0.09	0.4 ± 0.04	0.6 ± 0.14	0.4 ± 0.06

Results are expressed as Mean ± S.E.M, n=6

Liver Pathophysiological markers (Promotion)

Table 5: Liver pathophysiological markers for promotion

Particulars	Group I (Control)	Group II(DEN)	Group III (DEN + R)	Group IV(P)	Group IV(RP)	Group VI(R only)	Group VII(P only)	Group VIII(RP only)
AST	104.26 ± 9.81	399.71 ± 8.2	226.06 ± 10.61	219.20 ± 15.77	209.71 ± 19.76	111.83 ± 10.70	118.95 ± 18.8	103.61 ± 13.54
ALT	62.92 ± 12.37	165.51 ± 17.76	108.26 ± 7.96	85.63 ± 9.4	90.01 ±10.10	53.14 ± 8.78	56.77 ± 8.34	71.79 ± 13.26
ALP	110.69 ± 18.47	252.72 ± 19.54	209.81 ± 9.42	196.40 ± 17.23	120.45 ± 28.78	82.96 ± 8.55	92.38 ± 6.73	121.87 ± 7.13
ACP	1.66 ± 0.41	3.61 ± 0.74	3.46 ± 0.42	3.03 ± 0.34	2.67 ± 0.50	2.89 ± 0.55	2.22 ± 0.24	2.50 ± 0.34
LDH	108.11 ± 18.27	251.32 ± 20.59	154.50 ± 12.55	163.75 ±20.6	152.09 ± 22.93	96.99 ± 10.33	105.64 ± 4.3	117.92 ± 13.78
γ-GT	18.70 ± 1.83	50.98 ± 8.26	39.93 ± 2.69	39.68 ± 4.07	30.03 ± 2.59	20.74 ± 2.38	23.94 ± 2.62	23.77 ± 2.74
TBL(mg/100 ml)	0.46 ± 0.11	1.43 ± 0.21	0.77 ± 0.04	0.75 ± 0.23	0.46 ± 0.09	0.66 ± 0.08	0.74 ± 0.12	0.67 ± 0.09

Results are expressed as Mean ± S.E.M, n=6

Hepatic Enzymes and Tumor Markers

DEN administration produced marked alterations in liver function enzymes and tumor markers during both initiation and promotion phases[16].

Aspartate aminotransferase (AST) and **alanine aminotransferase (ALT):** DEN groups had significantly higher levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) than controls (P0.001). In both phases, treatment with piperine (P), rutin (R), and their combination (RP) significantly restored both enzymes to normal (P 0.001)[17].

Alkaline phosphatase (ALP) and **acid phosphatase (ACP):** In DEN-treated rats, both acid phosphatase (ACP) and alkaline phosphatase (ALP) significantly increased (P 0.001)[18]. R and P alone did not show significant restoration, whereas RP significantly normalized ALP (P<0.01, P<0.001) and ACP (P<0.001, P<0.01) in initiation and promotion respectively[19].

Lactate dehydrogenase (LDH): LDH levels were significantly elevated in DEN controls (P<0.001). LDH levels were significantly reduced in all treatment groups (P 0.001), indicating that protection was effective[20].

Gamma-glutamyl transferase (GGT): It a sensitive HCC marker, was markedly increased in DEN rats (P<0.001). R and P on their own were ineffective, but RP significantly decreased GGT during initiation and promotion (P 0.05, P 0.001) [21].

Total bilirubin: The DEN groups had higher total bilirubin levels (P0.001). Rutin had a moderate reduction (P 0.05–0.01), Piperine only had a positive effect (P 0.01), and RP had a significant reduction (P 0.01–0.001)[22].

AFP and CEA showed slight and significant elevations for tumor markers during initiation and promotion, respectively[23]. During promotion, R, P, and RP all significantly reduced these markers, with RP having the greatest impact. The DEN groups had significantly more DNA (P 0.001), but treatments reduced this increase in promotion (P 0.01–0.001). In DEN promotion groups, total protein levels decreased significantly (P 0.001) and were restored by all treatments, with RP performing the best[24]. Overall, DEN-induced hepatic injury was evident by elevated enzymes, tumor markers, and DNA changes. Co-administration of rutin and piperine demonstrated strong synergistic hepatoprotective and chemopreventive efficacy[25].

Aspartate amino transferase (AST)

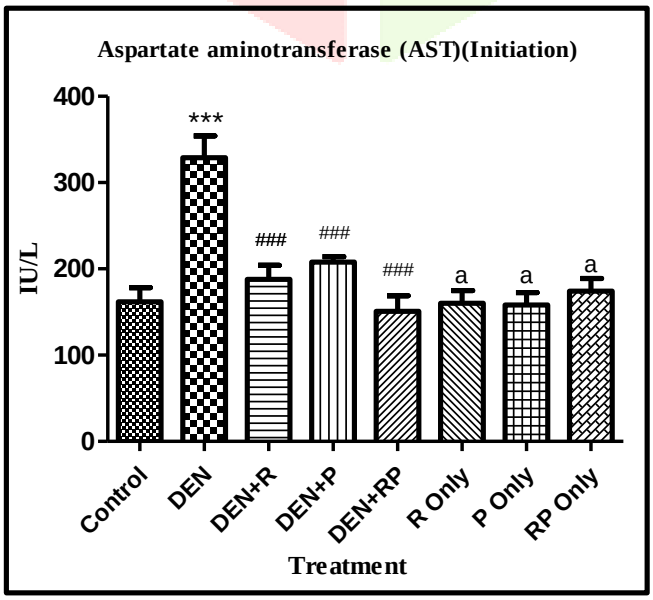

Figure 9 : Aspartate aminotransferase for initiation

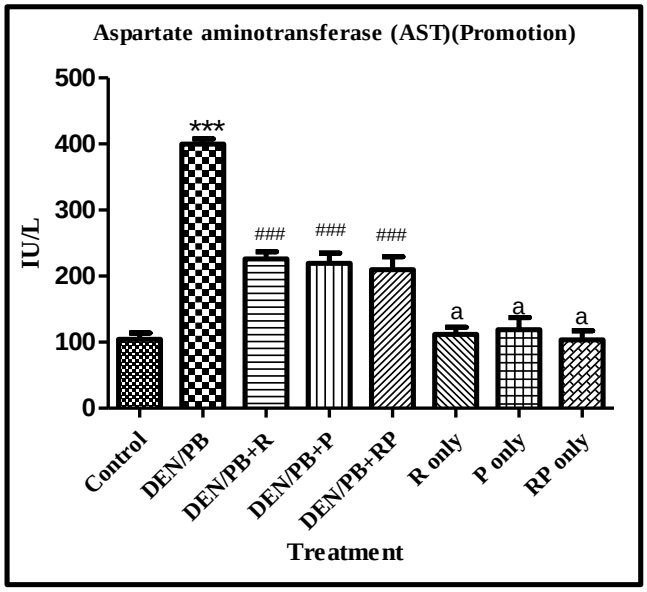

Figure 10: Aspartate aminotransferase for Promotion

Alanine aminotransferase (ALT)

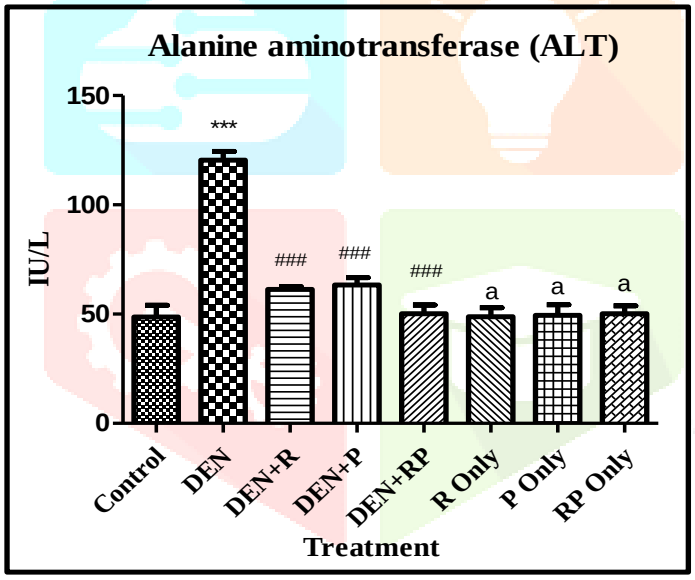

Figure 11: Alanine aminotransferase for Initiation

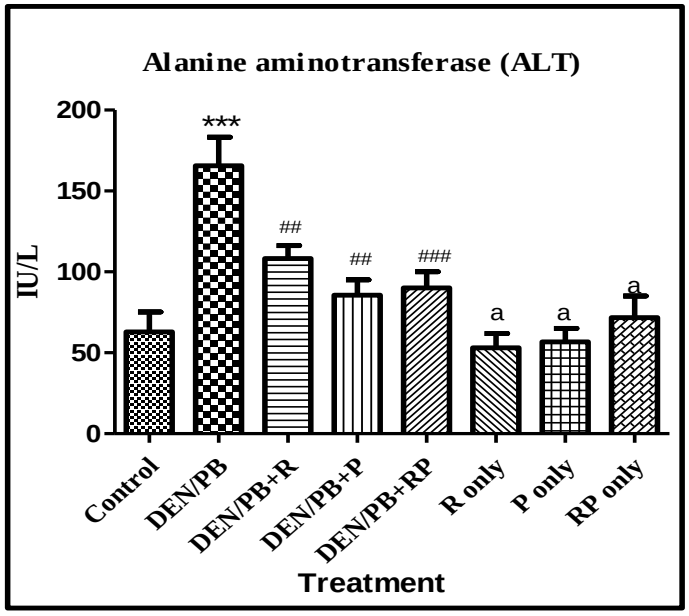

Figure 12: Alanine aminotransferase for Promotion

Alkaline phosphatase (ALP)

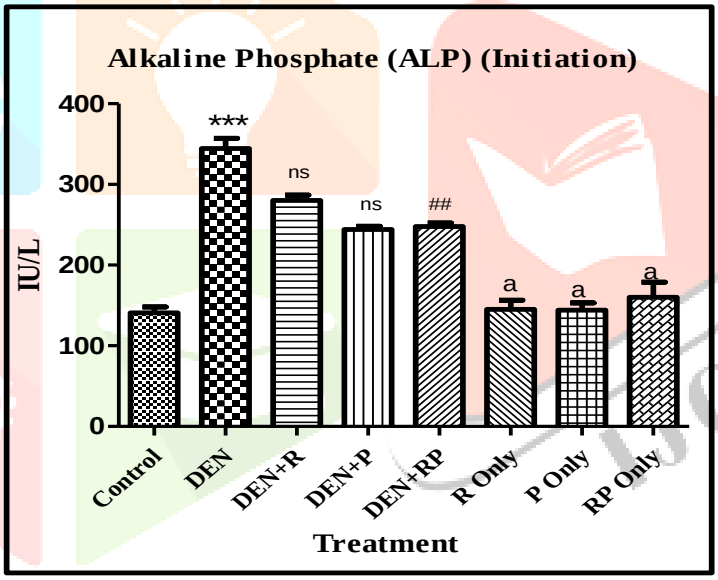

Figure 13: Alanine aminotransferase for Initiation

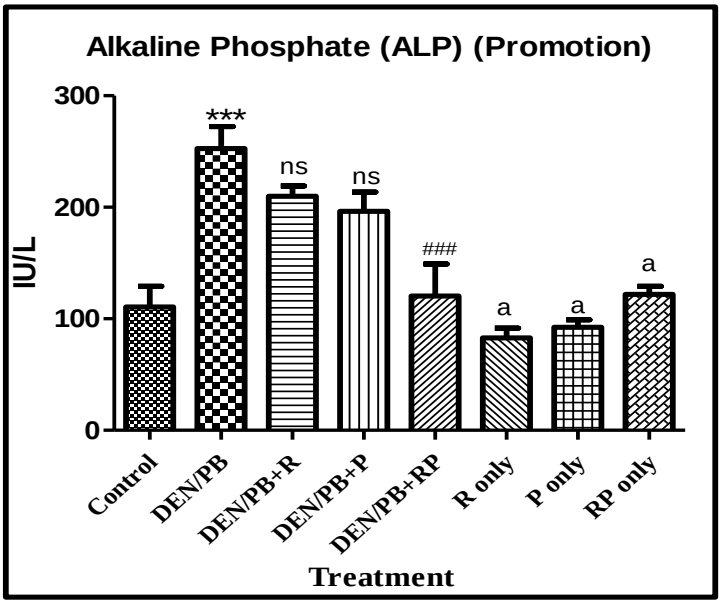

Figure 14: Alanine aminotransferase for promotion

Acid Phosphate (ACP)

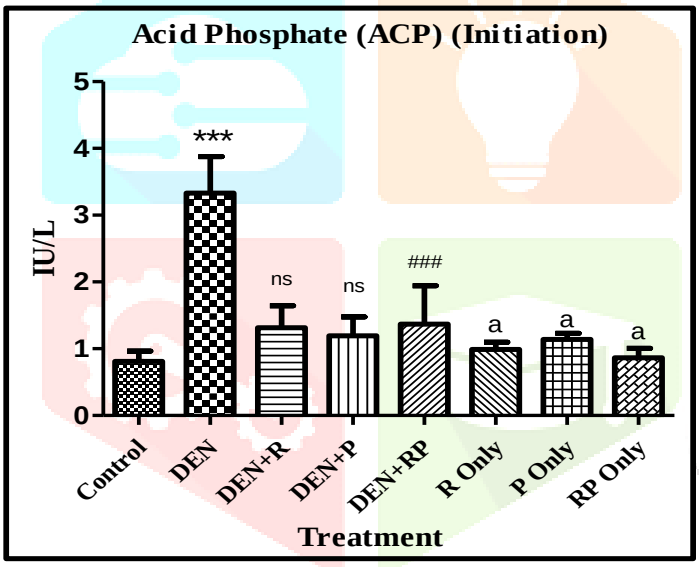

Figure 15: Acid phosphate for Initiation

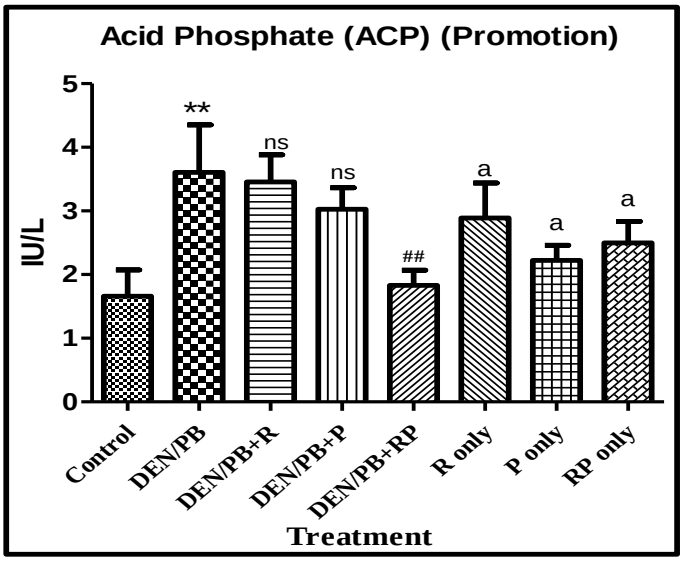

Figure 16: Acid phosphate for promotion

Lactate dehydrogenase (LDH)

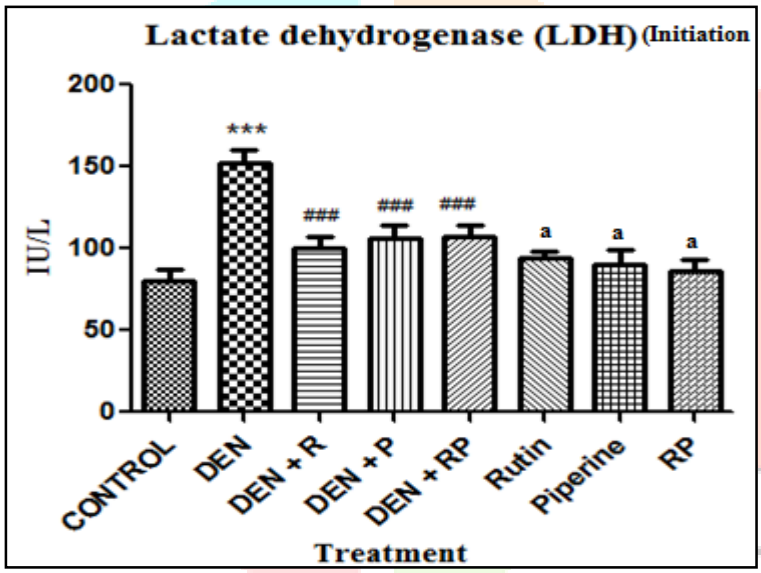

Figure 17: Lactate dehydrogenase for Initiation

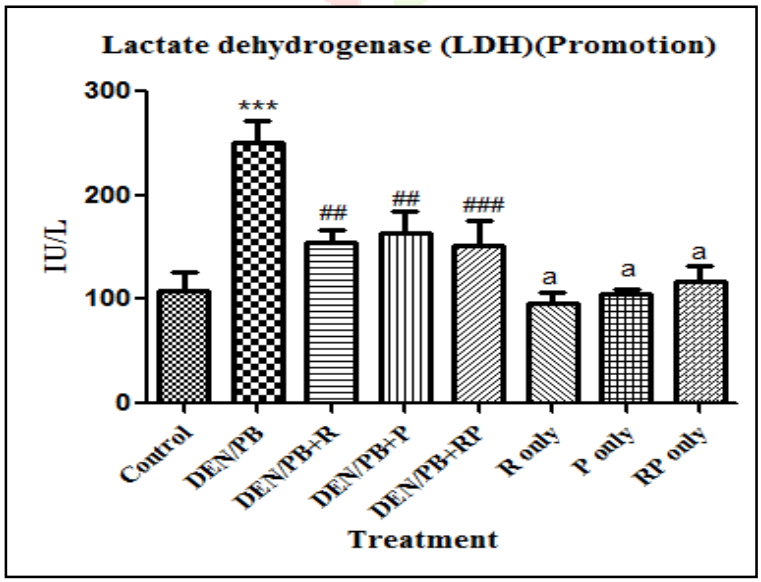

Figure 18: Lactate dehydrogenase for Promotion

Gamma-glutamyl Transferase (GGT)

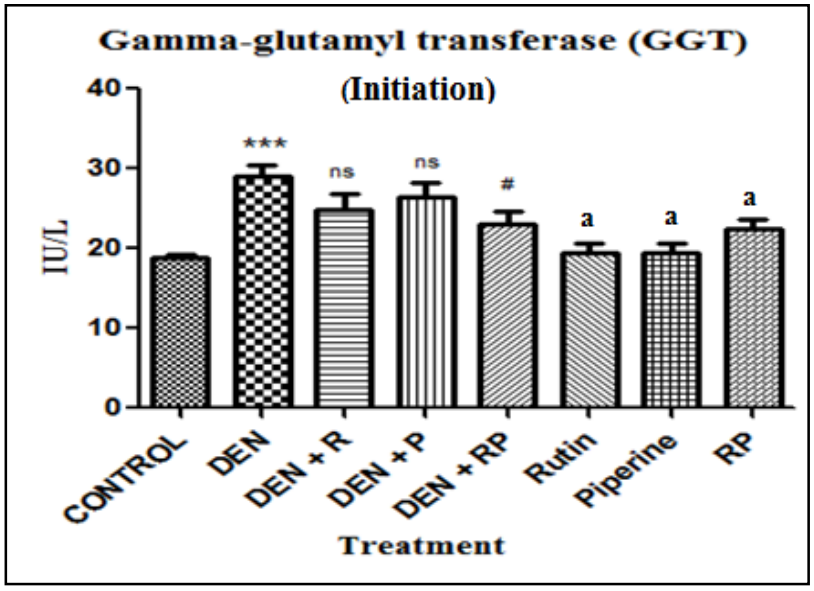

Figure 19: Gamma-glutamyl transferase (GGT) for initiation

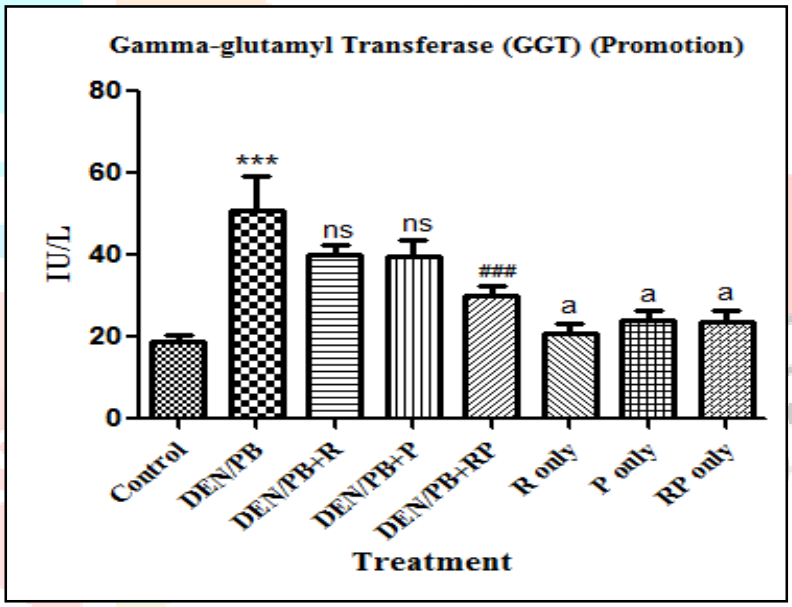

Figure 20: Gamma-glutamyl transferase (GGT) for Promotion

Total Bilirubin

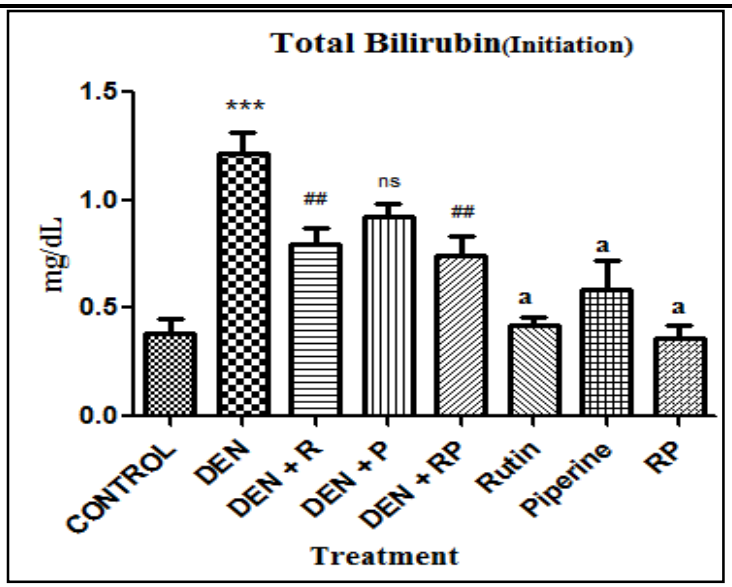

Figure 21: Total Bilirubin for initiation

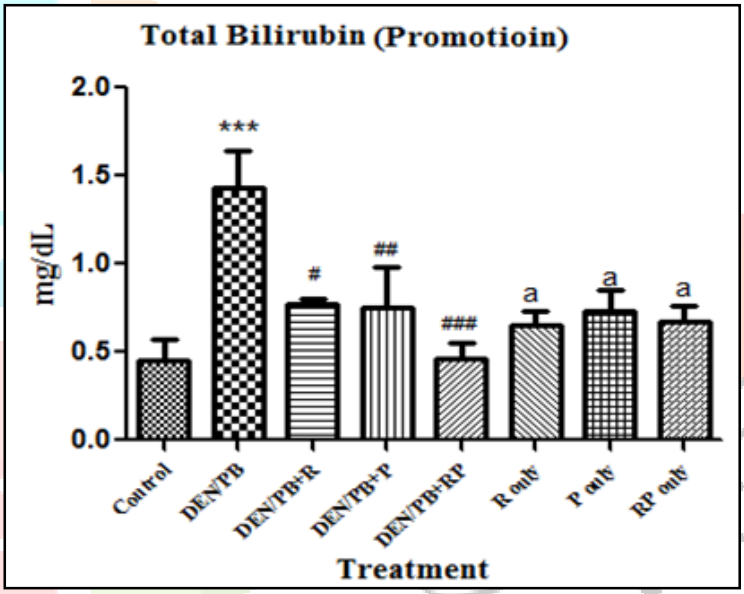

Figure 22: Total Bilirubin for Promotion

Liver Tumour markers

Table 6: Liver tumour markers (Initiation)

Particulars	Group I (Control)	Group II(DEN)	Group III (DEN + R)	Group IV(P)	Group IV(RP)	Group VI(R only)	Group VII(P only)	Group VIII(RP only)
AFP	0.25 ± 0.04	0.39 ± 0.03	0.34 ± 0.04	0.33 ± 0.05	0.33 ± 0.03	0.28 ± 0.04	0.25 ± 0.03	0.32 ± 0.03
CEA	0.17 ± 0.02	0.31 ± 0.07	0.42 ± 0.05	0.19 ± 0.05	0.18 ± 0.03	0.18 ± 0.01	0.20 ± 0.02	0.18 ± 0.02
DNA	0.80 ± 0.06	1.63 ± 0.16	1.37 ± 0.15	1.09 ± 0.15	1.15 ± 0.14	0.87 ± 0.05	0.83 ± 0.13	0.95 ± 0.14
Total Protein	10.77 ± 0.79	8.30 ± 0.55	9.72 ± 1.46	10.53 ± 1.34	10.07 ± 1.67	10.05 ± 0.75	10.14 ± 0.81	10.18 ±1.08

Results are expressed as Mean ± SEM, n=6

Table 7: Liver tumour markers (Promotion)

Particulars	Group I (Control)	Group II(DEN)	Group III (DEN + R)	Group IV(P)	Group IV(RP)	Group VI(R only)	Group VII(P only)	Group VIII(RP only)
AFP	0.18 ±0.02	0.62 ± 0.02	0.44 ± 0.03	0.42 ± 0.03	0.37 ± 0.04	0.22 ± 0.02	0.19 ± 0.02	0.22 ± 0.03
CEA	0.21 ±0.41	0.59 ± 0.74	0.47 ± 0.42	0.46 ± 0.34	0.41 ± 0.50	0.23 ± 0.55	0.24 ± 0.24	0.22 ± 0.34
DNA	39.60 ± 2.54	339.17 ± 28.98	239.99 ± 10.53	90.37 ± 8.82	47.55 ± 4.28	38.30 ± 2.53	40.52 4.61	38.02 ± 6.43
Total Protein	7.73 ± 0.41	3.09 ± 0.74	5.14 ± 0.50	5.70 ± 0.71	6.49 ± 0.29	7.77 ± 0.39	7.95 ± 0.52	7.40 ± 0.34

Results are expressed as Mean ± SEM, n=6

Tumor Markers, DNA, Proteins, and Electrolytes

The effect of rutin (R), piperine (P), and their combination (RP) on tumor markers, DNA, proteins, and electrolytes was evaluated in DEN-induced hepatocellular carcinoma (HCC) models during initiation and promotion phases[28].

α-Fetoprotein (AFP): During the DEN group's promotion phase, its evels were significantly higher (P0.001) than in the initiation phase (P0.05)[29]. Chemopreventive activity was demonstrated by the fact that R, P, and RP treatment had no effect on initiation but significantly reduced AFP during promotion (P0.001)[30].

Carcinoembryonic Antigen (CEA): Its levels showed similar trends, with a slight increase in initiation (P0.01) and a significant increase in promotion (P0.001). In the promotion phase, R, P, and RP significantly reduced CEA (P<0.05–0.001), with RP showing the strongest effect[31].

DNA content: During both phases, it was significantly higher (P 0.001) in the DEN-treated groups[32]. Treatments were ineffective at the beginning, but they significantly reduced DNA elevation during promotion (P0.01–0.001), indicating that abnormal DNA synthesis was inhibited[33].

Total proteins: It remained the same during initiation but significantly decreased during promotion (P 0.001). Protein levels were significantly (P 0.05–0.001) returned to normal following R, P, and RP treatments[34].

Serum electrolytes: These were also altered. During promotion, sodium decreased while potassium, calcium, and magnesium displayed abnormal fluctuations in the DEN group[35]. In the promotion phase, treatments significantly restored sodium, potassium, calcium, and magnesium, with RP demonstrating synergistic recovery, but they had no effect on initiation levels[36].

Overall, the findings demonstrate that DEN induces tumor marker elevation, DNA alterations, protein reduction, and electrolyte imbalance, while treatment with rutin and piperine, especially in combination, restores these biochemical parameters, supporting their synergistic chemopreventive efficacy[37].

α-fetoprotein (AFP)

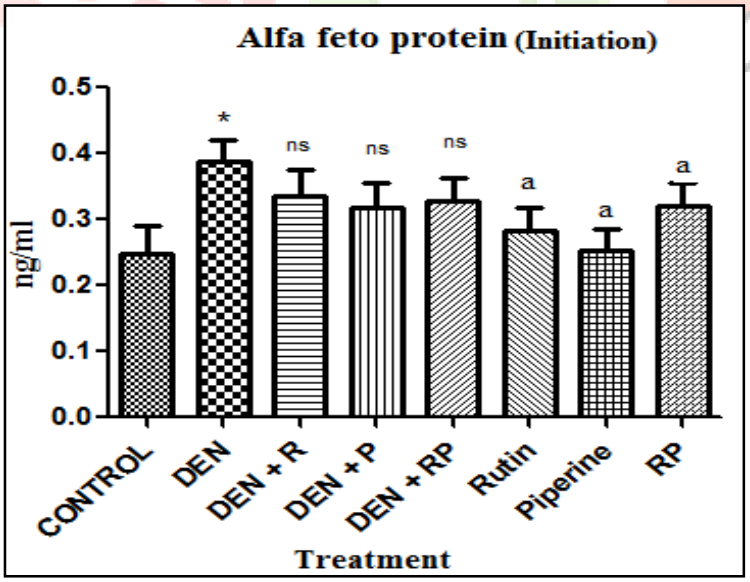

Figure 23: α-fetoprotein for Initiation

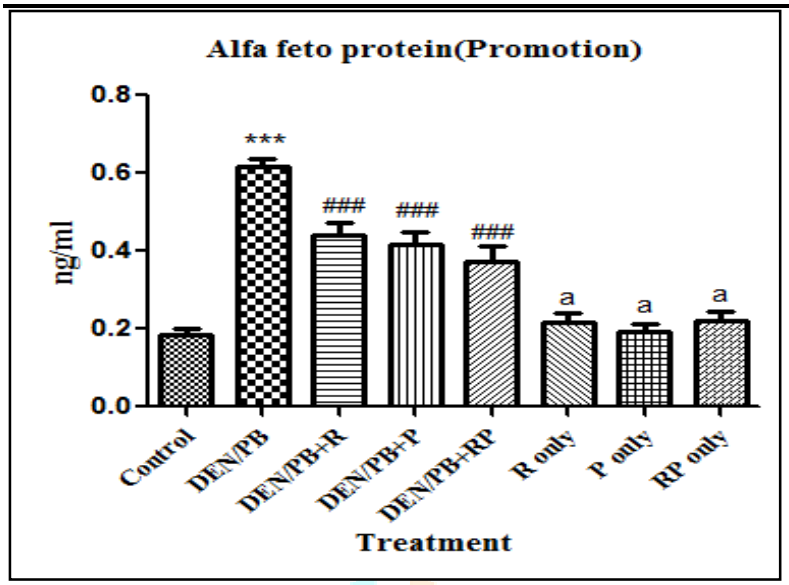

Figure 24: α-fetoprotein for Promotion

Carcino Embryonic Antigen (CEA)

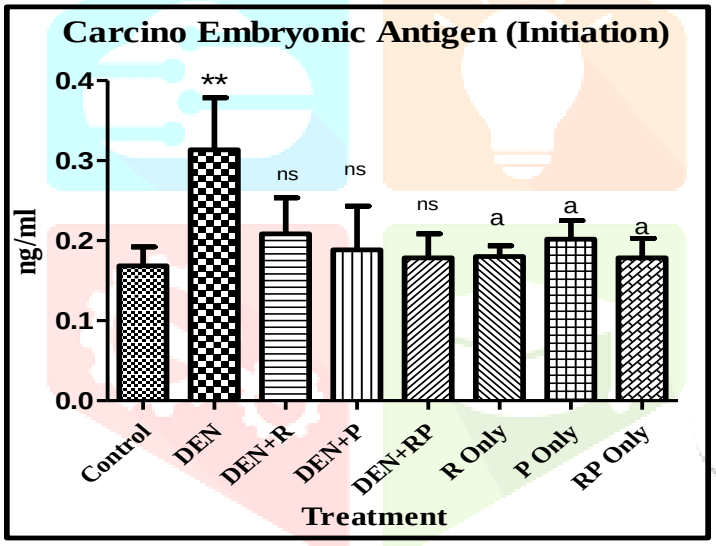

Figure 25: Carcino Embryonic Antigen for initiation

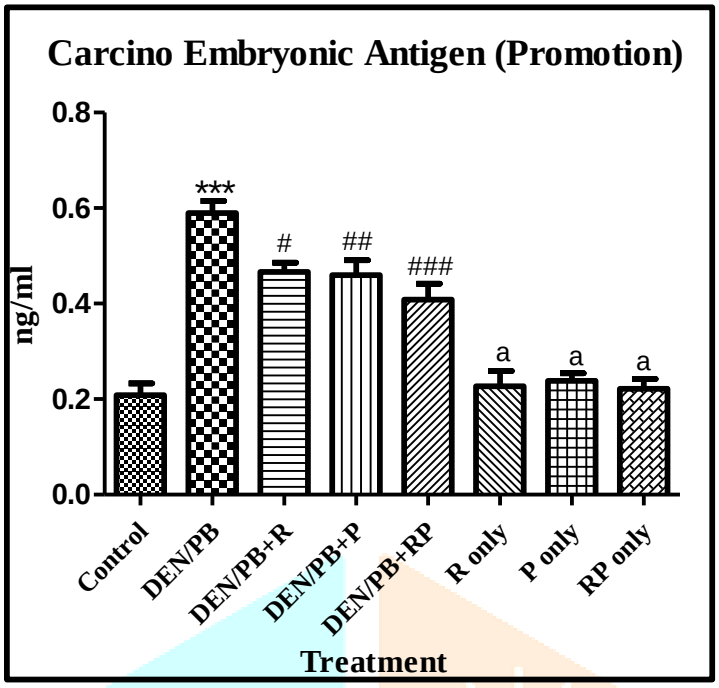

Figure 26: Carcino Embryonic Antigen for promotion

DNA levels

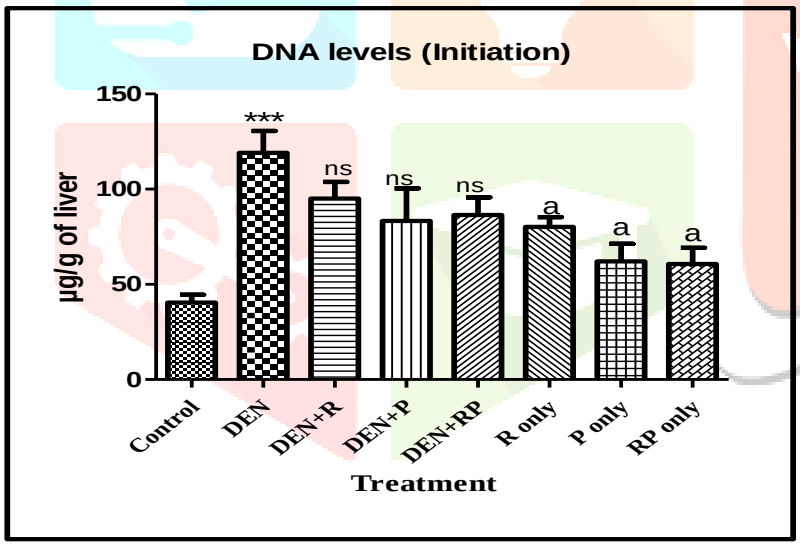

Figure 27: DNA levels for initiation

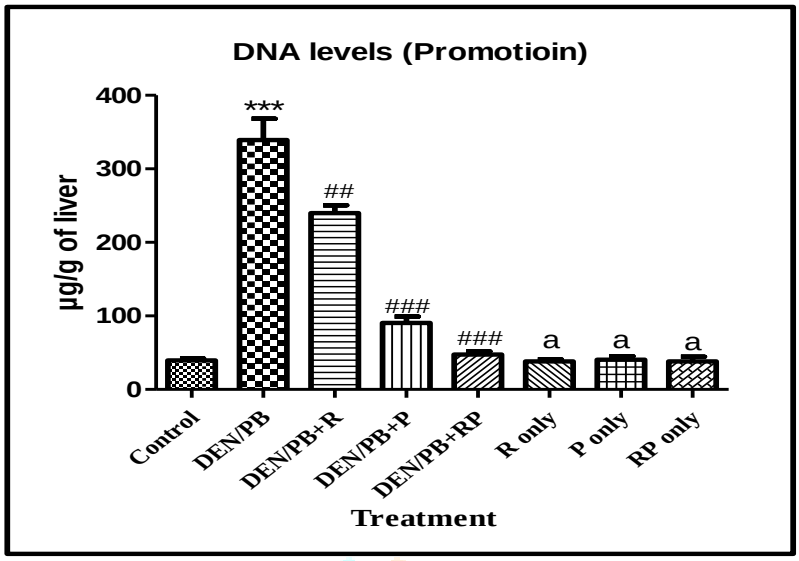

Figure 28: DNA levels for promotion

Total Proteins

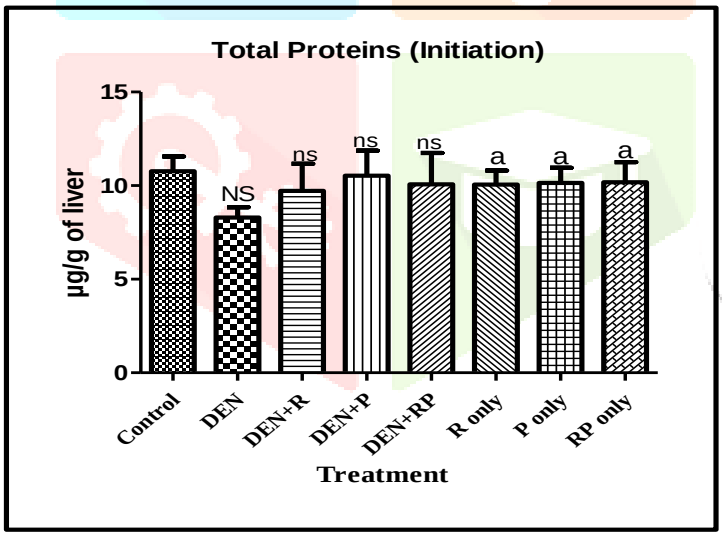

Figure 29: Total Protein level for initiation

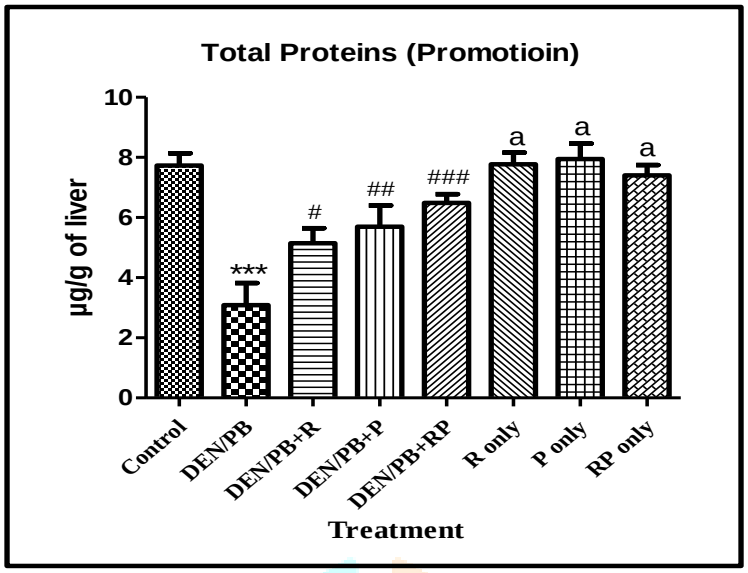

Figure 30: Total Protein level for promotion

Serum Electrolytes

Table 8: Estimation of electrolytes in serum (Initiation)

Particulars	Group I (Control)	Group II(DEN)	Group III (DEN + R)	Group IV(P)	Group IV(RP)	Group VI(R only)	Group VII(P only)	Group VIII(RP only)
Sodium	143.41 ± 4.12	109.17 ± 3.14	120.27 ± 7.34	129.82 ± 6.16	133.55 ± 4.30	148.97 ± 9.05	145.98 ± 7.84	147.42 ± 3.24
Potassium	6.00 ± 0.27	4.66 ± 0.37	5.01 ± 0.37	5.35 ± 0.51	5.57 ± 0.37	6.04 ± 0.39	6.14 ± 0.26	6.13 ± 0.46
Calcium	6.67 ± 0.7	10.94 ± 0.4	10.05 ± 1.0	10.63 ± 0.8	9.90 ± 0.5	6.35 ± 0.7	6.98 ± 0.7	6.80 ± 0.5
Magnesium	5.57 ± 0.4	4.00 ± 0.1	4.48 ± 0.5	4.72 ± 0.2	5.07 ± 0.3	5.93 ± 0.2	5.67 ± 0.3	5.78 ± 0.3

Results are expressed as Mean ±SEM, n=6

Table 9 : Estimation of electrolytes in serum (Promotion)

Particulars	Group I (Control)	Group II(DEN)	Group III (DEN + R)	Group IV(P)	Group IV(RP)	Group VI(R only)	Group VII(P only)	Group VIII(RP only)
Sodium	146.27 ± 2.83	92.20 ± 3.49	124.74 ± 7.14	134.86 ± 5.00	132.66 ± 5.21	146.22 ± 8.16	147.95 ± 6.79	148.72 ± 3.73
Potassium	6.01 ± 0.43	10.94 ± 0.49	6.75 ± 0.22	6.37 ± 0.43	6.14 ± 0.26	5.12 ± 0.30	5.24 ± 0.56	5.50 ± 0.36
Calcium	6.12 ± 0.89	14.59 ± 1.21	10.62 ± 1.12	8.54 ± 0.87	7.09 ± 0.76	6.04 ± 0.70	7.18 ± 0.78	7.16 ± 0.58
Magnesium	5.62 ± 0.52	10.71 ± 0.45	7.98 ± 0.67	7.56 ± 0.29	6.29 ± 0.84	5.17 ± 0.56	5.18 ± 0.54	5.07 ± 0.60

Results are expressed as Mean ±SEM, n=6

Electrolyte Balance and Membrane Bound Enzymes

In the present study, alterations in serum electrolytes and membrane bound enzymes were evaluated in DEN-induced hepatocellular carcinoma models under both initiation and promotion phases[38].

Serum sodium levels: They demonstrated a significant decrease in initiation and promotion in the DEN group (P0.01, P0.001), respectively[39]. Initiation of treatment with rutin (R) and piperine (P) alone did not alter sodium levels, whereas the combination (RP) resulted in a slight elevation (P0.05). In the promotion model, both R and P significantly restored sodium levels (P 0.05, P 0.01), and when compared to the individual treatments, the combination had a synergistic effect[40].

Serum potassium levels: They were higher in rats treated with DEN (P 0.05, P 0.001) No significant effect was observed in the initiation phase across treatments, while in promotion, R, P, and RP effectively normalized potassium levels (P<0.001)[41].

[42]

Serum magnesium levels: These were also increased in DEN-treated groups (P<0.01, P<0.001). No significant changes were observed in initiation, while in promotion, R, P, and RP significantly reduced elevated magnesium (P<0.05–0.001)[43].

Membrane bound enzyme activities: (Na+/K+ ATPase, Ca2+ ATPase, and Mg2+ ATPase) were significantly lower in DEN groups than in controls. R, P, and RP treatment resulted in partial to complete recovery, with RP playing a more protective role, particularly during the promotion phase[44].

Overall, the results show that DEN causes electrolyte imbalance and enzyme dysfunction. R and P, especially when used together, have restorative effects, suggesting that they work together to prevent cancer[45].

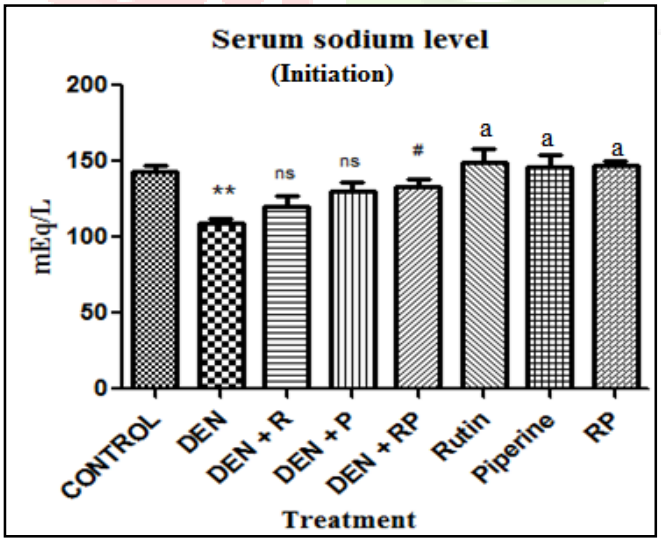

Figure 31: Serum sodium levels for initiation

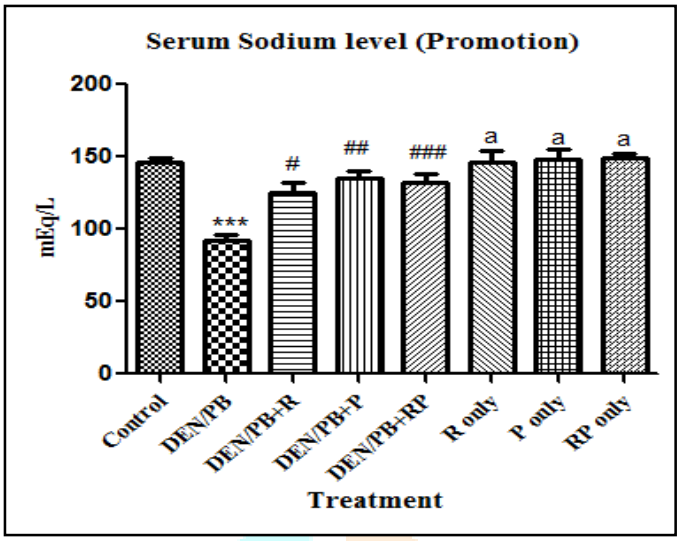

Figure 32: Serum sodium levels for promotion

Serum potassium levels

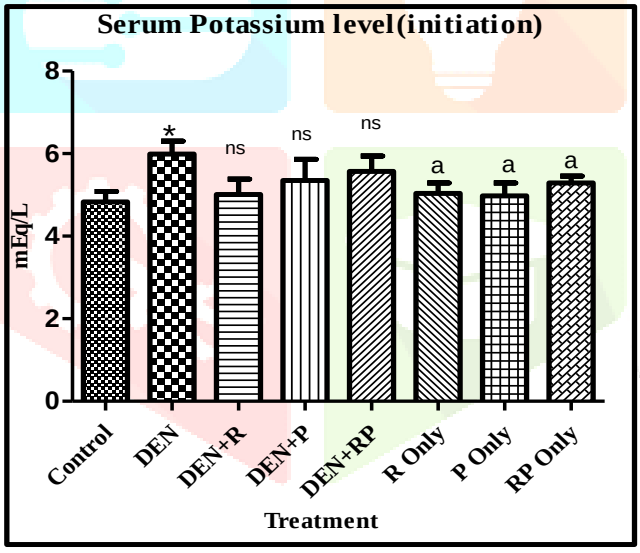

Figure 33: Serum potassium levels for initiation

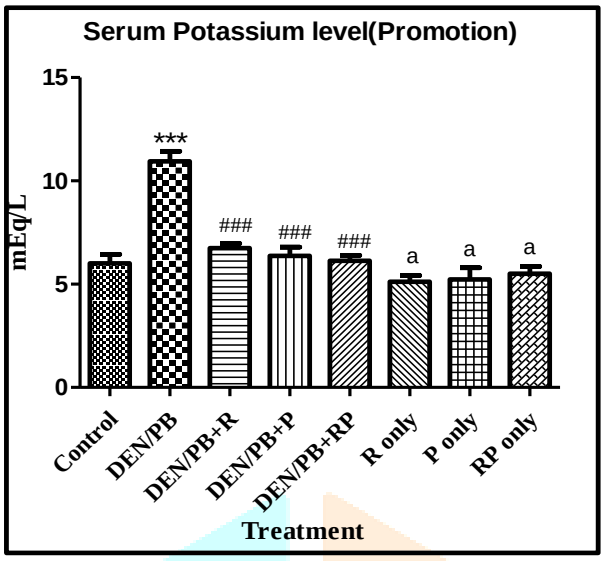

Figure 34: Serum potassium level for promotion

Serum Calcium levels

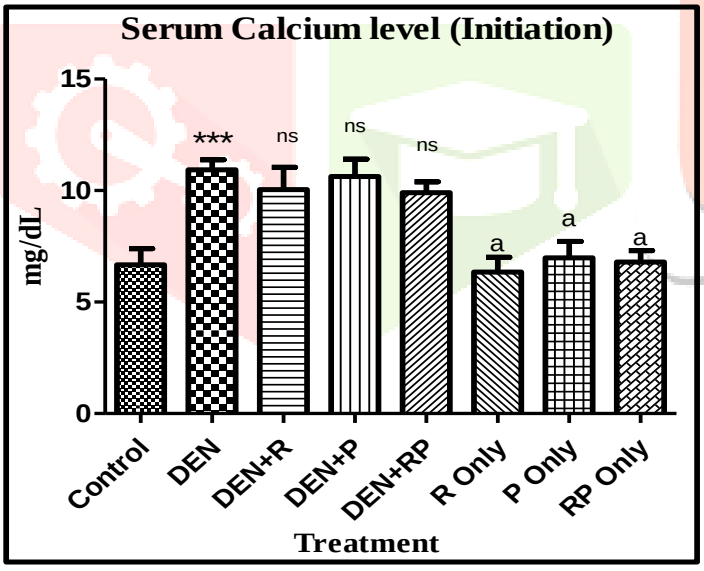

Figure 35: Serum calcium levels for initiation

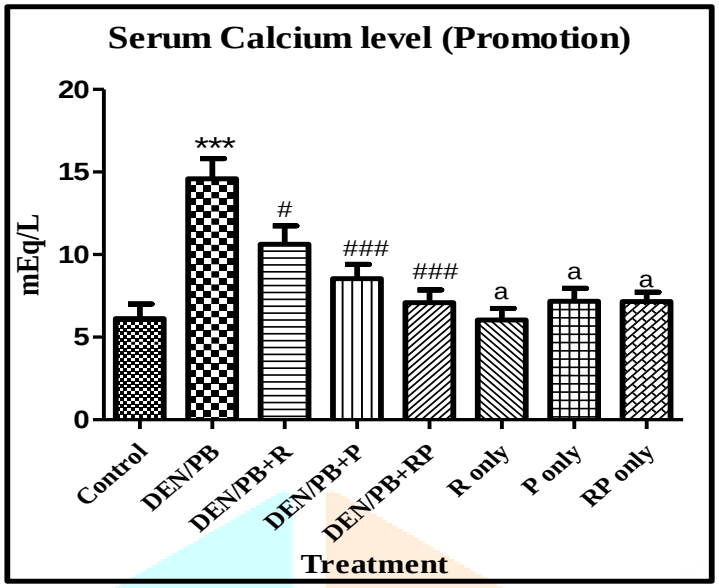

Figure 36: Serum calcium levels for Promotion

Serum magnesium levels

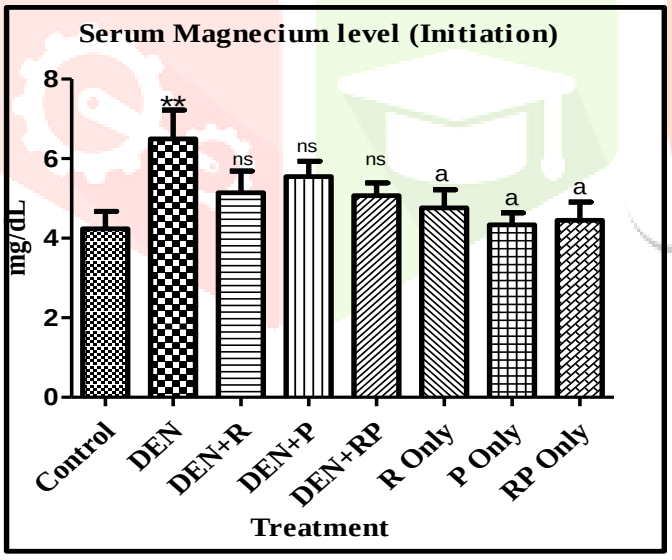

Figure 37: Serum magnesium level for Initiation

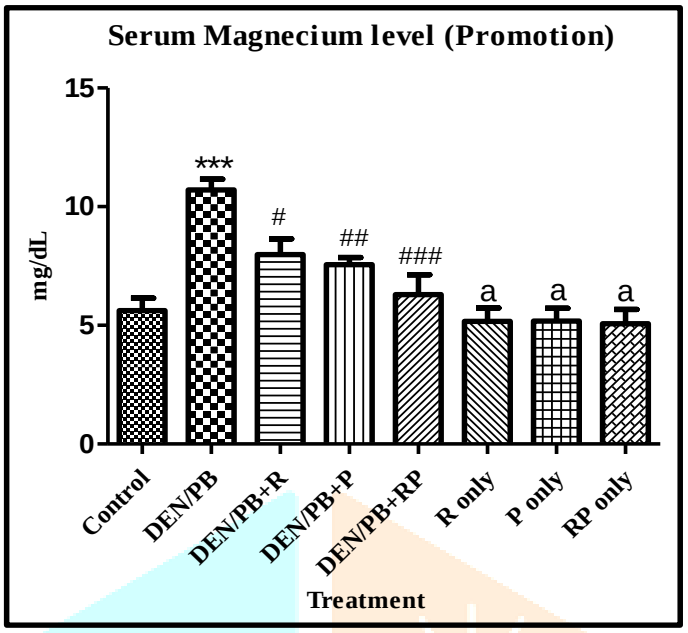

Figure 38: Serum magnesium level for Promotion

Estimation of membrane bound enzymes

Table 10: Estimation of membrane bound enzymes (Initiation)

Particulars	Group I (Control)	Group II(DEN)	Group III (DEN + R)	Group IV(P)	Group IV(RP)	Group VI(R only)	Group VII(P only)	Group VIII(RP only)
Na/K ATPase (µg/ml)	1.96 ± 0.22	1.16 ± 0.16	1.41 ± 0.16	1.70 ± 0.21	1.81 ± 0.21	2.14 ± 0.20	1.96 ± 0.18	2.02 ± 0.23
Ca/ATPase (µg/ml)	2.09 ± 0.10	1.07 ±0.10	1.32 ± 0.18	1.71 ±0.17	1.92 ± 0.22	2.30 ± 0.22	1.94 ± 0.25	1.97 ±0.20
Mg ATPase (µg/ml)	2.18 ± 0.16	1.41 ± 0.15	1.89 ± 0.13	1.92 ± 0.26	1.99 ± 0.36	2.14 ± 0.30	2.19 ± 0.37	2.30 ± 0.46

Results are expressed as Mean ± SEM, n=6

Table 12: Estimation of membrane bound enzymes (Promotion)

Particulars	Group I (Control)	Group II(DEN)	Group III (DEN + R)	Group IV(P)	Group IV(RP)	Group VI(R only)	Group VII(P only)	Group VIII(RP only)
Na/K ATPase (µg/ml)	2.09 ± 0.15	0.83 ± 0.09	0.93 ± 0.07	1.49 ± 0.29	1.41 ± 0.20	2.35 ± 0.19	2.30 ± 0.19	1.92 ± 0.20
Ca/ATPase (µg/ml)	2.33 ± 0.21	0.84 ± 0.10	1.11 ± 0.20	1.16 ± 0.16	1.22 ± 0.13	2.31 ± 0.46	2.31 ± 0.30	2.12 ± 0.26
Mg ATPase (µg/ml)	2.61 ± 0.28	0.74 ± 0.06	1.30 ± 0.17	0.96 ± 0.05	1.31 ±	2.19 ± 0.32	2.37 ± 0.28	2.27 ± 0.56

Results are expressed as Mean ± SEM, n=6

Sodium potassium dependent adenosine triphosphate (Na^+/K^+ATPase).

Results Exhibited that DEN treated groups showed reduction in Na^+/K^+ATPase compared with normal group in promotion model, No changes in were seen in initiation model but in promotion model & Promotion model.

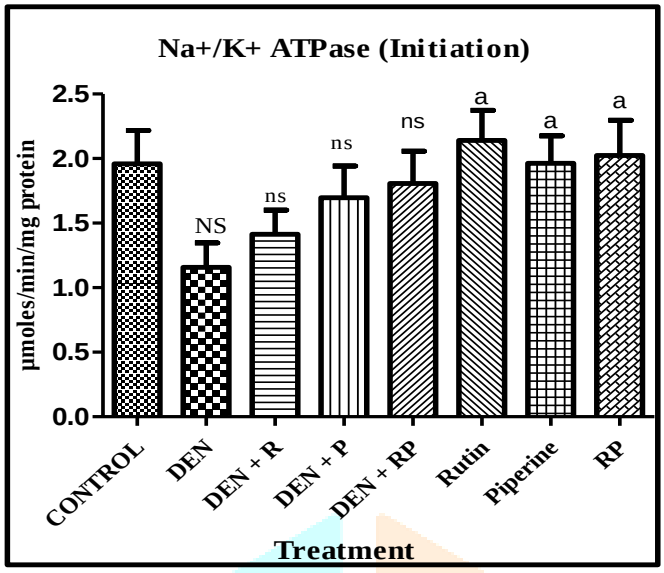

Figure 39: sodium potassium dependent adenosine triphosphate (Na^+/K^+ATPase) for initiation

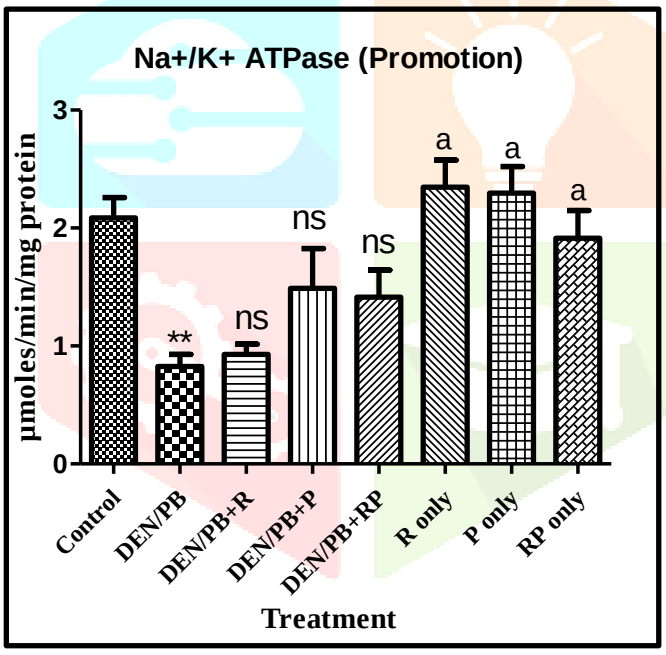

Figure 40: Sodium potassium dependent adenosine triphosphate (Na^+/K^+ATPase) for promotion

Values are expressed as Mean ± SEM (n=6), by one-way ANOVA followed by Bonferroni test. Where **< 0.01, compared with normal group ns, non significant of treatment groups compared with DEN treatment groups, a, is non significant compared to Normal group[46].

Calcium dependent adenosine triphosphate (Ca^{++}ATPase)

Results showed that in both the initiation and promotion models, the combination of the R&P group significantly increased the reduced levels at P0.05, while the DEN treated groups showed a reduction in Ca++ATPase compared to the normal group at p0.01 [47]. The R and P groups did not show any significant action Ca++ATPase levels. No changes in were seen in Positive control groups initiation and promotion model[48].

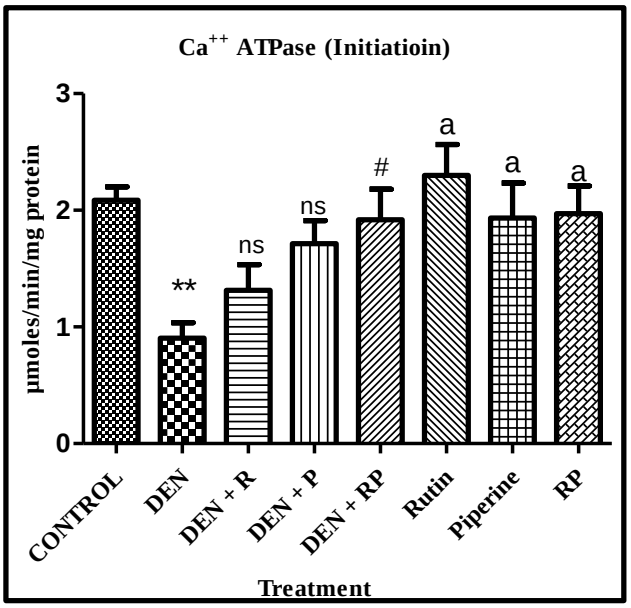

Figure 41: Calcium dependent adenosine triphosphate (Ca^{++}ATPase) for initiation

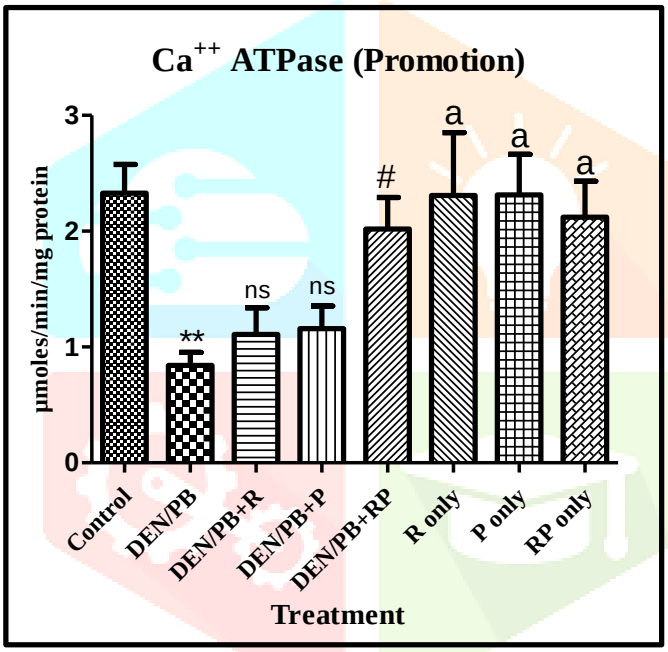

Figure 42: Calcium dependent adenosine triphosphate (Ca^{++}ATPase) for Promotion

Values are expressed as Mean ± SEM (n=6), by one-way ANOVA followed by Bonferroni test. Where ** is non-significant when compared to the Normal group and #p 0.05 when compared to the DEN group, ns stands for not significant when compared to the Normal group[49].

Magnesium dependent adenosine triphosphate (Mg^{++}ATPase)

Results showed that the DEN-treated groups had a p0.01 decrease in Mg++ATPase when compared to the untreated group, but that there were no significant changes in the initiation model[50]. On the other hand, the R and combination groups had a significant elevation (P0.05) of Mg++ATPase levels, but Piperine did not show any significant changes in the promotion model. In Initiation no significant action observed in initiation for all treatment groups[51]. No changes in were seen in Positive control groups initiation and promotion model [52].

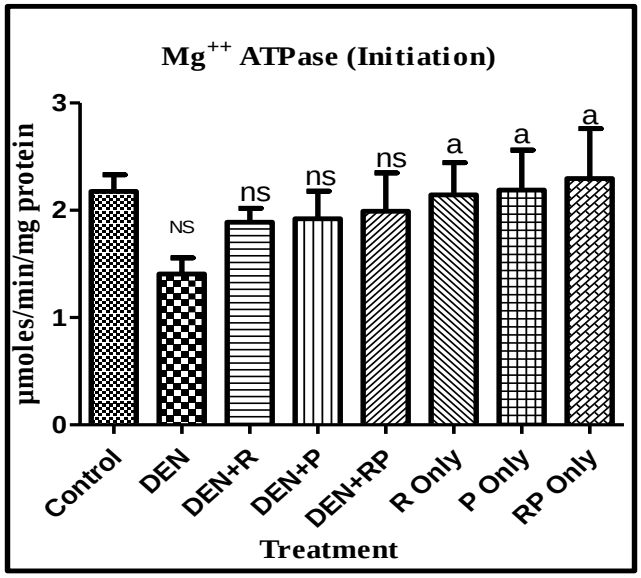

Figure 43: Magnesium dependent adenosine triphosphate for initiation

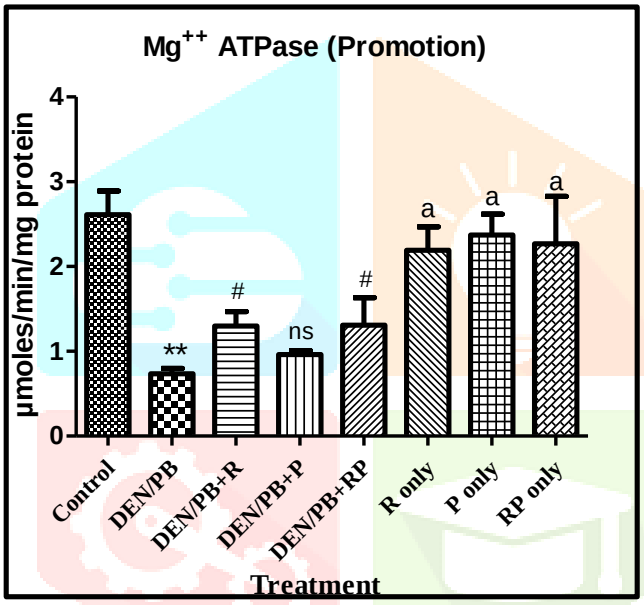

Figure 44: Magnesium dependent adenosine triphosphate for promotion

Values are expressed as Mean ± SEM (n=6), by one-way ANOVA followed by Bonferroni test. Where ** is non-significant when compared to the Normal group and #p 0.05 when compared to the DEN group, ns stands for not significant when compared to the Normal group[53].

Morphology of Liver

Morphology of liver Initiation

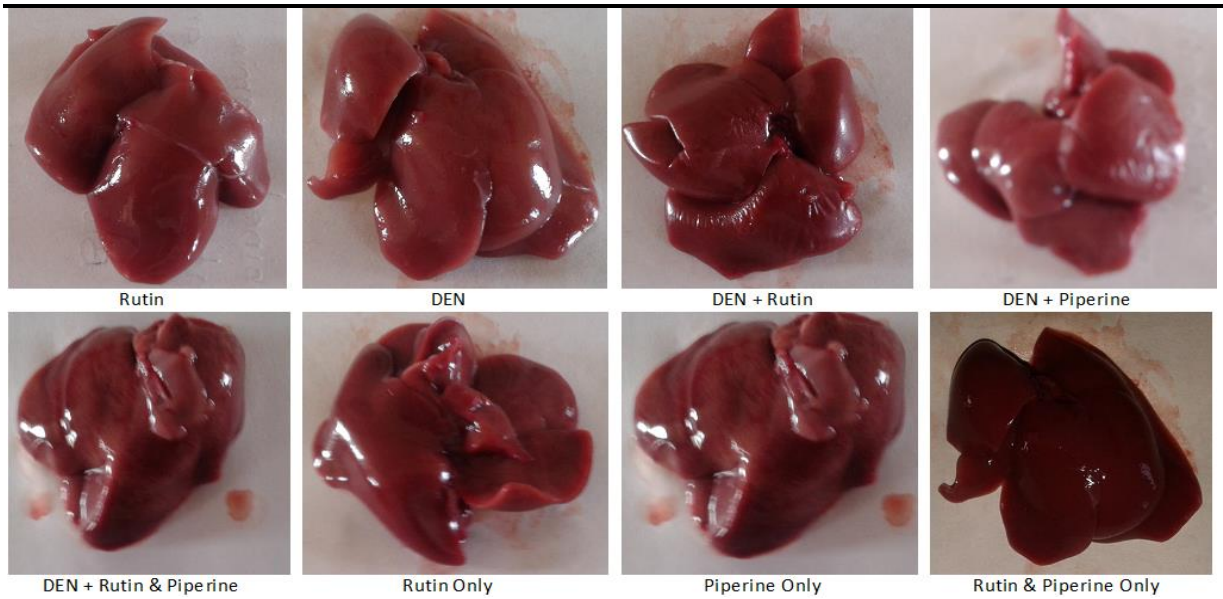

Figure 45: Morphology of Liver of all treatment groups for initiation model

Morphology of liver Promotion

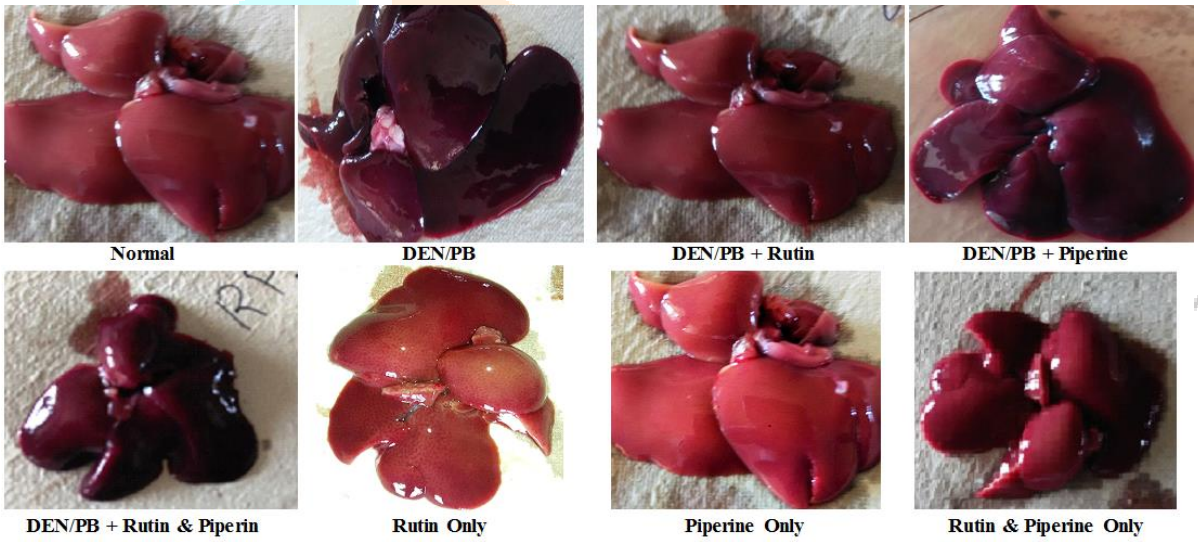

Figure 46: Morphology of Liver of all treatment groups for promotion model.

Histopathology of liver

Histopathology of liver for Initiation

Results showed normal architecture of liver with Central vein and sinusoids, DEN treated group showing that mild Spotty necrosis with ballooning of hepatocytes, and treatment groups showed much significant alterations in the normal architecture in initiation model [54].

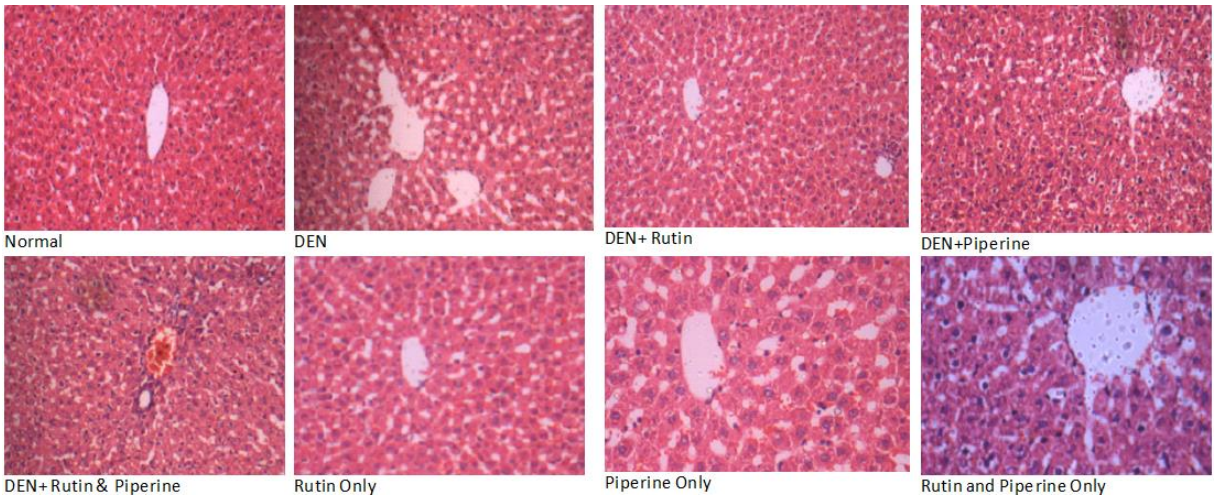

Figure 47: Histopathological examination of Liver for all treatment groups (Initiation model)

Histopathology of liver for Promotion

The results showed that the central vein and sinusoids were visible in the normal liver architecture; the DEN-treated group had spotty necrosis with ballooning of hepatocytes, centrilobular degeneration, vascular congestion, and kupffer cell hyperplasia; the treatment groups had significant improvements in the normal architecture and sinusoids; and the combination group had repairing of the cell architecture with clear visibility of the central vein. And groups that received only Rutin, Piperine, or a mixture of RP[55].

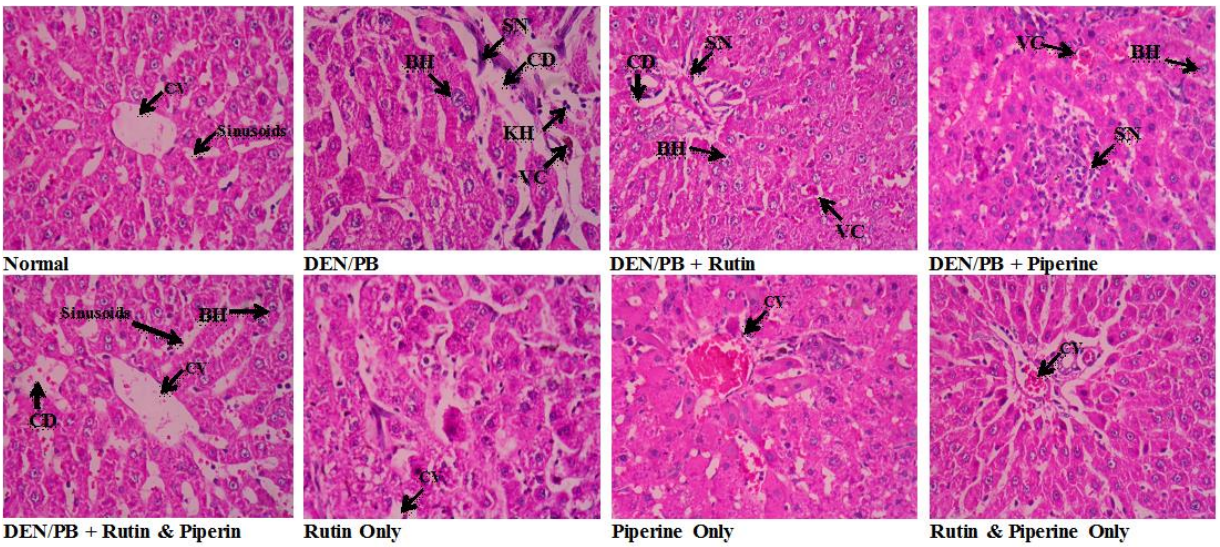

Figure 48 : Histopathological examination of Liver for all treatment groups (Promotion model)

CV: Central vein, CD: Centrilobular degeneration, BH: Ballooning of Hepatocytes, SN: Spotty Necrosis, VC: Vascular congestion, KF: Kupffercell hyperplasia[56].

Conclusion

The current study demonstrates that Rutin and Piperine have significant chemopreventive potential against DEN-induced hepatocellular carcinoma in rats, both on their own and in combination[57]. DEN administration resulted in marked hepatic injury, as evidenced by elevated liver enzymes, tumor markers, DNA content, and disrupted electrolyte balance. Treatment with Rutin and Piperine, especially in combination, effectively restored biochemical parameters, improved membrane-bound enzyme activity, and preserved liver histoarchitecture[58]. The RP combination showed superior efficacy in both initiation and promotion phases, indicating a synergistic interaction that enhances hepatoprotection[59]. The therapeutic potential of Rutin and Piperine as natural agents for the prevention of liver cancer is supported by these findings, which call for additional research into their molecular mechanisms and translational applicability[60].

References

1. El-Serag, H. B., & Rudolph, K. L. (2007). Hepatocellular carcinoma: Epidemiology and molecular carcinogenesis. *Gastroenterology, 132*(7), 2557–2576. https://doi.org/10.1053/j.gastro.2007.04.061

2. Surh, Y. J. (2003). Cancer chemoprevention with dietary phytochemicals. *Nature Reviews Cancer, 3*(10), 768–780. https://doi.org/10.1038/nrc1189

3. Srinivasan, K. (2007). Black pepper and its pungent principle-piperine: A review of diverse physiological effects. *Critical Reviews in Food Science and Nutrition, 47*(8), 735–748. https://doi.org/10.1080/10408390601062054

4. Ganeshpurkar, A., & Saluja, A. K. (2017). The pharmacological potential of Rutin. *Saudi Pharmaceutical Journal, 25*(2), 149–164. https://doi.org/10.1016/j.jsps.2016.04.025

5. OECD. (2008). *OECD Guidelines for the Testing of Chemicals: Section 423 – Acute Oral Toxicity*. OECD Publishing.

6. National Research Council (US). (2011). *Guide for the Care and Use of Laboratory Animals* (8th ed.). National Academies Press.

7. Pradeep, K., Raj Mohan, C. V., Gobianand, K., & Karthikeyan, S. (2010). Protective effect of Cassia fistula Linn. on diethylnitrosamine induced hepatocellular damage and oxidative stress in ethanol pretreated rats. *Biological Research, 43*(1), 113–125. https://doi.org/10.4067/S0716-97602010000100013

8. Pankaj, K., & Suresh, Y. (2015). Role of phenobarbital as a tumor promoter in hepatocarcinogenesis. *World Journal of Hepatology, 7*(3), 596–602.

9. Bancroft, J. D., & Gamble, M. (2008). *Theory and Practice of Histological Techniques* (6th ed.). Churchill Livingstone.

10. Khan, R. A. (2012). Protective effects of Launaea procumbens on oxidative liver injury induced by CCl_4. *BMC Complementary and Alternative Medicine, 12*, 114.

11. Aebi, H. (1984). Catalase in vitro. *Methods in Enzymology, 105*, 121–126. https://doi.org/10.1016/S0076-6879(84)05016-3

12. Recknagel, R. O., Glende, E. A., Jr., Dolak, J. A., & Waller, R. L. (1989). Mechanisms of carbon tetrachloride toxicity. *Pharmacology & Therapeutics, 43*(1), 139–154.

13. Chopra, K., Tiwari, V., & Arora, V. (2013). Protective role of Rutin against chronic liver injury in rats. *Journal of Pharmacy and Pharmacology, 65*(4), 518–524.

14. Al-Rejaie, S. S., et al. (2013). Protective effect of Rutin on the antioxidant defense system in CCl_4-induced hepatotoxicity in rats. *Experimental and Toxicologic Pathology, 65*(5), 513–521.

15. Pradeep, K., & Karthikeyan, S. (2011). Hepatoprotective and antioxidant effects of Rutin and quercetin in rats. *Indian Journal of Experimental Biology, 49*, 282–292.

16. Habig, W. H., Pabst, M. J., & Jakoby, W. B. (1974). Glutathione S-transferases: The first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry, 249*(22), 7130–7139.

17. Rajesh, M., et al. (2010). Protective effect of Piperine on hepatocellular carcinoma induced by DEN in rats. *Pharmacognosy Research, 2*(3), 163–170.

18. Rajesh, R., & Latha, M. S. (2011). Hepatoprotective activity of Piperine against DEN and CCl_4-induced liver damage. *Asian Journal of Pharmaceutical and Clinical Research, 4*(3), 84–88.

19. Bhatia, N., & Koul, A. (2014). Piperine inhibits DEN-induced hepatocarcinogenesis by modulating phase I and II enzymes. *Human & Experimental Toxicology, 33*(6), 574–583.

20. Lee, K. J., & Jeong, H. G. (2002). Protective effects of Rutin on liver damage induced by carbon tetrachloride. *Journal of Toxicology and Environmental Health, 65*(5–6), 419–431.

21. Pradeep, K., & Mohan, C. V. (2011). Biochemical markers of hepatic injury: Diagnostic and therapeutic significance. *Indian Journal of Clinical Biochemistry, 26*(1), 75–85.

22. Sahu, B. D., et al. (2013). Rutin attenuates oxidative stress and inflammation in rat liver injury. *Biomedicine & Pharmacotherapy, 67*(7), 619–627.

23. Lin, H., et al. (2014). Role of alpha-fetoprotein and CEA in liver carcinogenesis. *Clinical Biochemistry, 47*(7–8), 618–625.

24. Subramaniyan, V., et al. (2015). Chemopreventive effects of natural flavonoids on DEN-induced liver cancer. *Toxicology Reports, 2*, 654–663.

25. Dandekar, D. S., & Jain, A. (2013). Antioxidant and hepatoprotective effects of Rutin and Piperine combination. *Pharmacologyonline, 2*, 47–56.

26. Manna, P., et al. (2008). Protective role of Rutin in hepatocellular injury induced by oxidative stress. *Journal of Toxicology, 2008*, 1–9.

27. Oboh, G., et al. (2014). Piperine and its hepatoprotective mechanism against oxidative stress. *Journal of Functional Foods, 9*, 543–549.

28. Devi, S. A., et al. (2011). Chemopreventive efficacy of Rutin and Quercetin in DEN-induced hepatocellular carcinoma. *Nutrition and Cancer, 63*(7), 1036–1044.

29. Tietz, N. W. (2006). *Clinical Guide to Laboratory Tests* (4th ed.). Saunders.

30. Schutte, K., et al. (2009). Alpha-fetoprotein as a biomarker in hepatocellular carcinoma. *World Journal of Gastroenterology, 15*(29), 3450–3456.

31. Chauhan, S., et al. (2012). CEA as an indicator in liver tumor progression. *Journal of Clinical Pathology, 65*(8), 713–718.

32. Williams, G. M., & Whysner, J. (1996). Epigenetic carcinogenesis, synergism, and risk assessment. *Toxicologic Pathology, 24*(6), 787–798.

33. Singh, D., et al. (2014). Inhibition of DNA synthesis and tumor suppression by Piperine. *European Journal of Pharmacology, 742*, 124–132.

34. Kaur, G., et al. (2010). Protective role of flavonoids in hepatic protein synthesis during carcinogenesis. *Food and Chemical Toxicology, 48*(12), 3385–3390.

35. Linder, M. C. (1991). *Nutritional Biochemistry and Metabolism*. Elsevier.

36. Choudhury, S., et al. (2011). Modulation of serum electrolytes during chemical hepatocarcinogenesis. *Indian Journal of Experimental Biology, 49*(10), 749–757.

37. Khan, R. A., & Ahmed, S. (2015). Restoration of electrolyte balance by flavonoids in DEN models. *Toxicology Mechanisms and Methods, 25*(8), 620–630.

38. Datta, K., & Sinha, S. (2014). Role of ion homeostasis in hepatic oxidative stress and carcinogenesis. *Biological Trace Element Research, 160*(3), 350–357.

39. Chatterjee, S., et al. (2012). Sodium and potassium imbalance in hepatic injury. *Indian Journal of Medical Research, 136*(5), 812–818.

40. Lee, S. E., et al. (2013). Restoration of ionic equilibrium by Piperine and Rutin. *Journal of Biochemical and Molecular Toxicology, 27*(9), 399–406.

41. Satyanarayana, U., & Chakrapani, U. (2020). *Biochemistry* (5th ed.). Elsevier.

42. Abdelaziz, D. H., et al. (2014). Effects of DEN on liver enzyme activity and electrolyte profile. *Toxicology Reports, 1*, 688–695.

43. Mishra, S., et al. (2015). Restoration of magnesium-dependent enzymes by bioflavonoids. *Journal of Enzyme Inhibition and Medicinal Chemistry, 30*(4), 610–617.

44. Pandey, R., et al. (2018). Effect of Rutin and Piperine on membrane-bound ATPases in hepatic injury. *Pharmacognosy Magazine, 14*(55), 227–233.

45. Devi, K. P., et al. (2015). Molecular mechanisms of Rutin and Piperine synergy in hepatoprotection. *Life Sciences, 133*, 38–45.

46. Reitman, S., & Frankel, S. (1957). Determination of serum glutamic-oxaloacetic and glutamic-pyruvic transaminases. *American Journal of Clinical Pathology, 28*(1), 56–63.

47. Kim, Y. J., et al. (2011). Regulation of Ca^{2+} ATPase activity in DEN-induced HCC. *Toxicology Letters, 204*(1), 57–63.

48. Pal, R., & Nath, R. (2013). Antioxidant and enzyme modulating effects of Piperine. *Journal of Pharmacological Sciences, 121*(3), 308–315.

49. Sainath, S. B., et al. (2014). Modulation of Ca^{2+} ATPase and Mg^{2+} ATPase in hepatocellular carcinoma. *Indian Journal of Physiology and Pharmacology, 58*(2), 126–132.

50. Sharma, R., & Singh, D. (2016). DEN-induced hepatotoxicity and biochemical restoration by flavonoids. *BMC Pharmacology and Toxicology, 17*(1), 41.

51. Singh, A., et al. (2017). Role of Piperine in modulation of oxidative stress enzymes. *Pharmacological Reports, 69*(6), 1142–1149.

52. Wang, C. Z., et al. (2018). Protective effects of Rutin and Piperine against hepatotoxicity. *Frontiers in Pharmacology, 9*, 561.

53. Chen, X., et al. (2016). ATPase activity modulation during DEN-induced hepatocarcinogenesis. *Toxicology and Applied Pharmacology, 313*, 110–120.

54. Luna, L. G. (1968). *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology* (3rd ed.). McGraw-Hill.

55. Thirumalai, T., et al. (2011). Hepatoprotective effect of Rutin on DEN-induced liver damage. *International Journal of Pharmaceutical Sciences Review and Research, 9*(2), 110–116.

56. Rajesh, M., & Suman, S. (2013). Histopathological evaluation of Piperine and Rutin in DEN-induced hepatocellular carcinoma. *Asian Pacific Journal of Tropical Biomedicine, 3*(5), 400–406.

57. Bhatia, N., & Koul, A. (2016). Chemopreventive efficacy of Piperine and Rutin against hepatocarcinoma. *Chemico-Biological Interactions, 254*, 146–156.

58. Singh, D., et al. (2019). Combined administration of Rutin and Piperine exerts synergistic hepatoprotective effects. *Phytomedicine, 54*, 43–51.

59. Ahmed, O. M., et al. (2020). Synergistic antioxidant and anti-inflammatory effects of natural flavonoids. *Journal of Ethnopharmacology, 258,* 112906.

60. Yadav, D. K., et al. (2021). Translational potential of Rutin and Piperine co-administration in hepatocellular carcinoma. *Frontiers in Pharmacology, 12*, 642723. https://doi.org/10.3389/fphar.2021.642723