Anti-tumor activity of phloretin in treatment of induced hepatocellular carcinoma in rats.

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A R T I C L E I N F O

A B S T R A C T

Background: Hepatocellular carcinoma (HCC) is a major health problem, classified among the higheast five most widespread malignancies. **Objectives**: this experimental study aims to assess the anti-tumor activity of phloretin in treatment of induced HCC in rats. **Methods:** fifty adult male albino rats were classified into 5 groups (n=10, each). Group **I** (control group), Group **II** (DMSO group). Group **III** (preventive) (pre-treated with phloretin for 14 day 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 peroid). At the end of the experiment blood serum samples and liver tissues were collected. **Results:** the results showed that DENA caused liver damage as proved by significant high increase in (ALT), (AST), (ALP) (GGT) activities and marked significant decrease in albumin content. Also, induced oxidative stress as pointed out an increase in (MDA) and (NO) level and decrease in (GSH), (GST) and (CAT) activity compared with the control values. Also, it decreased apoptotic pathway by decrease in Caspase-3 and Caspase-8 concentration. Treatment with phloretin significantly reduced the elevation in 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.

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]**. Universally, it is recorded the fifth most common cancer and the second cause of cancer-related death **[2]**. The occurance rates of HCC are two to three fold higher than in developed countries **[3]**.

The mean causes of hepatocellular carcinoma (HCC) in human cases are hepatitis B and C viruses **[4]**. Moreover, other risk factors such as air pollution **[5]**, alcoholism **[6]**, as well as several ingested carcinogens, such as aflatoxins **[7]** and nitrosamines **[8]** also included.

Diethylnitrosamine(DENA) is a powerful hepatocarcinogenic nitrosamine, that is found in cheese, cured and oil cooked meals, alcoholic

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beverages, cosmetics, agricultural chemicals and pharmaceutical agents, also present in ground water having high level of nitrate [9].Nitrate and nitrite that are additionally added to meat and fish for the purpose of preservation, as color fixatives and as flavouring [10].

DENA is known to cause a wide numbers of tumors in all animal species [11] and cause oxidative stress during the metabolism that lead to cy-totoxicity, carcenogenicity and carcinogenicity [12,13]. DENA is known to cause destruction in many enzymes responsible for DNA repair and is normally used to induce liver cancer in experimental animal models [14]. DENA has been metabolized to its active ethyl radical metabolite that interacts with DNA causing mutation, which would lead to carcinogenesis [15, 16]. DENA produces the promutagenic adducts, O6-ethyl deoxy guanosine and O4- and O6-ethyl deoxy thymidine that causes DNA-chain damage and miscode gene sequences, paving the way to initiation of liver carcinogenesis[17 , 18].
Unfortunately, the available treatment for HCC is still disappointing [19, 20]. So, the prevention of HCC is of great importance.

Flavonoids are polyphenolic compounds and represent one of the most wide great ingredients in fruits, vegetables, nuts, tea and coffee [21], as well as in herbs[22]. Flavonoids are involved flavones, flavonols, flavanones, chalcones, anthocyanins, and isoflavones. Flavonoids are well known for their importance as an anti-inflammatory and antioxidant agents and shown to have health encouraging, chemopreventive, and disease-preventing properties [23, 24].

Phloretin, is a natural active compound which included in flavonoids, exists in sap of apple, pear, strawberries, other fruits and vegetables [25, 26, and 27]. Phloretin is established for its pharmacological and biological properties such as antioxidative [28, 29], antimicrobial [30] anticancer [31, 32] and anti-inflammatory activity [33, 34].Also, it has different biological finctions, including decreased human platelets activity, competitive inhibition of sodium-glucose cotransporters (SGLTs), reduced cardiovascular disease and anticarcinogenic activity [35, 36]. Phloretin has been known to inhibit cancer of liver [37], breast cancer [38] and colon cancer [39]. It can serve the functions of anti-oxidation, antitumor, anti-diabetes, antibiosis and para-hormone under physiological context [37, 40 and 41].
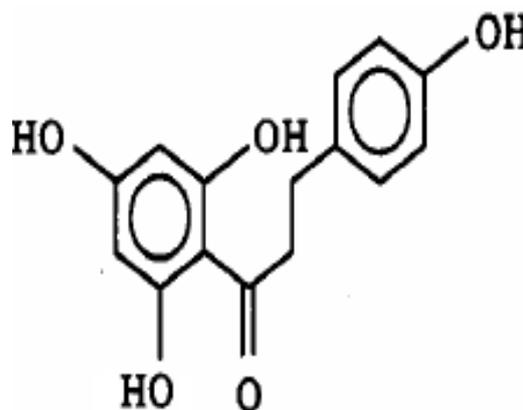
The molecular structure of phloretin (chemical formula: C15H14 O5, molecular weight: 274.27).

Materials and Methods:
Animal management

Adult male albino rats, weighing 80 - 120 g, were obtained from the Experimental Animal Care Center and were keeped in metabolic cages at the experimental animal house of the faculty of Science, Zagazig University under regulated environmental conditions (25°C and a 12 h

light/dark cycle) 7 days before starting the experiment. The animals were nourished 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 bought from Sigma Chemical Co (St. Louis, MO, USA). The animals intraperitoneally injected with Phloretin 10 mg/kg three times per week [37, 42].

Experimental design

To accomplish the ultimate goal of this study, after the acclimatization period of 7 days with standard basal diet, a total of 50 adult male albino rats were classified into five groups with 10 animals in each group.

Group I (Negative control): Animals were given an intraperitoneal injection with 1 ml saline single dose.

Group II (DMSO): Animals were given an injected intraperitoneal (i.p.) with 1 ml of 0.2% DMSO for the all experimental period of 6 weeks.

Group III (Preventive): Animals were pre-treated with phloretin (10 mg/kg three times per a week [37] in 1 ml PBS by intraperitoneal injection for 2 weeks) before they HCC induction and also co-administered with phloretin during induction period (6 weeks).

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 [43].

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 [37] in 1 ml PBS by intraperitoneal injection for 6 weeks).

Collection and sampling of blood

At the final of experimental period, the animals were fasted for 12 hours, anesthetized with ether, then they were killed by cervical decapitation and blood samples were gathered 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 37 ºC until clot, then centrifuged at 2000 rpm for 10 minutes for setting apart of serum which was transferred into Eppendorff tubes and stored frozen at -20 ºC 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 by ELIZA.

Histophathological analysis

Autopsy samples were gatherd from the liver tissues of rats in different studied groups and fixed in 10% neutral buffered formalin solution (PH=7.4) for 24 hours, washed with tap water then serial alcohols (methyl, ethyl and absolute ethyl) were used for dehydration . Tissue samples were cleared in xylene and embedded in paraffin at 56 degree in hot air oven for

24 hours. Paraffin bees wax tissue blocks were sectioned at 4 microns thickness by slidge microtome. The tissue sections were deparaffinized then stained with hematoxylin and eosin (HE) dyes then examinedthrough light electric microscope **[44].**

Biochemical analysis

Estimation of biochemical parameters

Estimation of serum albumin was performed according to modified bromocresol green binding assay colorimetric method (BCG) **Doumas et al., [45]**. The serum activities of ALT and AST were determined by colorimetric method of **Reitman and Frankle [46]** *using* assay colorimetric kit, while the activity of ALP was performed according to the method of **Belfield and Goldberg [47]**.Also, the activity of GGT was measured in the serum of rats following the method described by **Persijn and Vander Slik [48].**

The Level of hepatic MDA was estimated by the method of **Ohkawa et al., [49]** using colorimetric kit. The level of NO was analyzed using colorimetric kit according to the procedure of **Montgomery and Dymock [50]**.The liver content of GSH was evaluated by using colorimetric kit according to the method of **Beutler et al., [51]**.The activity of GST was determined by UV method according to **Habig et al., [52]**. Also activity of Catalase was determined by colorimetric method according to **Aebi [53]** and **Fossati et al., [54].**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 enzyme – linked immunosorbant assay (ELISA) ,SunRed Biotechnology Company.

Flow cytometry analysis of cell cycle and apoptosis (hepatic caspase -8)

Hepatic content of caspase-8 was investigated by flow cytometric technique**[55]**.The cells of controls and experimental samples were prepared sutabily and then the antibody was added to the suspensions separately, keep warm at room temperature for 30. After incubation, the cells were washed with cold PBS centrifuge at 1500 rpm for 5 minutes and throw out the resulting supernatant, re-suspended into PBS then data obtained by flow cytometry **[55].**

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 **[56]**.

Results:

Effect of Phloretin on liver function tests

Biochemical studies of liver enzymes activities in serum for different groups was shown in **table.1**. DENA treated group showed high elevation 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)** when compared to control group. Meanwhile, decreased serum concentrations of albumin was observed (**p<0.05**) when compared to control group.

On the other hand, treatment of HCC induced rats with phloretin in preventive and therapeutic group significantly decreased the elevated serum activities of ALT, AST (**p < 0.0001**) , GGT (**p < 0.01**).However, ALP (**p>0.05**) was also reduced, but this was statistically non- significant relative to DENA group. While it significantlly raised the lowered serum contents of albumin (**p<0.05**) in preventive but showed non- significant elevation in albumin content (**p>0.05**) in therapeutic group in comparison with HCC induced group.

Effect of Phloretin on antioxidant levels

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

Meanwhile, treatment of HCC induced rats 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 concequences 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.

Effect of Phloretin on apoptotic markers (caspase-3 and caspase -8)

Our results presented in **table 3** demonstrated that treatment of rats with DENA in HCC induced rats significantly decreased hepatic concentrations of caspase-3 and percentage of caspase -8(**p < 0.0001**) when compared to control group. However, treatment of HCC rats 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**) but in therapeutic caused statistically significant increase in percentage of caspase-8 (**p < 0.01**).

Phloretin induces cell cycle arrest

Our data of cell cycle analysis presented in **table (4)** , showed that 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. 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 cause cell cycle arrest at G0/G1 phase.

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).**

Discussion:

Hepatocellular carcinoma (HCC) is considered one of the famous health problems; the fifth widely spread cancer in the world **[57]**. HCC incidence and mortality have worledwide elevation over the last four decades [**58, 59**]. 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 **[60]**.

The present work investigate the role of phloretin in treatment of hepatic cell carcinoma (HCC) induced by DENA in rats.

It is established that the ALT, AST, ALP, GGT serum activities are indicative for hepatic function, their increase is correlated with the hepatic injury **[61].** 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., [62]** who said that DMSO at concentrations of 0.1% and 0.5% had little or no toxicity (table 1).

In DENA treated group the mean level of ALT, AST and GGT activities showed significant increase**(P < 0.001)** 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 evident by several research groups **Roy and Gadad [63], Glory and Thiruvengadam [64], Dhanasekaran et al., [65], Tanabe et al., [66], Rasha and Fares[67]** *and* **Amal et al., [68].**

Kumar et al., [69] found that DENA treatment caused significant elevation of liver serum markers ALT, AST, ALP and γ-GTP. Previous studies showed disturbances in the serum activities of transaminases in many models of DENA-induced HCC **[70, 71].**Also our results are in line with **Ramakrishnan et al., [72]** who ascribed the elevation 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. Moreover, our results agreed with who reported that the increase of ALT and AST

serum levels are specific to hepatocellular disturbance **Al- Rejaie et al., [73]**. In addition, **Abdel-Hamid et al., [74]** reported that DENA significantly increased serum of ALT, AST and gamma-glutamyl transferase activities in rats.

Another hepatic marker is ALP enzyme which is related to the membrane lipid in canalicular ducts. Increased activity of ALP in serum reflects the biliary flow disturbance. So, the extra or intra-hepatic interference with the bile flow leads to elevation of ALP serum levels **[61]**.

Also, GGT is a membrane-bound enzyme that is present mainly in the canalicular ducts. **Zhao et al., [61]** reported that the increase of serum GGT level in DENA group may be attributed to its liberation from the cellular membrane into blood indicating cellular membrane damage as a result of carcinogenesis.

As concerning to level of albumin our results agreed with **Metwally et al., [75]** 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., [76]** who revealed that phloretin significantly reduced **(p < 0.05)** the elevated activities of serum liver enzymes (ALT, AST, GGT and ALP).

Liver is the main site of DENA metabolism. DENA induces hepatic injury through the induction of disturbances in antioxidant defense systems, increases the reactive oxygen species (ROS) and membrane lipid peroxidation and therefore vital bio-membranes damage **[77,78]**.ROS can negativelly affect various cellular biomolecules as protein, RNA and DNA causing sincere damage to tissues and organs resulting in chronic disease such as cancer, heart disease, diabetes mellitus, arthritis and neurodegenerative disease **[79]** and peroxidation of lipids **[80]**, 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% **[62] (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 **Rajeshkumar and Kuttan[81], Ghosh et al., [82]** and **Bendong et al., [83]**. Also, **Pradeep et al., [13]** 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., [75]** who observed a highly significant increase in NO level in DENA treated

group and **Afifi et al., [84]** who showed significant increase in nitric oxide (NO) in rats induced with DENA**.**

In our study, MDA level was found to be significantly elevated by DENA. In agreement with our finding **Ahmedy et al., [85] and Kumar et al., [69]** who found that administration of DENA led to increase in the levels of lipid peroxidation**.** Also**, Jayaprakash et al., [86]** reported that animals treated with DENA showed increased lipid peroxide formation and cell membrane damage and decreased levels of antiperoxidative enzymes. This is also in harmony with **Ramakrishnan et al., [87]** and **Pradeep et al., [13]** who noticed that administration of DENA increases the level of lipid peroxides in rat liver.

Sivaramakrishnan et al., [88] 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., [69]** found that DENA administration led to decrease in (GST) enzyme activity in the liver homogenate.

GSH plays an important role in maintaining the normal reduced state of cells and counteracting the harmful effects of oxidative stress **[87]**. **Andre and Felley- Bosco [89]** 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. 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. In addition**, Ahmedy et al., [85]** observed a marked depletion of GSH level in DENA-induced HCC group.

Catalase acts as supporting antioxidant enzymes by transforming H2O2 to H2O, thereby providing protection against ROS **[87]**. **Ahmedy et al., [85]** found a significant decrease in the activity of catalase enzyme. Also, **Kumar et al., [69]** found that DENA administration led to decrease in catalase activity in the liver homogenate.

The depletion of GSH content and the reduction in the activity of catalase enzyme may be a result of excessive MDA production during DENA metabolism.

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., [76]** who approved that phloretin significantly inhibited lipid peroxidation at suitable concentrations. Moreover, **Xiao-yu et al., [90]** reported that NO production of macrophages was significantly inhibited by phloretin. Moreover, it was observed that phloretin significantly decreased levels of **NO [33]**. In addition, Phloretin treatment significantly (**p <0.05**) restored the GSH level **[91]**. It was illustrated that Phloretin is an important antioxidant for inhibiting the peroxidation of nitroso anions and lipids, and it has antitumor functions **[38]**. Also, it was illustrated that phloretin protect hepatocytes against oxidative stress **[29].**

Upon receiving a signal of apoptosis, a different of proteases including the group of proteases called caspases will be activated within the cells planned for this pathway **[92]**. Caspases are integrally expressed as carcinoma cases. These generally need proteolytic processing for their activation and are capable of self-activation as well as activating each other in a cascade-like

process [93]. In order to continuous a live, tumors always try to inhibit apoptosis by several mechanisms including downregulation or complete loss of caspase-3 expression. This disruption in caspase-3 expression is usually related to resistance to apoptosis as well as chemotherapy in different kinds of tumors [94].

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., [95]** and **Abouzied et al., [96]**.

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 results are in line with **Yang et al., [42]** who found 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 in accordance 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) [39].Also, our results agreed with **Liu et al., [97]** 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. Moreover, these changes coincide with the degradation of poly-ADP-ribose polymerase (PARP), a substrate of caspase-3, and the activation of caspase-9.

Currently considered, cell cycle arrest would induce apoptosis, and influence proliferation. A number of apoptotic signals have an effect on 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.

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 phase**& **G2/M** in DMSO group compared to control group (**p>0.05**) (table4).

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 [63] 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 [88] .

On the other hand, treatment with Phloretin in preventive and therapeutic group showed 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 are in line with **Xiao-yu et al., [90]** who demonstrated that exposure of T lymphocytes to phloretin caused the enrichment of G0/G1 phase, which then cause a decrease in S and G2/M phase so phloretin could induce cell cycle arrest at G0/G1 phase in T lymphocytes. Also, **Wu et al., [37]** showed that treatment of HepG2 cells with 200 μM phloretin for 24 h significantly induced HepG2 cell death, evidenced by an increase in the sub-G1 population. By cell cycle analysis after Phloretin treatment, **Wang et al., [98]** 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 .With elevated phloretin concentration, the proportion of cells in G1 phase, hypodiploid DNA (sub-G1 population) increased and that of cells in S phase decreased. Therefore, **Wang et al., [98]** 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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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)	GGT (U/L)
Negative control	Mean ± SD	4.38 ± 1.97	90.9 ± 22.2	169.8 ± 43.2	204.6 ± 58.6	18.3 ± 6.6
DMSO	Mean ± SD	4.6 ± 1.3	105.2 ±22.6	182.7 ± 31.5	208.6 ± 63.8	18.7 ± 7.6
	%*	4.1	15.7	7.6	2.0	2.2
	P*	0.814	0.170	0.455	0.886	0.902
Preventive	Mean ± SD	4.1 ± 0.9	129.0 ±29.7	198.4 ± 30.0	213.7 ± 84.3	18.9 ± 12.6
	%*	-7.1	41.9	16.8	4.4	3.3
	P*	0.658	0.004	0.103	0.782	0.896
	%**	55.9	-59.3	-52.9	-17.7	-49.9
	P**	0.012	< 0.0001	< 0.0001	0.433	0.001
DENA	Mean ± SD	2.6 ± 1.4	316.9 ±55.5	420.8 ± 65.2	259.7 ± 160.7	37.7 ± 7.4
	%*	-40.4	248.6	147.8	26.9	106.0
	P*	0.032	< 0.0001	< 0.0001	0.322	< 0.0001
Therapeutic	Mean ± SD	3.8 ± 1.5	181.9 ±35.9	257.3 ± 66.8	227.2 ± 70.4	20.0 ± 12.6
	%*	-13.2	100.1	51.5	11.0	9.3
	P*	0.472	< 0.0001	0.003	0.445	0.710
	%**	45.7	-42.6	-38.9	-12.5	-46.9
	P**	0.080	< 0.0001	< 0.0001	0.565	0.001

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

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	0.580	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	-30.0
	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	-51.0	-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
	%*	65.0	112.2	-13.8	-34.3	-42.0
	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* & P** in compared to negative control and HCC-induced group respectively, value considered significant at **p<0.05**. Also, %* & %** percent change in compared to negative control and HCC-induced group respectively.

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
	%*	-5.0	-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	-59.0
	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	24.0
	P**	0.825	< 0.0001

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

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)	G2/M (% of count)
Normal	*Mean ± SD*	11.4 ± 0.5	73.7± 1.4	7.8 ± 0.9	4.0 ± 1.9
DMSO	*Mean ± SD*	11.1 ± 0.3	69.5 ± 3.9	6.7 ± 0.66	2.2 ± 0.64
	%*	-2.6	-5.8	-14.5	-45.1
	P*	0.6	0.08	0.062	0.132
Preventive	*Mean ± SD*	24.8 ± 3.4	70.7 ± 5.2	5.6 ± 0.5	2.8 ± 0.6
	%*	116.9	-4.1	-28.6	-30.1
	P*	< 0.0001	0.464	< 0.0001	0.6
	%**	75.5	54.8	-83.9	-79.1
	P**	< 0.0001	< 0.0001	< 0.0001	< 0.0001
DENA	*Mean ± SD*	14.1 ± 0.4	45.7 ± 0.4	34.6 ± 0.5	13.5 ± 0.4
	%*	23.6	-38.1	344.7	234.2
	P*	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Therapeutic	*Mean ± SD*	24.3 ± 2.0	65.1±3.2	12.2 ± 1.3	0.6 ± 0.2
	%*	112.7	-11.7	57.4	-86.1
	P*	< 0.0001	< 0.0001	< 0.0001	0.003
	%**	72.1	42.5	-64.6	-95.8
	P**	< 0.0001	< 0.0001	< 0.0001	< 0.0001

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

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
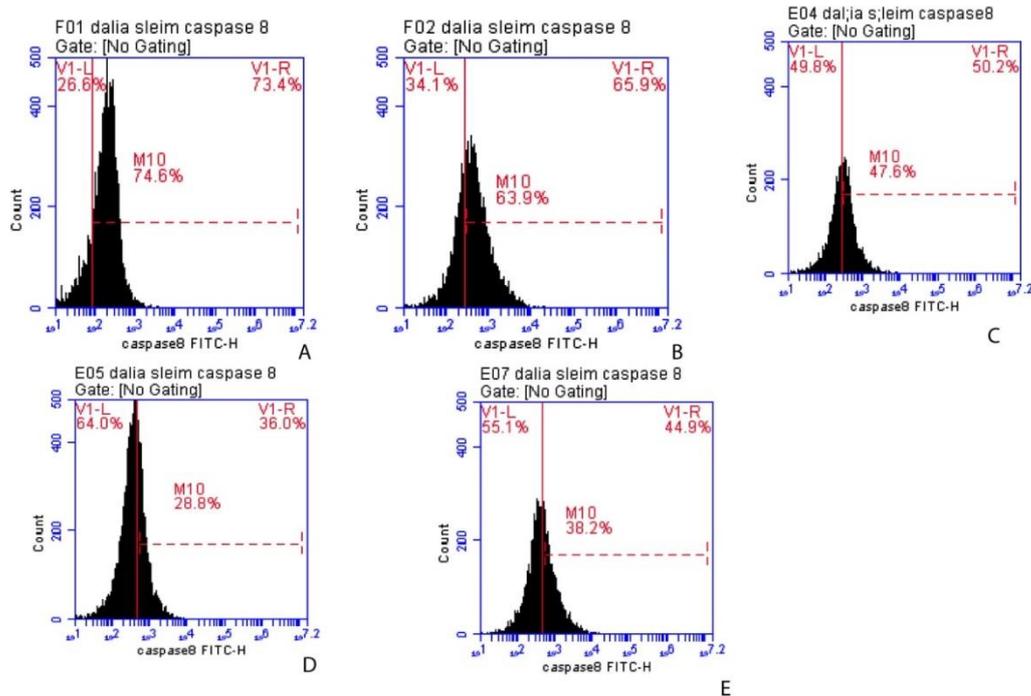
Figure 1: Flow cytometry histogram presented % of gated no. of +ve caspase 8 in different groups according to the FL1-detector of FITC caspase 8 flourochrome. FITC = Fluorescence Iso Thio Cyanate label.
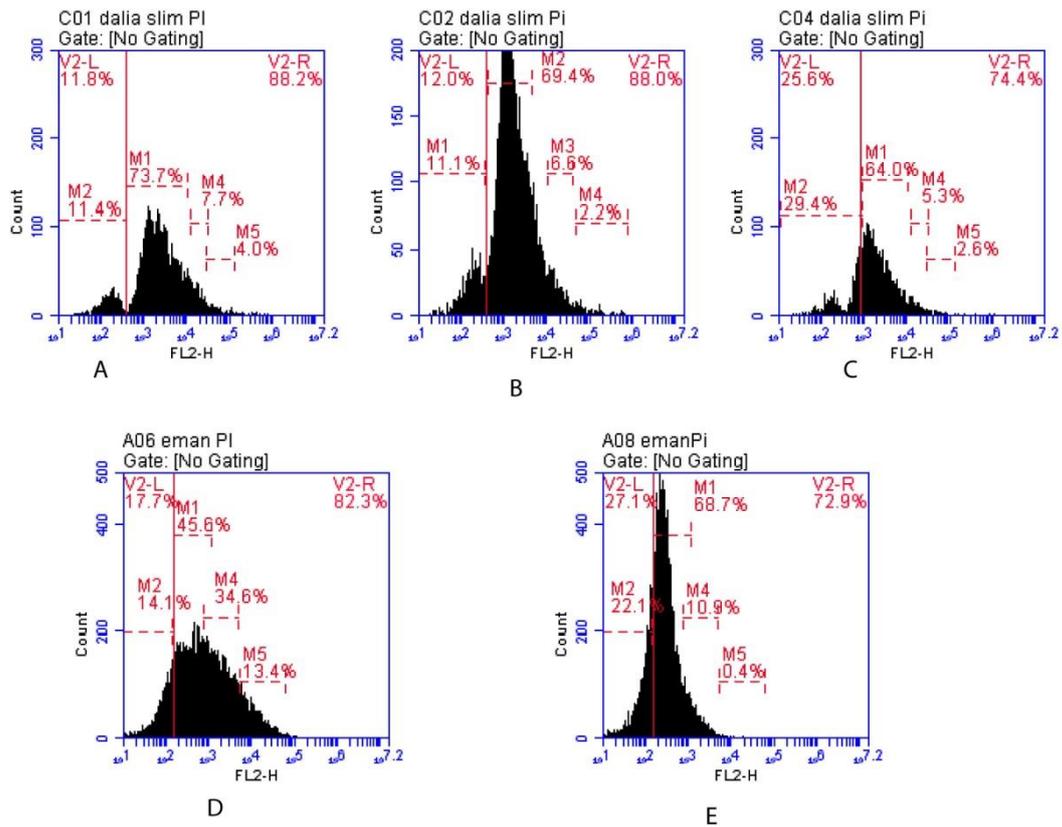
Figure 2: FACS analysis of cell cycle distribution histograms in all studied groups. A: negative control, B: DMSO, C: preventive, D: DENA (HCC-induced) and E: therapeutic.
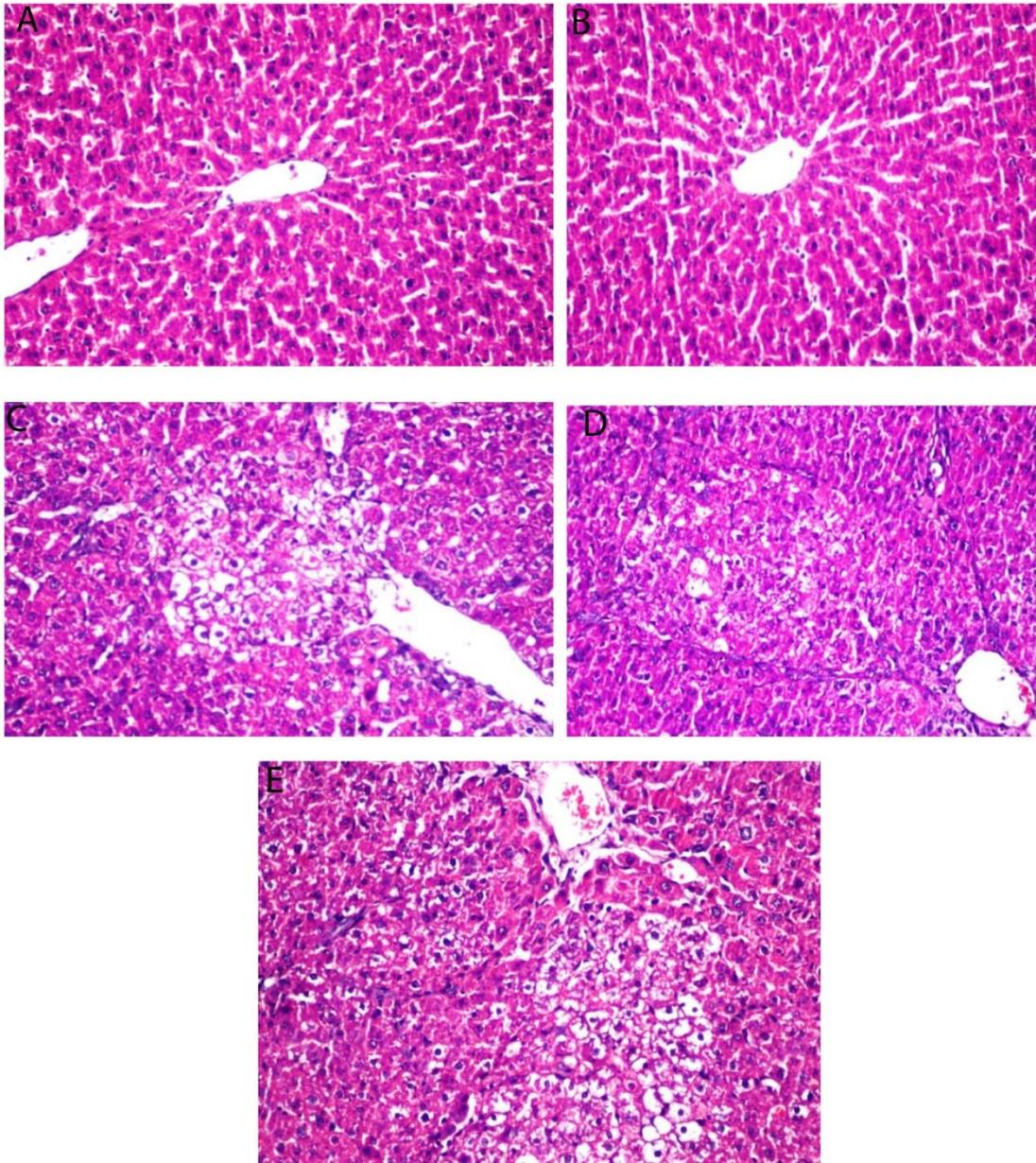
Figure 3: Histological architecture of rat liver tissue (A) normal control showing normal histology, (B) DMSO treated group showed apparently normal hepatocytes, (C) Preventive group showed the foci of altered hepatocytes (D) HCC-induced showing Showing the altered foci of hepatocytes (E) Therapeutic group showed that hepatocytes returned to nearly normal arrangement with less vacuolation and sinusoids with less dilatation.