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Anti-Tumor Activity of Phloretin in Treatment of Hepatocellular Induced Carcinoma in Rats

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ABSTRACT

This study aims to evaluate the anti-tumor activity of phloretin in treatment of induced HCC in rats. Fifty adult male albino rats were divided into five groups (n=10, each). Group I (control group), Group II (DMSO group). Group III (preventive) (pre-treated with phloretin for 2 weeks before HCC induction and continued during induction period). Group IV was HCC – Induced (DENA) group. Group V (therapeutic group) (treated with phloretin after HCC induction). Blood samples and liver tissues were collected at the end of the experiment. The results revealed that DENA induced liver damage as evidenced by significantly increase in (ALT), (AST), (ALP) (GGT) activities and decrease in albumin content. Also, caused oxidative stress as indicated by increase in (MDA) and (NO) level and decrease in (GSH), (GST) and (CAT) activity compared with the control values. Also, it decreased apoptosis by decrease in Caspase-3 and Caspase-8 concentration. Treatment with phloretin significantly reduced increased liver enzymes and oxidative stress; it also induced apoptosis by significant increase in Caspase-3 and Caspase-8 concentration compared to HCC group. Conclusions: this study suggests that phloretin plays an important role in protection against DENA induced HCC.

Keywords: Hepatocellular carcinoma; DENA; Phloretin; Apoptosis

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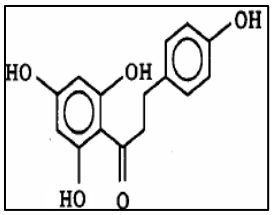
INTRODUCTION

Hepatocellular carcinoma (HCC) represents one of the most commonly tumors causing mortalities between populations all over the world, ranking the fifth of the most prevalent malignancies [1]. Globally, it is considered the fifth most common cancer and the second leading cause of cancer-related death [2]. The mean causes of hepatocellular carcinoma (HCC) in human cases are hepatitis B and C viruses [3]. Moreover, other risk factors such as air pollution [4], alcoholism [5], as well as several ingested carcinogens, such as aflatoxins [6] and nitrosamines [7] also included. Diethylnitrosamine (DENA) is a potent hepatocarcinogenic nitrosamine that is found in cheddar cheese, cured and fried meals, alcoholic beverages, cosmetics, agricultural chemicals and pharmaceutical agents, also present in ground water having high level of nitrate [8]. DENA is known to induce a wide range of tumors in all animal species [9] and causes oxidative stress during the metabolism that lead to cytotoxicity, mutagenicity and carcinogenicity [10,11]. DENA is known to induce damage in many enzymes involved in DNA repair and is normally used to induce liver cancer in experimental animal models [12]. DENA has been shown to be metabolized to its active ethyl radical metabolite and the reactive product interacts with DNA causing mutation, which would lead to carcinogenesis [13,14]. Unfortunately, the available treatment for HCC is still disappointing [15,16]. So, the prevention of HCC is of great importance. Flavonoids are polyphenolic compounds in a natural manner and represent one of the most extensive ingredients in fruits, vegetables, nuts, tea and coffee [17], as well as in herbal medicine [18]. Flavonoids are consisted of flavones, flavonols, flavanones, chalcones, anthocyanins, and isoflavones.

Phloretin, is a natural active compound which belongs to flavonoids and mainly present in apples and strawberries [19-21]. Phloretin is known for its pharmacological and biological properties such as antioxidative [22,23], antimicrobial [24] antitumor [25,26] and anti-inflammatory activity [27,28]. Also, it has numerous biological properties, including reduction of human platelets activity, competitive inhibition of sodium-glucose cotransporters (SGLTs), inhibition of cardiovascular disease and anticarcinogenic activity [29,30]. Phloretin has been known to inhibit liver cancer [31], breast cancer [32] and colon cancer [33]. It can serve the purposes of anti-oxidation, antitumor, anti-diabetes, antibiosis and para-hormone under physiological context [31,34,35].



Molecular structure of phloretin (chemical formula: C15H14 O5, molecular weight: 274.27).

MATERIALS AND METHODS

Animal Management

Adult male albino rats, weighing 80 -120 g, were obtained from the Experimental Animal Care Center and were housed in metabolic cages at the experimental animal house of the faculty of Science, Zagazig University under controlled environmental conditions (25°C and a 12 h light/dark cycle) one week before starting the experiment as acclimatization period. The animals were fed on a standard diet and tap water.

Hepatocarcinogenesis Model

The hepatocarcinogenesis was induced by DENA which was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Phloretin Dose Selection and Treatment

Phloretin was purchased from Sigma Chemical Co (St. Louis, MO, USA)**.** The animals received intraperitoneal injections of Phloretin 10 mg/kg three times per week [31,36].

Experimental Design

After the acclimatization period, a total of 50 adult male albino rats were divided into five groups with 10 animals in each group.

Group I (Negative control):

Animals were injected intraperitoneal (i.p.) with 1 ml saline single dose.

Group II (DMSO):

Animals were injected intraperitoneal (i.p.) with 1 ml of 0.2% DMSO for the entire experimental period.

Group III (Preventive):

Animals were pre-treated with phloretin (10 mg/kg three times a week [31] in 1 ml PBS by intraperitoneal injection for 2 weeks) before they were induced for HCC and also co-administered with phloretin during induction period.

Group IV (HCC – Induced) (Positive control):

Animals were induced for HCC by intraperitoneal (i.p.) injection with 75 mg/kg b.w of diethyl nitrosamine (DEN) once/week for 3 weeks, then 100 mg/kg b.w for another successive 3 weeks [37].

Group V (Therapeutic):

Animals were induced for HCC (as group 4). After the induction of HCC by DENA (after 6 weeks), animals were post treated with phloretin (10 mg/kg three times a week [31] in 1 ml PBS by intraperitoneal injection for 6 weeks).

Collection and Sampling of Blood

At the end of experimental period, the animals were fasted for 12 hours, anesthetized with ether, then they were killed by cervical decapitation and blood samples were collected in centrifuge tubes for separating the serum. The serum was prepared by collection of blood in anticoagulant –free tube, then left for 10 minutes in water bath at 37ºC until clot, then centrifuged at 2000 rpm for 10 minutes for separation of serum which was transferred into eppendorff tubes and kept frozen at -20ºC until analysis**.**

Liver Tissue Sampling

After blood collection, liver tissues were quickly excised from the animals, rinsed with ice-cold phosphate-buffered saline (pH 7.4) to flush out any blood, hepatic tissue samples were used for homogenate preparation, for estimation levels of NO, MDA and GSH and also, activities of CAT and GST**.** Also used for estimation of caspase-3 concentration.

Histophathological Analysis

Autopsy samples were taken from the liver of rats in different groups and fixed in 10% neutral buffered formalin solution (pH=7.4) for 24 hours, washing was done in tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration . Specimens were cleared in xylene and embedded in paraffin at 56 degree in hot air oven for 24 hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness by slidge microtome. The obtained tissue sections were collected on glass slides, deparaffinized and stained with hematoxylin and eosin (HE) dyes for examination through the light electric microscope [38].

Biochemical Analysis

Estimation of biochemical parameters:

Estimation of serum albumin was measured by colorimetric method of Doumas et al. [39]. The serum activities of ALT and AST were determined by colorimetric method of Reitman and Frankle [40], while the activity of ALP was performed according to the method of Belfield and Goldberg [41]. Also, the serum activity of GGT was measured by the method of Persijn and Vander Slik [42].

The level of hepatic MDA was estimated by the colorimetric method of Ohkawa et al. [43]. The level of NO was analyzed by the colorimetric method of Montgomery and Dymock [44]. The liver content of GSH was evaluated by colorimetric method of Beutler et al. [45]. The activity of GST was determined by UV method of Habig et al. [46]. Also activity of Catalase was determined by colorimetric method of Aebi [47] and Fossati et al. [48]. Kits of MDA, NO, GSH, GST and CAT were purchased from Biodiagnostic Company (Biodiagnostic, Egypt).

Estimation of caspase -3:

Caspase -3 was determined by Rat/Mouse caspase -3 Immunoassay Kit, (Catalog Number 201-12-0970**)** by ELIZA technique, SunRed Biotechnology Company.

Flow cytometry analysis of cell cycle and apoptotic marker (hepatic caspase -8):

Hepatic content of caspase-8 was evaluated by flow cytometric analysis using the method of Dean and Jett [49]. The cells of controls and experimental samples were prepared appropriately and then the antibody was added to the suspensions separately, incubated at room temperature for 30. After incubation, the cells were washed with cold PBS centrifuge at 1500 rpm for 5 minutes and discard the resulting supernatant, re-suspended into PBS then data acquired by flow cytometry [49].

Statistical Analysis

All results were analyzed by SPSS software (SPSS, ver.16.00, USA). Data were expressed as mean ± SD. Comparison of mean values of studied variables among different groups was done using ANOVA test. $P<0.05$ was considered to be significant [50].

RESULTS

Effect of Phloretin on Liver Function Tests

Biochemical analysis of liver enzymes activities in serum for different studied groups was shown in Table 1. DENA treated group showed marked elevations in the serum activities of ALT, AST and GGT ($p<0.0001$) and showed increase in ALP activity which was statistically non- significant ($p>0.05$), also, decreased serum concentrations of albumin was observed ($p<0.05$) when compared to control group.

Treatment with phloretin in preventive and therapeutic group significantly decreased the elevated serum activities of ALT, AST ($p<0.0001$), GGT ($p<0.01$) and ALP ($p>0.05$) in compared to DENA group. While the treatment markedly raised the lowered serum contents of albumin ($p<0.05$) in preventive but the induced effects were less potent in therapeutic group ($p>0.05$) in comparison with HCC induced group.

Table 1: The effect of phloretin on liver function tests in different studied groups

Group		ALB. (g/dl)	ALT (U/L)	AST (U/L)	ALP (U/L)
Negative control	Mean ± SD	4.38 ± 1.97	90.9 ± 22.2	169.8 ± 43.2	204.6 ± 58.6
DMSO	Mean ± SD	4.6 ± 1.3	105.2 ± 22.6	182.7 ± 31.5	208.6 ± 63.8
	%*	4.1	15.7	7.6	2
	P*	0.814	0.17	0.455	0.886
Preventive	Mean ± SD	4.1 ± 0.9	129.0 ±29.7	198.4 ± 30.0	213.7 ± 84.3
	%*	-7.1	41.9	16.8	4.4
	P*	0.658	0.004	0.103	0.782
	%**	55.9	-59.3	-52.9	-17.7
	P**	0.012	<0.0001	<0.0001	0.433
HCC-induced	Mean ± SD	2.6 ± 1.4	316.9 ±55.5	420.8 ± 65.2	259.7 ± 160.7
	%*	-40.4	248.6	147.8	26.9
	P*	0.032	<0.0001	<0.0001	0.322
Therapeutic	Mean ± SD	3.8 ± 1.5	181.9 ±35.9	257.3 ± 66.8	227.2 ± 70.4
	%*	-13.2	100.1	51.5	11
	P*	0.472	<0.0001	0.003	0.445
	%**	45.7	-42.6	-38.9	-12.5
	P**	0.08	<0.0001	<0.0001	0.565

P* and P** in compared to negative control and HCC-induced group respectively, value considered significant at **p<0.05**. Also, %* and %** percent change in compared to negative control and HCC-induced group respectively.

Effect of Phloretin on Antioxidant Levels

Our results presented in Table 2 showed that DENA treated group produced significant increase in the levels of hepatic MDA ($p<0.0001$) and NO ($p<0.01$) accompanied with marked decrease in the activity of antioxidants including GST, CAT ($p<0.01$) and also, significant decrease in GSH content ($p<0.0001$) in liver homogenate when compared to the control group.

Meanwhile, treatment with phloretin in preventive group caused significant decrease in the hepatic contents of both MDA ($p<0.01$) and NO ($p<0.05$), but the induced effects were less potent in therapeutic group which caused decrease in MDA and NO levels which was non-significant ($p>0.05$) when compared to HCC group. Moreover, preventive and therapeutic group caused significant increase in the activity of GST ($p<0.05$) ($p<0.01$), CAT ($p<0.0001$) ($p<0.05$) respectively, accompanied by major increase in hepatic GSH content ($p<0.0001$), ($p<0.05$) respectively in comparison with HCC induced group.

Table 2: Effect of phloretin on hepatic oxidative and anti-oxidative parameters in different studied groups

Group		NO (µmol /L)	MDA (nmol/g)	GST (U/g)	GSH (mg /g)	CAT (U/g)
Normal	Mean ± SD	31.5 ±17.2	23.7 ± 4.8	8.9 ± 0.95	6.6 ±1.9	2.4 ± 0.8
DMSO	Mean ± SD	39.6 ± 18.8	24.2 ± 5.2	9.0 ± 0.8	6.2 ± 1.4	2.0 ± 0.4
	%*	25.5	2.2	0.1	-6.3	-14.5
	P*	0.332	0.817	0.974	0.58	0.255
Preventive	Mean ± SD	45.3 ± 30.8	31.4 ± 7.6	8.0 ± 2.6	5.9 ± 2.0	1.7 ± 0.04
	%*	43.6	32.6	-10.5	-11.1	-30
	P*	0.234	0.014	0.295	0.404	0.013
	%**	-48.4	-54.1	82.7	117.4	32.5
	P**	0.022	0.002	0.014	< 0.0001	< 0.0001
DENA	Mean ± SD	87.7 ± 43.8	68.4 ± 32.0	4.4 ± 3.3	2.7 ± 1.2	1.3 ± 0.2
	%*	178.1	188.9	-51	-59.1	-47.2
	P*	0.001	< 0.0001	0.001	< 0.0001	0.001
Therapeutic	Mean ± SD	52.0 ± 41.1	50.3 ± 25.9	7.7 ± 1.3	4.4 ± 1.9	1.4 ± 0.2
	%*	65	112.2	-13.8	-34.3	-42
	P*	0.163	0.005	0.025	0.017	0.001
	%**	-40.7	-26.5	76.1	60.8	9.8
	P**	0.077	0.181	0.008	0.038	0.208

P* and P** in compared to negative control and HCC-induced group respectively, value considered significant at $p<0.05$. Also, %* and %** percent change in compared to negative control and HCC-induced group respectively.

Effect of Phloretin on Apoptotic Markers

Our results presented in Table 3 demonstrated that HCC induced group showed significantly decreased hepatic concentrations of caspase-3 and percentage of caspase -8 ($p<0.0001$) when compared to control group. However, treatment with phloretin in preventive group significantly increased hepatic concentrations of caspase-3 and

percentage of caspase -8 ($p<0.0001$) when compared to HCC group, but the induced effects were less potent in therapeutic group that caused slight increase but statistically non-significant in caspase-3 concentration ($p>0.05$) however, therapeutic group caused significant increase in percentage of caspase-8 ($p<0.01$) (Figure 1).

Table 3: Effect of phloretin on apoptotic markers in all studied groups

Group		Caspase-3(ng/ml)	Caspase-8(% of count)
Normal	Mean ± SD	14.2 ± 0.6	75.1± 8.91
DMSO	Mean ± SD	13.4 ± 0.8	63.6 ± 7.99
	%*	-5	-15.4
	P*	0.2955	0.06
Preventive	Mean ± SD	13.8 ± 0.6	47.9 ± 7.99
	%*	-2.6	-36.2
	P*	0.779	0.0001
	%**	26.4	55.7
	P**	<0.0001	<0.0001
DENA	Mean ± SD	10.9 ± 1.3	30.8 ± 7.99
	%*	-22.9	-59
	P*	<0.0001	<0.0001
Therapeutic	Mean ± SD	11.8 ± 1.5	38.2 ± 7.99
	%*	-16.3	-49.2
	P*	0.002	<0.0001
	%**	8.6	24
	P**	0.825	<0.0001

P* and P** in compared to negative control and HCC-induced group respectively, value considered significant at $p<0.05$. Also, %* and %** percent change in compared to negative control and HCC-induced group respectively

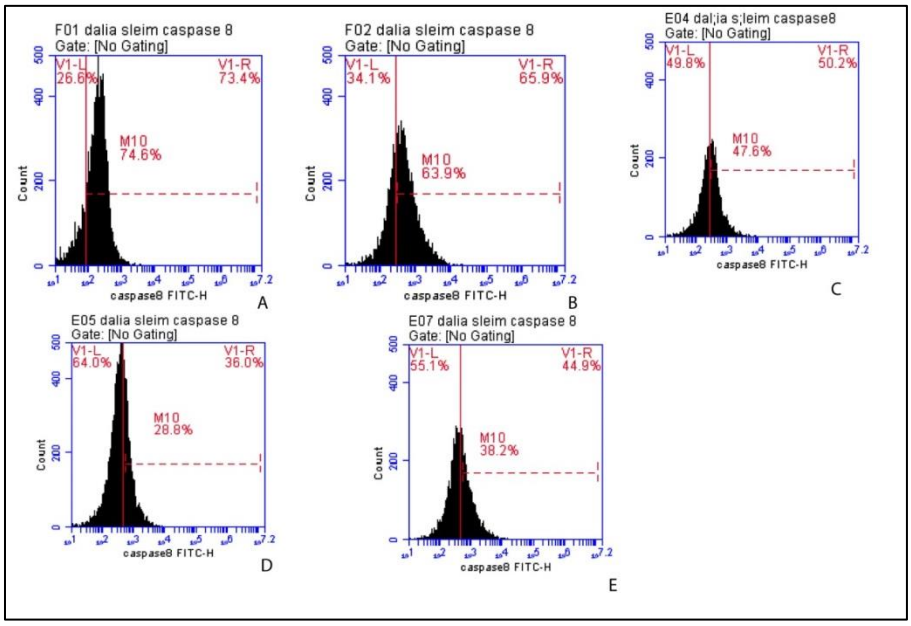


Figure 1: Flow cytometry histogram presented % of gated no. of +ve caspase 8 in different groups according to the FL1-detector of FITC caspase 8 flourochrome. FITC = Fluorescence Iso Thio Cyanate label.

Phloretin Induced Cell Cycle Arrest

Our data of cell cycle analysis presented in Table 4 , showed that DENA treated group produced a slight significant increase in Sub G apoptosis ($p<0.0001$) and major significant increase in S Phase and G2/M ($p<0.0001$) which was accompanied by significant decrease in G0/G1 ($p<0.0001$) when compared to control group. On the other hand treatment with Phloretin in preventive and therapeutic group showed significant increase in Sub G apoptosis and G0/G1 ($p<0.0001$) which was accompanied by significant decrease in S Phase and G2/M ($p<0.0001$) in comparison with HCC- induced group. These results indicate that phloretin could induce cell cycle arrest at G0/G1 phase (Figure 2).

Table 4: Effect of phloretin on cell cycle in different studied groups

Group		sub G (% of count)	G0/G1 (% of count)	S phase (% of count)
Normal	Mean ± SD	11.4 ± 0.5	73.7± 1.4	7.8 ± 0.9
DMSO	Mean ± SD	11.1 ± 0.3	69.5 ± 3.9	6.7 ± 0.66
	%*	-2.6	-5.8	-14.5
	P*	0.6	0.08	0.062
Preventive	Mean ± SD	24.8 ± 3.4	70.7 ± 5.2	5.6 ± 0.5
	%*	116.9	-4.1	-28.6
	P*	<0.0001	0.464	<0.0001
	%**	75.5	54.8	-83.9
	P**	<0.0001	<0.0001	<0.0001
DENA	Mean ± SD	14.1 ± 0.4	45.7 ± 0.4	34.6 ± 0.5
	%*	23.6	-38.1	344.7
	P*	<0.0001	<0.0001	<0.0001
Therapeutic	Mean ± SD	24.3 ± 2.0	65.1±3.2	12.2 ± 1.3
	%*	112.7	-11.7	57.4
	P*	<0.0001	<0.0001	<0.0001
	%**	72.1	42.5	-64.6
	P**	<0.0001	<0.0001	<0.0001

P* and P** in compared to negative control and HCC-induced group respectively, value considered significant at p<0.05. Also, %* and %** percent change in compared to negative control and HCC-induced group respectively

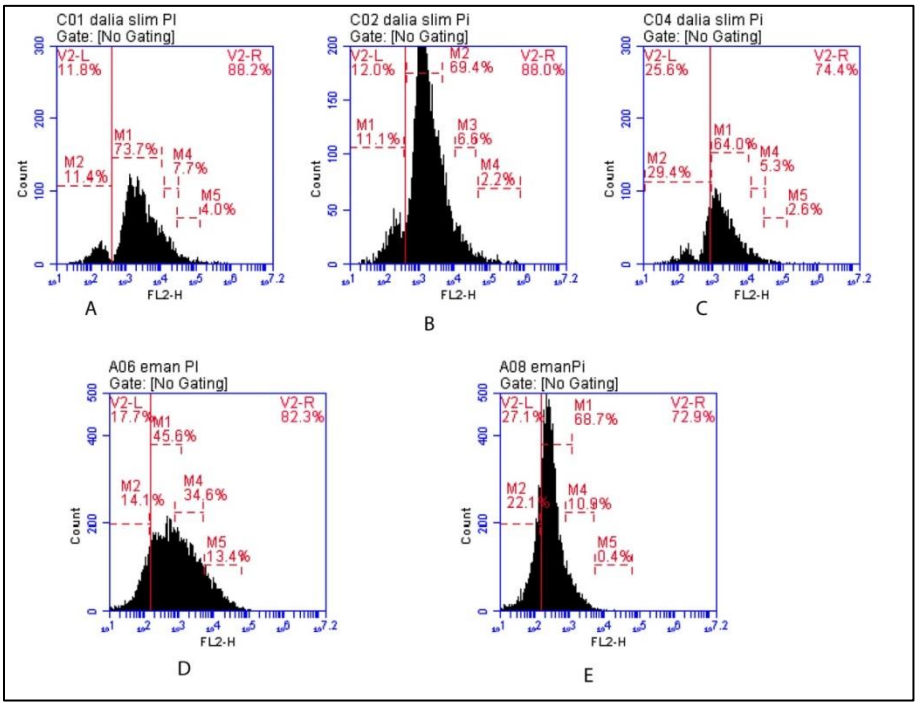


Figure 2: FACS analysis of cell cycle distribution histograms in all studied groups. A: negative control, B: DMSO, C: preventive, D: DENA (HCC-induced) and E: therapeutic

Histopathological Examination of the Liver Tissue

Histopathological examination of liver sections of normal control and DMSO group**s** showed that there was no histopathological alteration and the normal histological structure of the central vein and surrounding hepatocytes in the hepatic parenchyma was recorded in respectively**.** In preventive group sever dilation was noticed in the central vein associated with degeneration in the surrounding adjacent hepatocytes as well as appearance of other altered foci of degenerated hepatocytes in**.** However**,** in HCC- induced group fibroblastic cells proliferation was dividing the hepatic parenchyma into nodules of degenerated and necrotic hepatocytes as well as altered foci of dysplastic degenerated one in**.** In therapeutic group mild fibrosis was dividing the degenerated hepatocytes into nodules as well as formation of altered dysplastic foci and congestion in the portal veins in Figure 3 (Table 5).

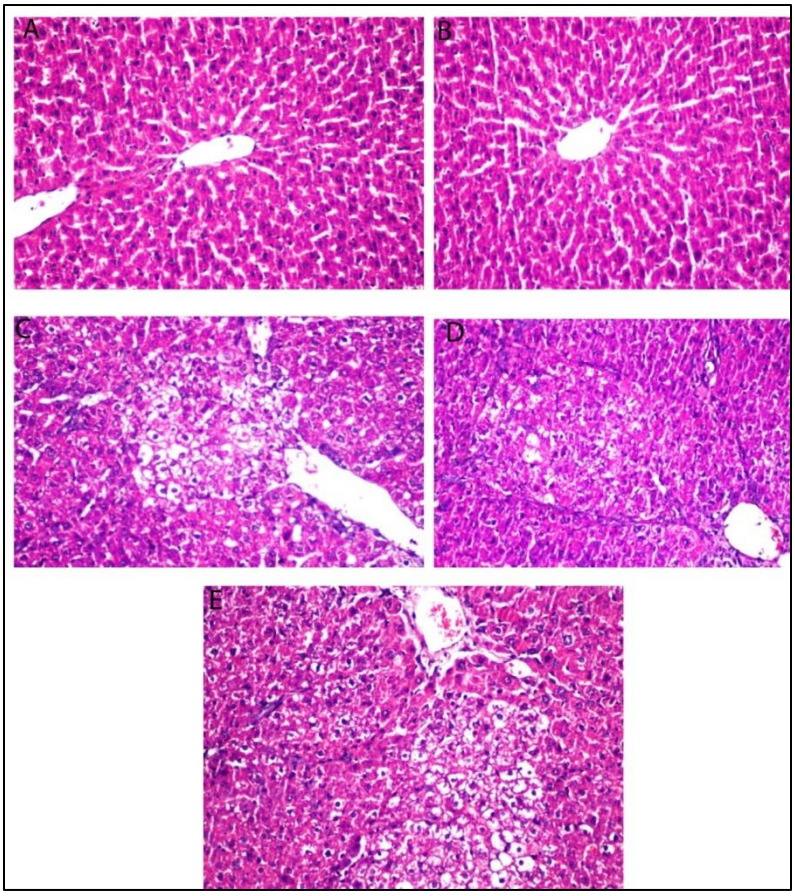


Figure 3: Histological architecture of rat liver tissue (A) normal control showing normal histology, (B) DMSO treated group showed apparently normal hepatocytes, (C) Preventive group showed the foci of altered hepatocytes (D) HCC-induced showing the altered foci of hepatocytes (E) Therapeutic group showed that hepatocytes returned to nearly normal arrangement with less vacuolation and sinusoids with less dilatation

Table 5: The severity of histopathological alteration in hepatic tissue of different studied groups

Histopathological Alteration	Negative control	DMSO	Preventive	DENA	Therapeutic.
Fibroblastic cell proliferation with nodule formation	_	_	+	+++	+
Foci of altered dysplastic hepatocytes. (precancerous)	_	_	+	+++	++

Where +++ → Sever, ++ →Moderate, + → Mild, - → Nil

DISCUSSION

Hepatocellular carcinoma (HCC) is considered one of the famous health problems; the fifth widely spread cancer in the world [51]. DENA is a genotoxic compound which forms alkyl DNA adducts and initiates several nuclear aberrations in the rat liver that finally lead to the development of HCC [52].

The present work investigate the role of phloretin in treatment of hepatic cell carcinoma (HCC) induced by DENA in rats. It is known that the ALT, AST, ALP, GGT serum activities are indicative for hepatic function, their increase is correlated with the hepatic injury [53]. DENA hepatic injury is related to the disturbance in hepatocytes membrane instability and metabolism resulting in alterations of the serum levels of these enzymes.

Our data illustrated that the mean level of ALT, AST, ALP and GGT activities showed to be slightly increased but statistically non-significant ($P>0.05$) in DMSO group when compared to normal control indicating that DMSO is safe and relatively nontoxic in agreement with Jamalzadeh et al. [54] who said that DMSO at concentrations of 0.1% and 0.5% had little or no toxicity. In DENA treated group the mean level of ALT, AST and GGT activities showed significant increase and ALP activity showed increase but statistically non-significant ($p>0.05$) along with significant decrease in serum content of albumin ($p<0.05$) when compared to normal control group which indicates

hepatocellular damage and impairment of liver function (Table 1). These elevations were also observed by several research groups Roy and Gadad [55], Rasha and Fares [56] and Amal et al. [57].

Kumar et al. [58] found that DENA treatment caused significant elevation of liver serum markers ALT, AST, ALP and GGT. Also our results are in line with Ramakrishnan et al. [59] who attributed the increases in serum aminotransferase enzyme activities to their intracellular location in the cytosol, so toxicity affecting the liver with subsequent breakdown in membrane architecture of the cells leads to their spillage into serum where their concentration rises. As concerning to level of albumin our results agreed with Metwally et al. [60] who showed a marked depletion in albumin level in DENA treated group as compared to healthy control group.

Phloretin treatment in preventive and therapeutic group significantly decreased the elevated serum activities of liver enzymes and raised albumin content when compared to HCC group suggesting that phloretin may have a potential protective effect against DENA induced liver damage. These results agreed with Zuo et al. [61] who revealed that phloretin significantly reduced ($p<0.05$) the elevated activities of serum liver enzymes (ALT, AST, GGT and ALP). DENA induces hepatic injury through the induction of disturbances in antioxidant defense systems, increases the reactive oxygen species (ROS) and membrane lipid peroxidation and consequently vital bio-membranes damage [62,63]. ROS can adversely affect various cellular biomolecules like protein, RNA and DNA causing serious damage to tissues and organs resulting in chronic disease such as cancer, heart disease, diabetes mellitus, arthritis and neurodegenerative disease [64] and peroxidation of lipids [65] , so to prevent cellular damage induced by ROS, the organism has a lot of antoxidative defense system, including the non-enzymatic (mainly GSH) and enzymatic antioxidant defenses such as (GST, CAT, SOD, GR, and GPx) which consider the key enzymes in elimination of free radicals. Our data showed that there was no significant change in the mean values of NO, MDA, GSH and GST, CAT activity concentration in DMSO group compared to control group ($p>0.05$) which indicate that DMSO is safe at 0.2% [54] (Table 2).

Our present study approved hepatic oxidative stress as indicated by increased production of hepatic MDA and NO, accompanying with decreased hepatic activities of antioxidants including GST, CAT and GSH level in rats treated with DENA compared to control group which indicates liver damage (Table 2). Our data are in agreement with previous findings Ghosh et al. [66] and Bendong et al. [67]. Also, Pradeep et al. [11] demonstrated that such subsequent decrease in the antioxidant defense is a result of the decreased expression of these antioxidants during hepatocellular damage.

As concerning to liver NO level, our results revealed a highly significant increase in DENA-treated rats as compared to the normal group. Our result is in line with Metwally et al. [60] and Afifi et al. [68] who observed a highly significant increase in NO level in DENA treated group**.**

Also, MDA level was found to be significantly elevated by DENA. In agreement with our finding Ahmedy et al. [69] and Kumar et al. [58] who found that administration of DENA led to increase in the levels of lipid peroxidation**.** Sivaramakrishnan et al. [70] revealed a reduction in (GST) enzyme activity in rat liver after induction with DENA which may be due to the excessive utilization of this enzyme in scavenging free radicals in the body. Also, Kumar et al. [58] found that DENA administration led to decrease in (GST) enzyme activity in the liver homogenate. Also, Ahmedy et al. [69] observed a marked depletion of GSH level in DENA-induced HCC group. Andre and Felley-Bosco [71] found that GSH conjugates with nitric oxide (NO) to form s-nitroso glutathione adduct, which is cleaved by the thioredoxin system to release GSH and NO so an increase in NO production by cytotoxicity caused an inhibition of glutamyl cysteine synthetase, a cytosolic enzyme help in GSH synthesis, leading to GSH depletion.

Ahmedy et al. [69] and Kumar et al. [58] found a significant decrease in the activity of catalase enzyme.

On the other hand, Phloretin treatment in preventive and therapeutic group decreased the elevation in oxidative stress markers (NO and MDA) and increased the activity of antioxidants including GST, CAT and GSH content when compared to HCC- induced group. These results agreed with Zuo et al. [61] who approved that phloretin significantly inhibited lipid peroxidation at suitable concentrations. Moreover, Xiao-yu et al. [72] reported that NO production of macrophages was significantly inhibited by phloretin. Moreover, it was observed that phloretin significantly decreased levels of NO [27]. In addition, Phloretin treatment significantly ($p<0.05$) restored the GSH level [73]. It was demonstrated that Phloretin is an effective antioxidant for inhibiting the peroxidation of nitroso anions and lipids, and it has antitumor functions [32]. Also, it was demonstrated that phloretin protect hepatocytes against oxidative stress [23].

Upon receiving a signal of apoptosis, a variety of proteases including caspases (cysteinyl aspartate-specific proteases) become activated within the cells planned for this pathway [74]. Caspases are expressed as carcinoma cases. These generally require proteolytic processing for their activation and are capable of self-activation as well as activating each other in a cascade-like process [75]. In order to survive, tumors always try to escape apoptosis by several mechanisms including downregulation or complete loss of caspase-3 expression. This disruption in caspase-

3 expression is usually associated with resistance to apoptosis as well as chemotherapy in different kinds of tumors [76].

Our data showed that there was a slight decrease which was statistically non- significant in the mean values of Caspase-3 concentration and Caspase-8 percentage in DMSO group compared to control group ($p>0.05$) (Table 3).

Our investigation approved that apoptotic markers including caspase-3 and caspase-8 proteins were significantly decreased in the rat liver of DENA group (HCC induced- rats) in comparison to control group and these findings were in agreement with those obtained by Mahfouz et al. [77] and Abouzied et al. [78].

On the other hand, treatment with phloretin in preventive and therapeutic group elevated the apoptotic markers (caspase-3 and Caspase-8) when compared to HCC induced group. These data are in agreement with Yang et al. [36] who demonstrated the activation of caspases 3, 8, and 9 and his observations indicates that caspase activation is involved in drug-induced apoptosis of human liver cancer cells and his results were Consistent with another study that detected that caspase activation, DNA fragmentation, and cleavage of poly(ADP ribose) polymerase in Phloretin-induced colon cancer cells (HT 29) [33]. Also, our results agreed with Liu et al. [79] who observed that after treatment with phloretin, a significant increase in the expression of the pro-apoptotic factors Bax and Bak and a decrease in the anti-apoptotic factor Bcl-2, suggesting that changes in the ratio of pro-apoptotic and antiapoptotic Bcl-2 family proteins might contribute to phloretin- induced apoptosis.

Cell cycle arrest would induce apoptosis, and influence proliferation. Many apoptotic signals affect apoptotic machineries as well as cell cycle progression at the same time. Therefore cell cycle analysis is one of the most important evaluations in apoptotic research. Furthermore, blocking cell cycle to induce apoptosis now serves as a new target for anticancer drugs.

CONCLUSION

Our data showed that there was a slight decrease which was statistically non- significant in the mean values of sub G apoptosis, G0/G1, S phaseand G2/M in DMSO group compared to control group ($p>0.05$) (Table 4).

Treatment of rats with DENA in DENA treated group produced a slight significant increase in Sub G apoptosis ($p<0.0001$) and major significant increase in S Phase and G2/M ($p<0.0001$) which was accompanied by significant decrease in G0/G1 ($p<0.0001$) when compared to control group. Roy and Gadad [55] reported that DENA‑treated group showed significant ($P<0.001$) increase in DNA and RNA when compared to normal group that was in harmony with previous studies which revealed that DNA and RNA levels are elevated in DENA‑treated animals [70]. On the other hand, treatment with Phloretin in preventive and therapeutic group showed a major significant increase in Sub G apoptosis and G0/G1 ($p<0.0001$) and showed marked significant decrease in S Phase and G2/M ($p<0.0001$) compared to HCC- induced group ,so these results indicated that phloretin could induce cell cycle arrest at G0/G1 phase.

These results agreed with Xiao-yu et al. [72] who found that exposure of T lymphocytes to phloretin resulted in the enrichment of G0/G1 phase, which was accompanied by a decrease in S and G2/M phase. By cell cycle analysis after Phloretin treatment, Wang et al. [80] found that the cells were arrested in G1 phase (G1 phase increased) with decreased proportion of S and G2 phases, causing a reduction in M phase and inhibited cell division. Therefore, Wang et al. [80] speculate that with the aromatic ring structure phloretin can insert into the DNA double helix, thereby preventing DNA synthesis, affecting cell cycle progression and leading to apoptosis.

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