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Beyond its antioxidant properties: Quercetin targets multiple signalling pathways in hepatocellular carcinoma in rats

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ABSTRACT

Aims: Hepatocellular carcinoma (HCC) pathogenesis involves the interplay of multiple signalling pathways. Notch and Hedgehog (Hh) are two major developmental pathways that act in concert to regulate adult cell repair. CK2α -serine-threonine kinase-down-regulation enhanced apoptotic activity and was proven beneficial for HCC patients. Quercetin is a bioactive flavonoid and has been shown to protect against HCC through its antioxidant activity. This study was carried out to elucidate the antineoplastic effect of quercetin through regulating both Notch and Hh pathways, apoptosis, cell proliferation and CK2α activity.

Main methods: Hepatocellular carcinoma was induced in male Sprague Dawley rats by thioacetamide. Quercetin was administered in both protective and curative doses. Parameters of liver function and oxidative stress were assessed. CK2α, Notch and Hh pathways were evaluated using RT-PCR and ELISA. Apoptosis was investigated by detecting caspase-3, caspase-8 and p53. Proliferative and cell cycle markers as cyclin D1 and Ki-67 were detected immunohistochemically.

Key findings: Quercetin inhibited CK2α and downregulated mRNA and protein expression of Notch1 and Gli2. Quercetin also suppressed caspase-3 expression but not caspase-8. Quercetin elevated p53 expression whereas proliferative and cell cycle markers cyclin D1 and Ki-67 were downregulated. Markers of hepatic cellular integrity such as AST, ALT, ALP, GGT, albumin and bilirubin were significantly ameliorated. This was confirmed by histological examination. Quercetin also alleviated oxidative stress as shown by SOD, GSH, MDA and NO levels.

Significance: We can conclude that in addition to its antioxidant power, quercetin blocked Notch, Hedgehog, regulated the apoptotic and proliferative pathways and inhibited CK2α in HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is of a major concern as it ranks the sixth among most commonly occurring cancers worldwide [47]. Mortality caused by HCC has also increased significantly [98]. HCC is an aggressive malignancy and its prognosis is poor. Curative options are either palliative or not possible for many patients as in the case of resection or liver transplantation [7]. This has prompted exploring signalling pathways that are implicated and altered during hepatocarcinogenesis to identify novel therapeutic targets [99].

Hepatocellular carcinoma (HCC) involves the interplay of various signalling pathways that regulate proliferation, apoptosis, and angiogenesis. Pathways such as Hedgehog, Notch and Wnt regulate most developmental embryonic processes [8,69,88].

Apoptosis is also implicated in the progression of HCC [22]. Apoptosis is a highly sophisticated process that involves an energy-dependent cascade of molecular events. Activation of the caspase cascade regulates apoptosis, mainly though caspase-3 which is responsible for the majority of the events [28]. p53 is a tumor suppressor gene that is involved in cell cycle regulation. Activation of the p53 family is involved in the DNA-damage response [25], chemosensitivity [24] and prognosis of HCC [29]. p53 family-mediated apoptosis signalling can be detected at different levels of HCC [27]. Uncontrolled cell proliferation causes the tumor cells to aquire damaged genes that directly regulate the cell cycle [77]. Cell cycle-related proteins were used in the assessment of the neoplastic cells behavior such as Ki-67 and cyclin D1. Cyclin D1 is involved in the transition through the G1 phase of the cell cycle [77].

Many studies revealed the oncogenic role of Notch signalling where it is activated in human HCC samples and in mice [26,95]. Notch

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Abbreviations

HCC	Hepatocellular carcinoma
NICD	Notch Intracellular Domain
RBP-J	Recombination Signal Binding protein
CSL CBF-1	Suppressor of Hairless, Lag-1
Hh	Hedgehog
PTCH	Patched
Smo	Smoothened
Gli	Glioma-associated oncogene
CK2α	Casein Kinase-2α
TAA	Thioacetamide
H&E	Hematoxylin & Eosin
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
NO	Nitric oxide
MDA	Malondialdeyhde
GSH	Glutathione
SOD	Superoxide dismutase

signalling pathway is a key regulator in the development and homeostasis of most tissues [81]. The classical canonical pathway involves a cell-cell communication system where a membrane-tethered ligand in one cell interacts with one of the four Notch receptors (Notch1-4) in the neighboring cell, releasing Notch intracellular domain (NICD). Afterwards, NICD is translocated into nucleus where it interacts with DNA-binding proteins RBP-J (Recombination Signal Binding protein). This culminates in target gene expression [46].

Notch signalling positively crosstalks with other pathways such as Hedgehog pathway (Hh) [8]. Notch signalling regulates ciliary trafficking of Hh components, Patched (PTCH) and Smoothened (Smo) [45]. The Hh signaling cascade is initiated by Hh family ligands binding to the PTCH receptor on the target cell. When the ligand is absent, PTCH represses the activity of Smo protein. Activated Smo induces the transcription factor Gli (glioma-associated oncogene) [69].The family of Gli transcription factors include Gli1, Gli2, Gli3. Many studies showed that although Gli1 is dispensable for animal development, Gli2 is the major mediator of Hh signalling and is required for embryogenesis. Gli2 is essential for hepatocellular carcinoma (HCC) growth and blocking Gli2 was found to be sufficient to repress HCC growth in cell lines [44]. Gli2 was also shown to directly control key cell cycle regulators in the G1 phase [41]. Aberrant activation of Hh has been observed in HCC [103]. Hh pathway has been shown to be abnormally activated in human HCC [37,79]. Moreover, HCC cell growth and motility decreased by blocking Hh signaling pathway [90].

Quercetin is a naturally-occurring flavonoid known for its antioxidant activity, proliferative and pro-apoptotic effects [35]. It has been recently shown to directly block the activity of casein kinase-2α (CK2α) [56]. CK2α is a ubiquitous protein serine/threonine kinase with oncogenic properties. CK2α was found overexpressed in HCC [106]. Similarly, CK2α positively regulates the activity of Hh signalling through phosphorylation and activation of Smo in Drosophila [38]. Inhibition of CK2α decreased Gli1 transcriptional activity in human lung cancer cells [109]. In addition, many studies pointed to the role of CK2α in apoptosis by removal of growth factors [89,104] and through activation of the death receptor pathway [17]. Moreover, CK2α was found to play a role in the repair of chromosomal DNA strand breaks [51]. This has prompted increasing interest in targeting CK2α in HCC therapy.

Herein, we investigated the anticancer activity of quercetin in an *in vivo* model of HCC. We show that in addition to its antioxidant activity, quercetin inhibited CK2α, blocked Notch and Hh pathways, induced apoptosis and suppressed cell proliferation.

2. Material and methods

2.1. Drugs and chemicals

Quercetin and Thioacetamide (TAA) were purchased from Sigma-Aldrich. (St. Louis, MO, USA). RNAlater RNA stabilization reagent and QIAzol lysis reagent from QIAGEN (QIAGEN Sciences, United States of America).

2.2. Animals

Sixty adult male Sprague Dawley rats 7 weeks old (180–200 g) were purchased from "Medical Experimental Research Centre" (MERC) (Faculty of medicine, Mansoura University, Mansoura, Egypt). Rats were provided with free access to food and water, at 12-h light/dark cycle, at 25 ± 2 °C. Animals were kept one week at these conditions for acclimatization. The experimental work adhered to the ethical principles and guidelines for the care and use of laboratory animals as instructed by the Scientific Research Ethical Committee in Faculty of Pharmacy, Mansoura University (code of approved protocol: 2016–75, code of approved manuscript: 2018-106) and ARRIVE guidelines.

2.3. Animal grouping and experimental design

Sixty rats were randomly divided into five groups each comprised of 12 rats: a)**normal control** group received PBS *p.o* once daily b) **drug control** received (quercetin 100 mg/kg dissolved in saline, *p.o*) once daily c) **HCC** group TAA (200 mg/kg, *i.p.*) solubilized in PBS and administered twice per week for sixteen weeks [19] d)**quercetin** (quercetin 100 mg/kg dissolved in saline, *p.o*) once daily for 21 days starting on week 17 after TAA administration. The dose and duration were selected based on our preliminary tests and previous study [84] e)**protection with quercetin** received quercetin (25 mg/kg) which was dissolved in normal saline and administered once 5 days/week *p.o* for eight weeks concurrently with TAA treatment. The dose and duration were selected based on our preliminary tests and previous study [31].

Blood samples were collected by retro-orbital puncture. Blood samples were centrifuged at 3000 × *g* for 20 min at 4 °C and sera were separated, divided into aliquots and stored at -80 °C for serum biomarkers analysis. Liver tissue was isolated and divided into three parts. The first part was immediately submerged in RNAlater RNA solubilizing solution stored in -80 °C for quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). The second part (about 0.5 g) was homogenized in ice-cold phosphate-buffered saline (PBS) (0.02 M, pH 7.4) (10% w/v) with an electric homogenizer on ice. The resulting suspension was centrifuged at 3000 × *g* for 20 min at 4 °C and supernatant was kept at -80 °C until further analysis. The third part was saved in 10% phosphate-buffered formalin (pH 7.2) for histopathological examination using Hematoxylin & Eosin (H&E) and immunohistochemical examination.

2.4. Assessment of alpha-fetoprotein

Alpha-fetoprotein was assessed in liver homogenates using ELISA based on the single step sandwich technique, where antigen present in the 25 µl sample is allowed to react with the two antibodies during the incubation period for 30 min at 37 °C. After washing, a complex is generated from immunological reaction. Chromogen solutions were added and allowed for catalysis by the formed complex. The concentration of the sample is directly proportional to the produced color intensity. The absorbance of each well was read using a microplate reader (Biotek-USA) set at 450 nm within 15 min (BioCheck Company,

South San Francisco, CA, USA).

2.5. Assessment of liver function biomarkers

Serum levels of liver biomarkers were assessed using colorimetric commercial kits as per the manufacturer's instructions. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were detected by kinetic methods where the reduced coenzyme activity is proportional to ALT and AST activities by mixing 100 µl of serum sample to 1000 µl of working solution and absorbance measured sperctrophotometrically at 546 nm (BioMed diagnostics Company, South San Francisco, CA, USA). Alkaline phosphatase (ALP) activity was determined by measuring the rate of hydrolysis of *p*-nitrophenyl phosphate where the colorless *p*-nitrophenyl phosphate is hydrolyzed by ALP to yield *p*-nitrophenol in alkaline solution; absorbance measured spectrophotometrically at 405 nm, the rate of increased absorbance is proportional to enzyme activity. 25 µl of serum sample were used following kits protocol (BioMed diagnostics Company, South San Francisco, CA, USA). Serum albumin was measured according to Bromocresol green method where bromocresol green forms colored complex with albumin. 25 µl of serum sample were mixed with 1000 µl color reagent, then absorbance was measured at 590 nm. The absorbance of this complex is proportional to the albumin concentration in the sample (Diamond Diagnostics Company, Hannover, Germany). Total bilirubin is converted to colored azobilirubin measured spectrophotometrically at 580 nm where 100 µl of serum sample were mixed with kit reagent. The intensity of the color formed is proportional to the bilirubin concentration in the sample (Diamond Diagnostics Company, Hannover, Germany). Gamma glutamyl transferase (GGT) catalyses the gamma-glutamyl group transport from gamma-glutamyl-p-nitroanilide to an acceptor glycylyglycine compound to form 2-nitro-5-aminobenzoic acid. This compound formed is measured spectrophotometrically at 412 nm and is directly proportional to the catalytic concentration of GGT present in the sample. 30 µl of serum sample were used following kits protocol (Spinreact Company, Spain).

2.6. Evaluation of oxidative stress markers

Liver homogenate was used for assessment of levels of nitric oxide (NO) concentration by employing the Griess reaction method to form a deep purple azo dye that can be spectrophotometerically quantified based on its absorbance at 540 nm. 0.1 ml of sample was added to 1 ml of Greiss reagent. Malondialdehyde (MDA) forms with thiobarbituric acid a product that is measured at 532 nm by mixing 0.2 ml of homogenate to 1 ml (Thiobarbituric acid) (BIODIAGNOSTICS assay kits, Giza, Egypt). Glutathione (GSH) was detected using BIODIAGNOSTICS assay kits Giza, Egypt. Where 5, 5'dithiobis 2- nitrobenzoic acid (DTNB) is reduced by glutathione (GSH) to give a yellow compound. The reduced compound is directly proportional to GSH content and its absorbance can be determined at 405 nm. 0.1 ml of sample was allowed to react with reagents provided in the kits and following the protocol. Superoxide dismutase (SOD) - BIODIAGNOSTICS assay kits (Giza, Egypt), activity was determined by its ability to inhibit nitro blue tetrazolium to an insoluble formazan. 0.1 ml of sample was mixed with 1 ml of reagent following kit's protocol. The absorbance was measured at 560 nm immediately.

2.7. Assessment of Notch 1 and Gli2 concentration levels

Liver homogenate was used for measurement of hepatic Notch1 using MyBioSource ELISA kit, (Sandiego, CA, USA) and Gli2 protein levels concentration using (Cloud-CloneCorp, Fernhurst, Katy, USA) where the microtiter plate provided in the kit has been coated with antibody specific to Notch 1 and Gli2 respectively. 100 µl of sample and Standards are then added to the microtiter plate well with a biotin-conjugated polyclonal antibody preparation specific for Notch 1. Upon adding HRP-streptavidin conjugate (SABC) and TMB substrate, a color is produced in wells containing Notch 1 or Gli2. The color change is measured at wave length of 450 nm. The concentration of Notch1 and Gli2 in samples is then calculated by comparing absorbance values of the samples to the standard curve.

2.8. Quantitative real-time PCR (qPCR)

RNA was extracted using QIAzol lysis reagent (QIAGEN Sciences, United States of America) as instructed by the manufacturer. Concentration and purity of RNA samples were assessed using NanoDROP2000 spectrophotometer ® thermoscientific. Synthesis of cDNA from RNA population was carried out using RevertAid First strand cDNA Synthesis kit (Thermo scientific). The product of the first strand cDNA synthesis was used directly in qPCR: 1 µl of the first strand cDNA synthesis reaction mixture is used as template for subsequent PCR in 50 µl total volume. qPCR was performed using SYBERGREEN kit and using StepOne™ Real Time PCR System, Applied Biosystems. The thermo cycler program used for real-time polymerase chain reaction: PCR initial activation step: at 95 °C for 10 min followed by a three-step 40 cycle of: denaturation at 95 °C for 15 s, annealing at 58:60 ^{O}C for 30 s, extension at 72 °C for minute. And ended by a final extension step at 72 °C for 6 min. The primers were designed by the aid of Primer3 (v. 0.4.0) software and were further verified with net primer software (Premier Biosoft, CA, USA). Target specificity was confirmed using BLAST software (National Library of Medicine, MD, USA). Table (1) shows primer sequences. Relative quantification of mRNA expression was calculated via the $2^{-\Delta\Delta CT}$ method. The data were presented as relative quantity of target mRNA, normalized respect to GAPDH mRNA and relative to a calibrator sample. Normal control group samples were used as calibrators.

2.9. Histopathological and immunohistochemical examination of liver sections of different groups

Liver tissues were kept in neutral buffered formalin and were then embedded in paraffin. To examine the cell structure by light microscope, 5 µm thick sections were prepared and stained with H&E stain. Microscopic pictures were captured using a digital camera aided computer system (Nikon digital camera, Japan).

For immunohistochemical examination, tissue sections were depraffanized and rehydrated. Slides were washed then incubated overnight with primary antibody against caspase-3 (Thermo Fisher Scientific, catalog # PA5-77887; 1:300 dilution), cyclin D1 (Thermo Fisher Scientific, catalog # PA5 -16777; 1:300 dilution), Ki-67 (Master Diagnóstica, Ref: MAD-004072 R/D; 1:50 dilution) and p53 (Dako p53 Protein Autostainer Clone DO-7, Ready to use, Code IS616) at 4 °C

Table 1
Primer sequence used for qPCR.

Gene name	Accession number	Forward primer	Reverse primer	Amplicon size
Notch 1	NM_001105721	GTGGGATGGACTGGACTGTG	CGCAGGAAGTGGAAGGAGTT	117
Gli 2	NM_001107169	GGCACCAACCCTTCAGACTA	GAGATGGATAGAGCCCGCTT	161
Casein kinase 2α	NM_053824.2	ATTGTCACAGCATGGGGATT	ACCTTTGAAATATCGGGAAGCA	200
Caspase-8	NM_022277.1	CCTTTCTCCTCCCTCTGACCTC	GTAACCTGTCGCCGAGTCCC	193
GAPDH	NM_017008.4	AAGTTCAACGGCACAGTCAAGG	CATACTCAGCACCAGCATCACC	165

overnight. Slides were washed and visualized using horseradish peroxidase labeled streptavidin-biotin detection system (GBI Labs, Mukilteo, WA, USA) following supplied instructions. Light microscope (Leica Microsystems, Wetzlar, Germany) was used for visualizing stained sections.

2.10. Statistical analysis

Data were expressed as means ± standard error of mean (SEM) in each group. Statistical evaluation of the results was carried out by means of one way analysis of variance (ANOVA) followed by post-hocTukey's test. Differences were considered significant when the p value was < 0.05. Statistical tests and graphs were generated using GraphPad Prism software (Graphpad Software Inc., San Diego, USA).

3. Results

3.1. Quercetin treatment ameliorated hepatic function

In order to test the effect of quercetin treatment on liver function, a number of routine tests were conducted to evaluate its activity. Serum aminotransferases (ALT and AST), GGT and ALP activities were assessed. Concentrations of bilirubin, total protein and albumin were measured and compared to the normal control and HCC groups. Both quercetin curative and protective groups lowered the levels of aminotransferases, GGT and ALP that were elevated in HCC group. Serum bilirubin levels were lowered in both curative and protective groups. However, albumin and total protein concentrations were significantly higher when compared with HCC group ($p < 0.05$) as shown in (Fig. 1).

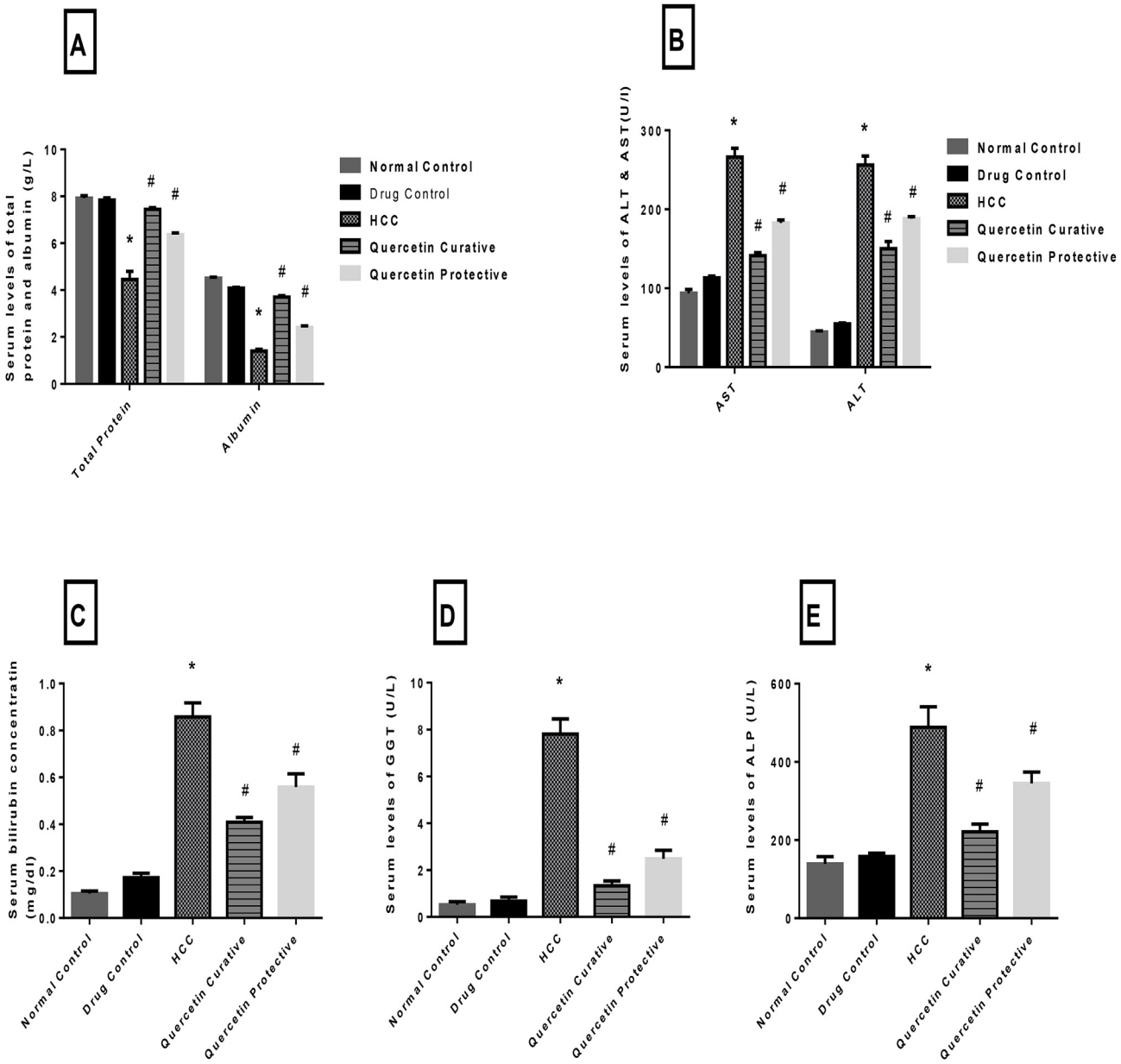

Fig. 1. Serum levels of A) Total protein and albumin B) Aminotransferases (ALT and AST) C) Bilirubin D) GGT E) ALP in normal control, Drug control, HCC, Quercetin curative and Quercetin protective groups. Data is expressed as mean ± SEM, n = 12 Different symbols represent indicate statistical significance of $p < 0.05$ (* compared to normal control group, # compared to HCC group) using regular one-way ANOVA followed by Tukey's multiple comparisons test.

Table 2
Levels of superoxide dismutase (SOD) activity, reduced glutathione (GSH), malondialdehyde (MDA) and nitric oxide (NO), concentrations (mean ± SEM) in normal control, drug control, HCC, quercetin curative and quercetin protective groups.

	SOD(U/g)	GSH (mg/g tissue)	MDA(nmol/g tissue)	NO (μ mol/L)
Normal Control	168.21 ± 2.44	18.53 ± 0.82	15.47 ± 0.41	1.659 ± 0.20
Drug Control	172.7 ± 1.37	19.31 ± 0.61	14.73 ± 0.59	1.979 ± 0.14
HCC	55.17 ± 2.46	1.942 ± 0.49	67.50 ± 3.03	66.79 ± 1.59
Quercetin curative	96.54 ± 3.92	5.868 ± 0.52	43.37 ± 0.72	37.08 ± 0.74
Quercetin protective	171.06 ± 4.91	4.243 ± 0.58	25.20 ± 1.62	18.61 ± 1.54

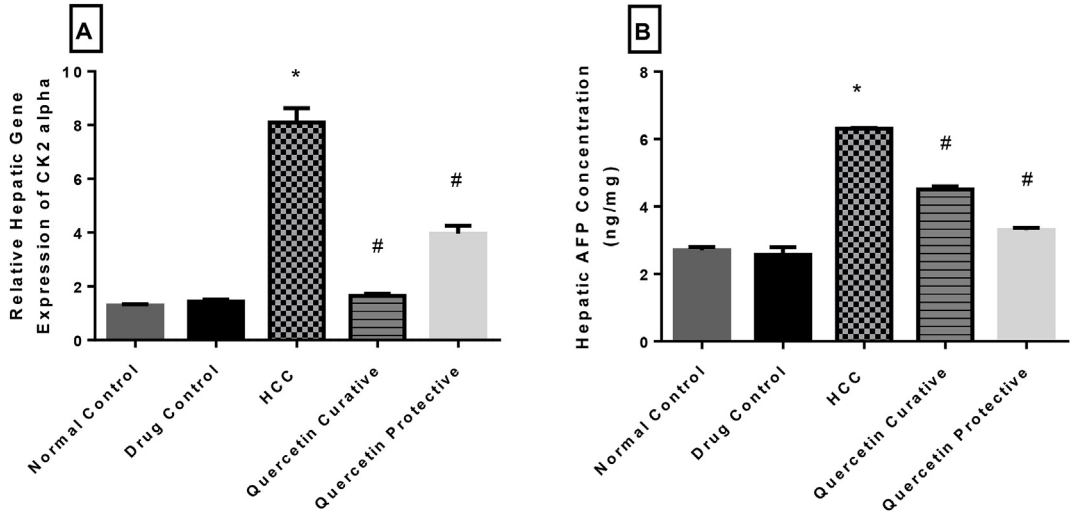

Fig. 2. A) Relative hepatic mRNA levels of CK2-α expressed as n-fold change B) protein levels of AFP(ng/mg) in normal control, drug control, HCC, quercetin curative and quercetin protective groups. Data is expressed as mean ± SEM, n = 12. Different symbols represent indicate statistical significance of $p < 0.05$ (* compared to normal control group, # compared to HCC group) using regular one-way ANOVA followed by Tukey's multiple comparisons test.

3.2. Quercetin treatment and protection promotes the antioxidant machinery

We tested the antioxidant activity in both curative and protective groups by evaluating superoxide dismutase (SOD) activity and glutathione reduced (GSH), nitric oxide (NO) and malondialdehyde (MDA) concentrations in liver homogenate (Table 2).

Quercetin curative and protective groups suppressed the increased oxidative damage that was present in the TAA-treated group resulting in elevated SOD levels 3.1 and 1.7 fold of HCC group in protective and curative groups respectively. It also raised GSH levels 2.2 and 3 fold of HCC group in protective and curative groups respectively. On the other hand, quercetin significantly reduced NO levels 0.27 and 0.5 fold of HCC group and lowered as well MDA levels 0.37 and 0.6 fold of HCC group in protective and curative groups respectively at ($p < 0.05$).

3.3. Evaluation of α-fetoprotein (AFP) levels

Alpha-fetoprotein (AFP) has been the standard diagnostic and in many cases a prognostic biomarker for HCC for many years [55]. Treatment and protection with quercetin have both significantly reduced serum levels of AFP 0.7 and 0.5 fold of HCC group respectively which have been elevated in HCC groups (2.3 fold of normal control group)(Fig. 2B).

3.4. Quercetin inhibited CK2-α hepatic gene expression in both prophylactic and treatment groups

Flavonoids have been shown to be potent inhibitors of CK2-α. Quercetin directly inhibits CK2-α in chronic lymphocytic leukemia [70]. CK2-α mRNA levels were significantly elevated in HCC group about 6 folds of that in the normal control group. Protective and curative groups have significantly lowered its levels to 0.48 and 0.2 fold of the HCC group (Fig. 2A).

3.5. Estimation of Notch1 protein and mRNA levels in hepatic tissue

Upregulation of Notch-1 activity is highly implicated in the progression of HCC [9]. We tested the inhibitory effect of quercetin on Notch1 expression protein and mRNA in both protective and curative groups.

Notch 1 protein levels of HCC group were elevated by 2.3 folds of normal control group. The protein levels of protective and curative groups decreased by 0.69 fold and 0.68 fold respectively in comparison to HCC groups (Fig. 3C).

In HCC, mRNA levels were elevated 2.4 fold of normal control group (Fig. 3A).The mRNA levels of protective and curative groups decreased by 0.6 fold and 0.4 fold respectively in comparison to HCC groups (Fig. 3A).

3.6. Quercetin suppressed hepatic Gli2 protein and mRNA expression

In order to evaluate the effect of CK2α inhibition on Hh signalling, we measured hepatic downstream Gli2 protein and mRNA levels. Hepatic Gli2 protein levels were elevated 1.2 fold in HCC group compared to normal control group. Administration of quercetin reduced Gli2 protein levels 0.8 fold and 0.9 fold in protective and curative groups respectively compared to HCC group (Fig. 3B). Similarly, mRNA levels of Gli2 were also affected. HCC caused a drastic elevation of 8 fold of normal group. Quercetin dramatically suppressed mRNA levels in protective and curative groups 0.3 and 0.4 of HCC group respectively (Fig. 3D).

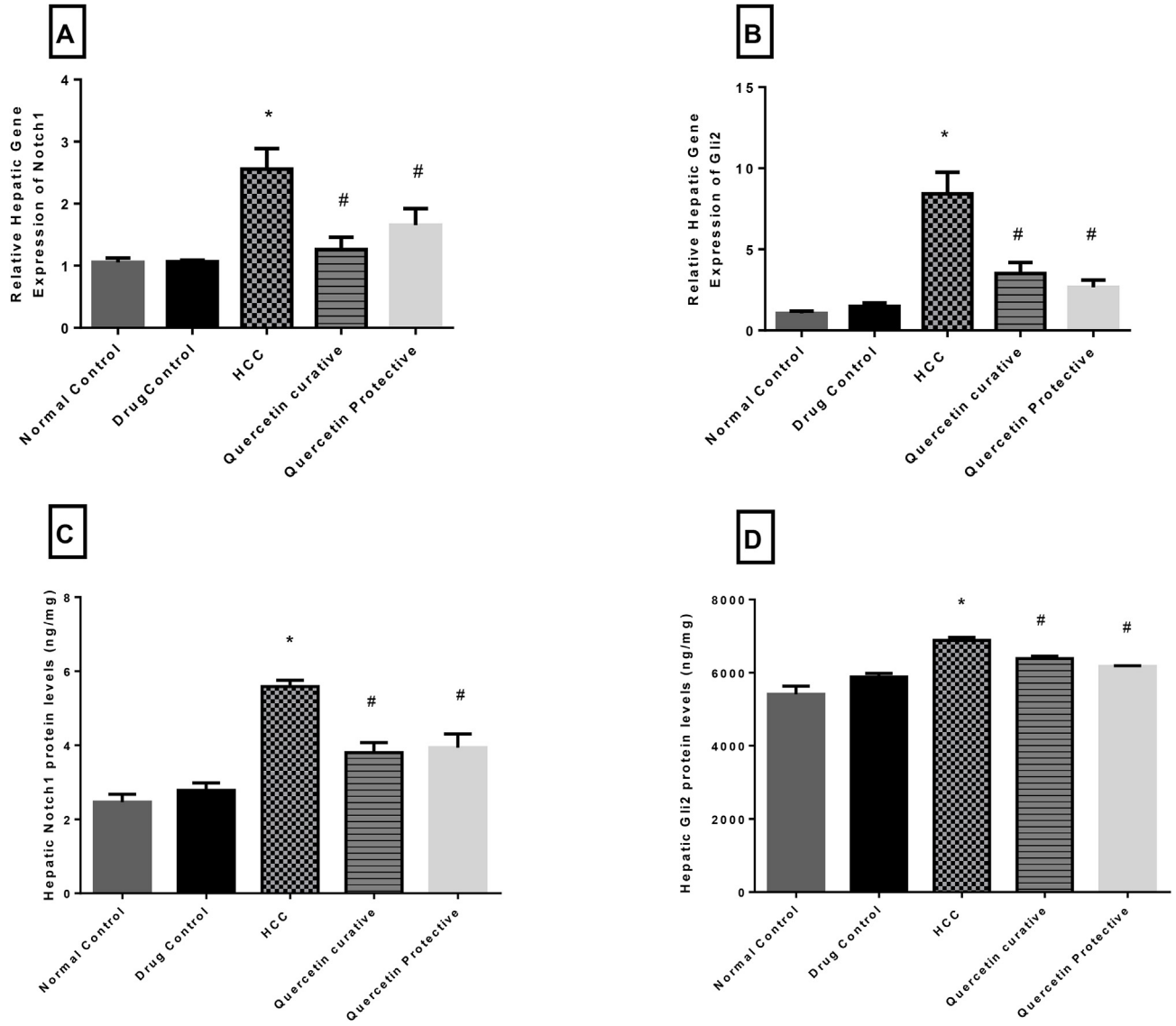

Fig. 3. Hepatic mRNA levels (expressed as n-fold) of A) Notch1 B) Gli2 and hepatic protein levels of C) Notch1 D)Gli2 in normal control, drug control, HCC, quercetin curative and quercetin protective groups. Data is expressed as mean ± SEM, n = 12. Different symbols represent indicate statistical significance of $p < 0.05$ (* compared to normal control group, # compared to HCC group) using regular one-way ANOVA followed by Tukey's multiple comparisons test.

3.7. Quercetin suppressed caspase-8 hepatic mRNA expression

In order to evaluate the role of quercetin in modulating the extrinsic apoptotic pathway we measured the mRNA levels of caspase-8. HCC mRNA expression showed an elevation 2.6 folds of normal group. Quercetin suppressed mRNA levels in protective and curative groups 0.5 and 0.57 fold of HCC group respectively (Fig. 4).

3.8. Histopathological examination

Microscopic pictures of H&E stained liver sections showed normal hepatic architecture with radially arranged hepatic cords around central veins in both normal and drug control groups (Fig. 5A and E). In HCC, well-differentiated cells showed cellular atypia characterized by swelling of hepatocytes, microvesicular steatosis with enlarged vesicular hyperchromatic nuclei, prominent nucleoli and eosinophilic intracytoplamic inclusions (Fig. 5B). Mild inflammatory cell infiltration was observed in quercetin curative and protective groups (Fig. 5C and D).

3.9. Immunohistochemical examination of hepatic cyclin D1, caspase-3, Ki-67 and p53

We conducted the immunohistochemistry analysis to compare the differences in cyclin D1 expressions before and after quercetin treatment. The nucleus of tumor cells was positively stained. Compared to the HCC group, cyclin D1 staining in the tumor cells of both groups exhibited a significant decrease in intensity and the reduction on the percentage of positively stained cells. Hepatic tissue of normal control animals showed mild expression of both cytoplasmic and nuclear caspase-3 whereas cytoplasmic and nuclear caspase-3 were markedly expressed in HCC rats. Protection and treatment with quercetin suppressed cytoplasmic and nuclear caspase-3 in hepatic tissue (Fig. 6). Microscopic pictures of immunostained liver sections against Ki-67 showed negative reaction in normal control group (Fig. 7A and B). Positive brown staining was detected in proliferated fibrous tissue in HCC group (Fig. 7C and D). Positive brown staining was detected in cenrtilobular degenerated hepatocytes (Fig. 7E and F) in curative group. Meanwhile, the positive brown staining disappeared in protective group (Fig. 7G and H).

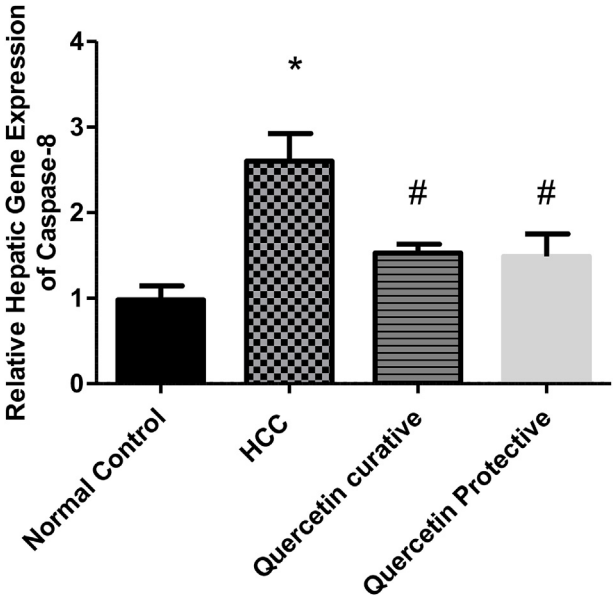

Fig. 4. Hepatic mRNA levels (expressed as n-fold) of caspase-8 in normal control, HCC, quercetin curative and quercetin protective groups. Data is expressed as mean ± SEM, n = 12. Different symbols represent indicate statistical significance of $p < 0.05$ (* compared to normal control group, # compared to HCC group) using regular one-way ANOVA followed by Tukey's multiple comparisons test.

Microscopic pictures of immunostained liver sections against p53 showing very mild positive brown staining in very few hepatocytes in control group (Fig. 8A). Almost no staining was observed in HCC group (Fig. 8B). Protective group showed moderate positive brown staining (Fig. 8C) and curative group showed mild positive brown staining (Fig. 8D).

4. Discussion

Quercetin is a dietary anticancer flavonoid whose activity is primarily attributed to its antioxidant properties. We here sought to elucidate the role of quercetin in modulating key pathways that are involved in hepatocellular carcinoma (HCC) pathogeneses such as Notch, Hh signalling, apoptosis and through regulating the cell cycle and casein kinase 2 (CK2α) activity. We here attempted to test the activity of quercetin to block Notch and Hh signalling by inhibiting casein kinase 2 (CK2α) as one of many mechanisms that regulate both pathways. CK2α has been shown to be a key regulator for both Notch and Hedgehog activities [2,107,108]. The histological examination of the liver sections showed loss of normal hepatic architecture confirming the hepatotoxic effect of TAA. Quercetin treatment restored hepatic morphology and cellular integrity of rats in both protective and curative groups as indicated by corrected AST, ALT, ALP, GGT, albumin and total protein levels. Serum alpha-fetoprotein levels were also declined in both protective and curative groups confirming the anti-tumor activity of quercetin.

Quercetin suppressed hepatic CK2α mRNA expression in both

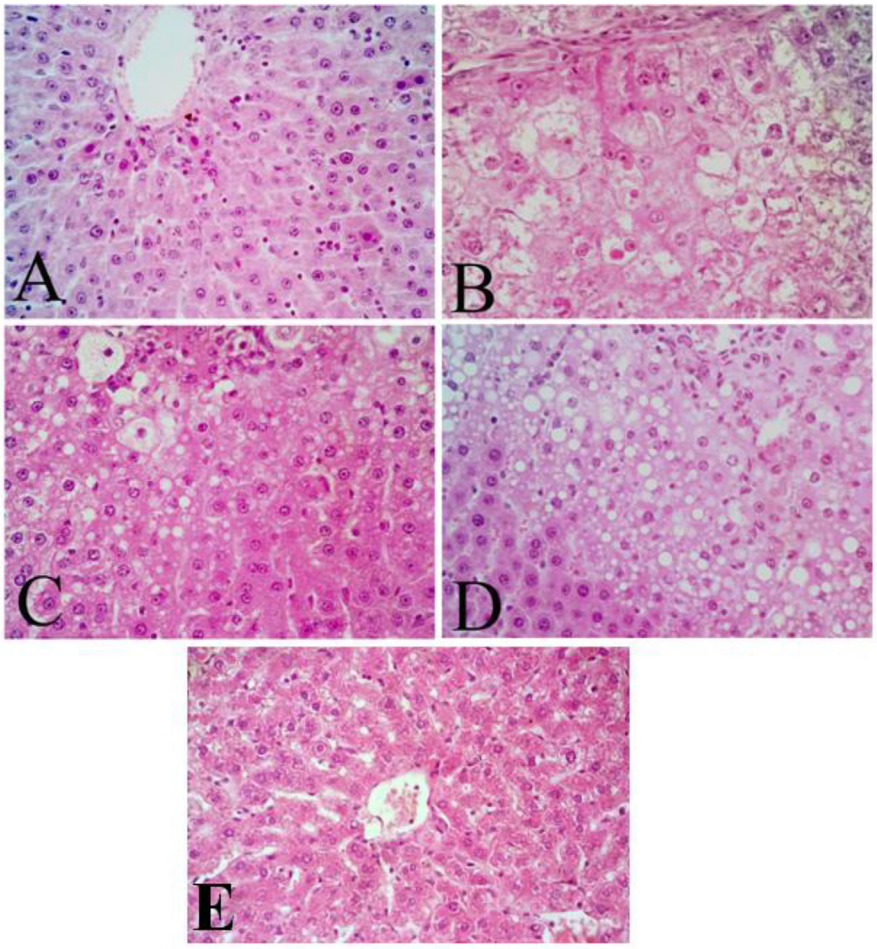

Fig. 5. Histopathological examination of liver sections stained with (H&E) of A) normal control, B) HCC, C) quercetin curative, C) quercetin protective groups and E) drug control groups.

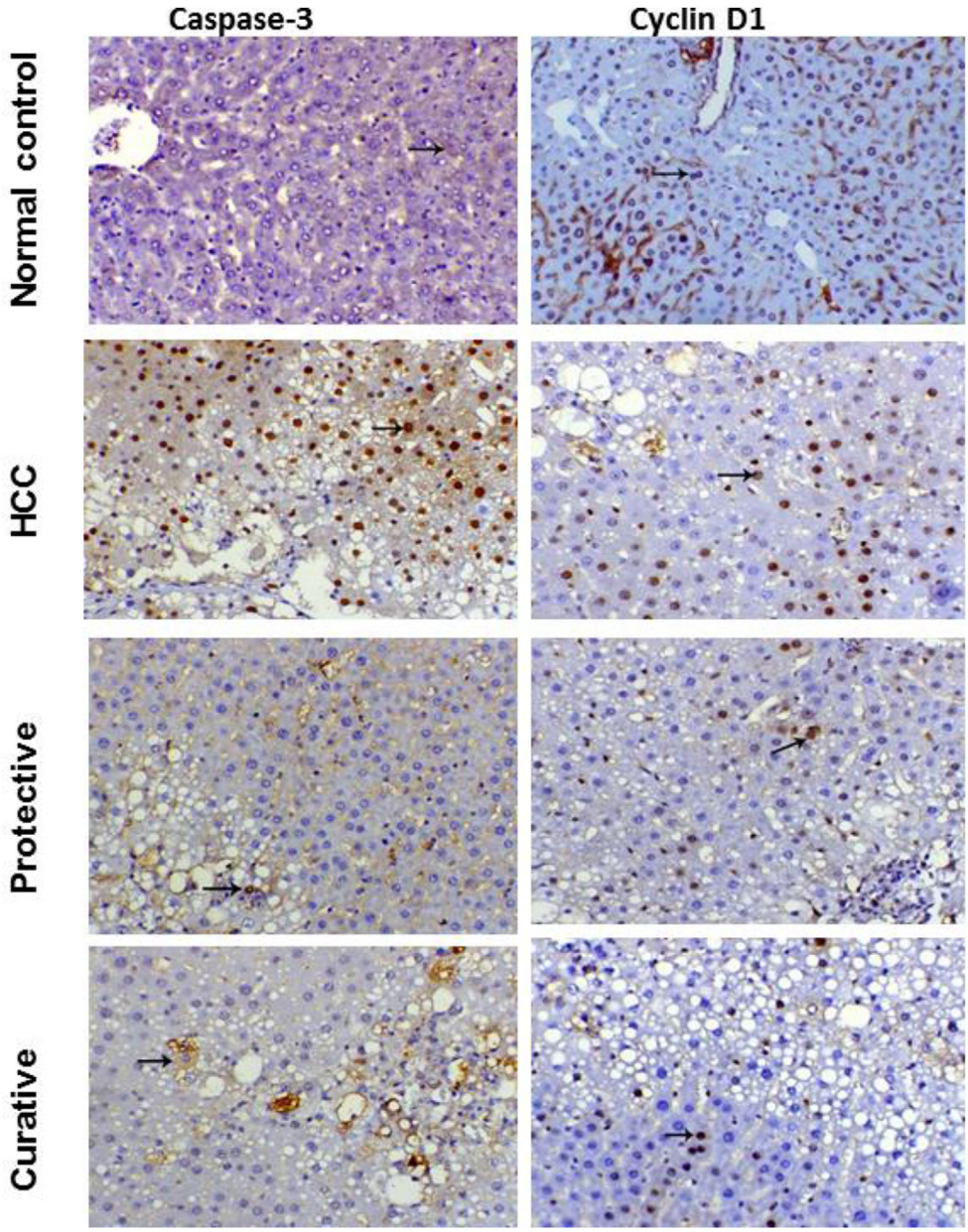

Fig. 6. Immunohistochemical examination of hepatic caspase-3 and cyclin D1 expression(indicated by arrows) in hepatic tissue in normal control, Drug control, HCC, quercetin protective and quercetin curative groups (bar = 50 μm). Arrows point to caspase-3 and cyclin D1 in their respective panels.

protective and curative groups. Levels of CK2α transcripts and proteins were proposed as prognostic markers for HCC [14]. Quercetin directly inhibited CK2α in chronic lymphocytic leukemia *in vitro* [70]. In fact, quercetin as well as related alkaloids with a common flavone scaffold exhibited inhibitory activities against kinases. Crystal structures of CK2α in complex with some of the alkaloids were solved, but the crystal structure of CK2α-quercetin complex has not been elucidated yet [52,61]. However, recent docking experiments have revealed that quercetin binds in a similar mode as other flavonoids [5].

Notch signalling pathway is involved in tumorigenesis, where disrupted Notch genes are frequently found in HCC tissues in comparison to normal liver tissues [36]. Notch1 signalling has been shown to facilitate HCC growth in HCC cell lines and genetically engineered mouse model [60,95]. Notch signalling is also involved in cancer angiogenesis [59], invasion and metastasis [96,102]. We indeed found that Notch signalling was activated as indicated by increased Notch1 protein and transcript levels in HCC rats. These results are in accordance with a previous study [95]. This might be in response to the change in biochemical characteristics in tumor cells resulting in abnormal signalling that maintains and promotes cancer invasion [83]. Different classes of Notch inhibitors have been developed such as gamma secretase inhibitors which block the upstream proteolytic cleavage processes [63,92] or antibodies that inhibit ligand binding [50,101]. Initial attempts for designing inhibitors that interfere with transcriptional protein-protein interactions have also been reported [1,57]. Previous studies reported the inhibitory effect of quercetin on Notch1 signalling in leukemia cells [62] and in human leukemia cell lines [13]. Quercetin upregulated microRNAs that directly targeted Notch and suppressed Notch signalling in pancreatic cancer [61]. Quercetin, in both protective and curative groups, suppressed hepatic Notch1 protein and mRNA levels. One explanation could potentially be through direct inhibition of CK2α by quercetin. CK2α positively regulates Notch signalling pathway through phosphorylation of NICD resulting in decreased binding of NICD to DNA and consequently a lower transcriptional activity [66]. Mapping and mutational studies identified multiple CK2α phosphorylation sites located in the ankyrin domain of Notch [66]. CK2α was

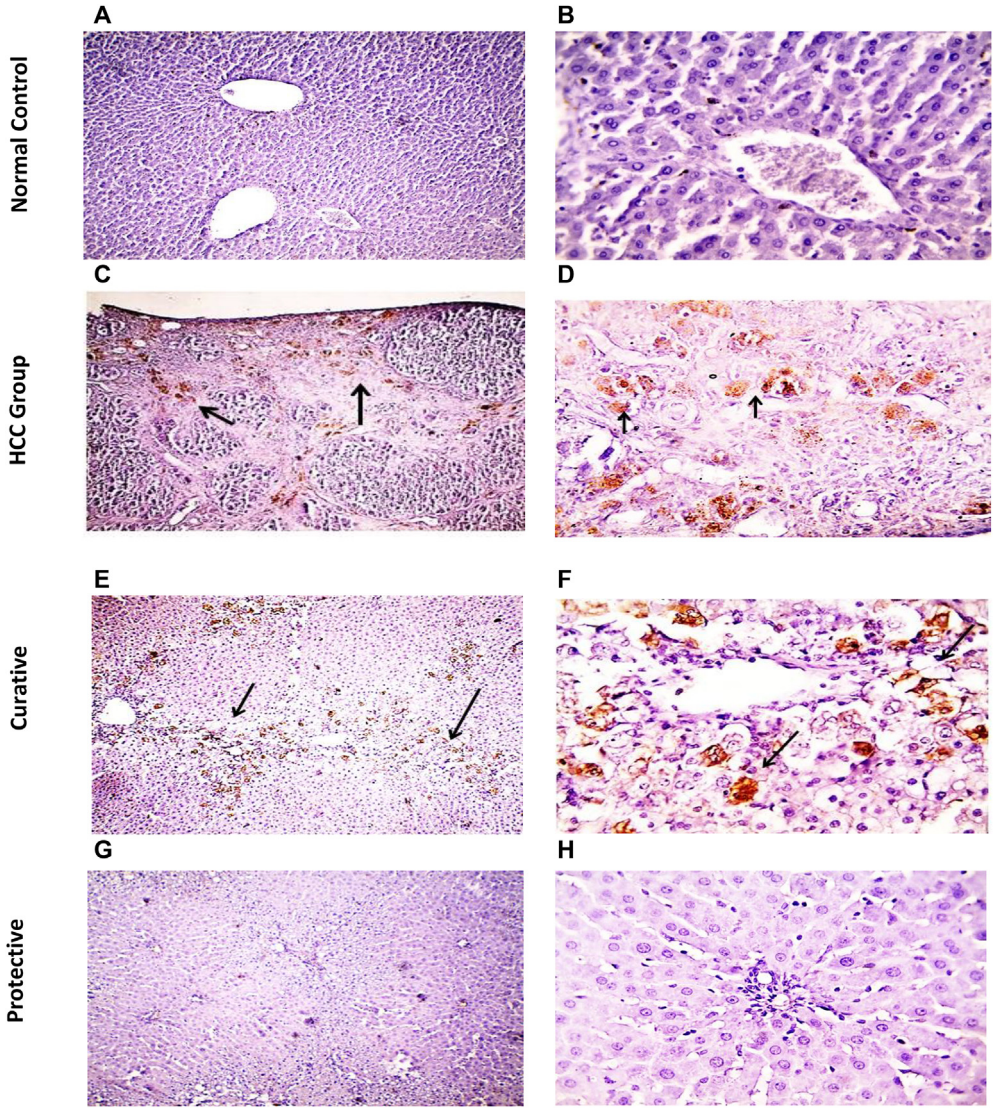

Fig. 7. Microscopic pictures of immunostained liver sections against Ki-67 (arrows point to positive reaction) in normal control, HCC, quercetin protective and quercetin curative groups. IHC counterstained with Mayer's hematoxylin. X: 100 bar 100 (A,C,E,G) and X: 400 bar 50 (B,D,F,H) (bar = 50 μm).

also found to positively regulate Notch1 in lung cancer cells [110].

Hedgehog signalling pathway is another highly conserved pathway that controls cell proliferation and differentiation [85]. Aberrant activation of Hh signalling has been associated with the initiation and progression of different cancers [33]. Mutations of different components of the pathway have led to deregulated Hh signalling causing increased cell proliferation [4]. Hh signalling is important for angiogenesis [58] and for sustaining cancer cell growth and reducing apoptosis [110]. Hh upregulation has been linked to HCC proliferation and aggressiveness [20,80]. Previous studies revealed the role of Gli1 in tumorigenicity [113] and Gli2 in the progression of HCC [44,90]. Increasing evidence confirmed that Gli2 regulates Gli1 transcription and is involved in the carcinogenesis of several carcinomas [6,23]. Moreover, Gli2 was reported to have a dominant role over Gli1 and Gli3 in regulating target genes and HCC proliferation [44]. Gli2 was markedly overexpressed in HCC tissues and could be considered an independent prognostic factor [105]. We here showed that quercetin downregulated Gli2 in both curative and protective groups compared to HCC group possibly through inactivation of CK2α. CK2α regulates Hh signalling pathway through phosphorylating Smo [38]. CK2α also prevents the proteosomal degradation of Gli1 and Gli2 leading to its accumulation and activation of Hh signalling in Drosophila [38]. Hepatic Gli2 protein and mRNA levels in both protective and curative groups were significantly reduced compared to HCC group indicating its Hh inhibitory activity. These results are in accordance with studies where quercetin inhibited Hh signalling in prostate cancer [82] and chronic myeloid leukemia [49]. Silencing of CK2α in human lung cancer cell lines inhibited Gli1 expression and transcriptional activity [108]. Moreover, inhibition of CK2α inactivated Hh signalling pathway *in vitro* and targeted inhibition of CK2α has been beneficial to patients with HCC [100].

CK2α is not only linked to dysregulated cell growth but also to apoptosis as a mechanism of cell death. Many studies demonstrated that CK2α is a key suppressor of apoptosis [3]. Overexpression of CK2α protected against chemical, heat and UV-mediated apoptosis [91]. Increasing evidence has shown that modulation of CK2α impacts the apoptotic machinery and alters the activity of caspases-2,-3,-8 and -9 [78,97]. Caspase-3 is one of the executioner caspases and is activated by any of the initiator caspases (caspase-8, caspase-9, or caspase-10). Quercetin also possesses proapoptotic activity through activating caspase-3 and caspase-9 [93]. Caspase-3 was overexpressed in HCC cell lines compared to non-tumor liver tissue [64]. Similarly, we have shown that caspase-3 activity was significantly elevated in HCC group and was suppressed in both protective and curative groups. Yet,

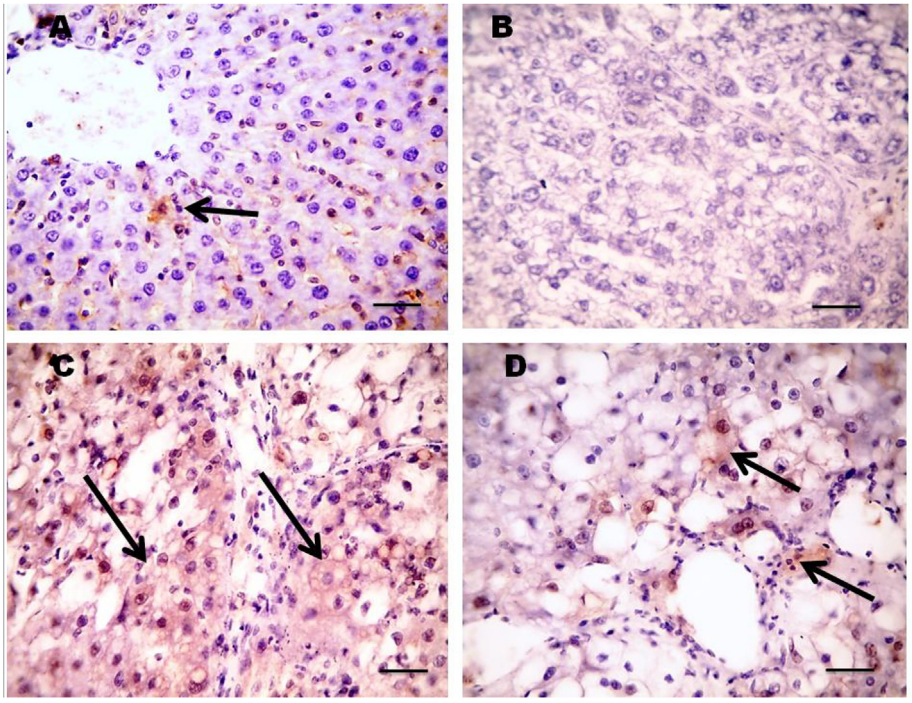

Fig. 8. Microscopic pictures of immunostained liver sections against p53 (arrows point to positive reaction) in A) normal control B) HCC C) quercetin protective and D)quercetin curative groups. IHC counterstained with Mayer's hematoxylin (arrows point to positive reaction). X: 400 bar 50.

caspase-3 levels of both quercetin-treated rats still remained higher than the normal rat group. Rates of apoptosis after quercetin administration are higher in the preneoplastic liver than in the normal tissue. This could be attributed to decreased cytotoxicity by the proapoptotic effect of quercetin [12,18,28]. CK2α down-regulation enhanced apoptotic activity and this was proven beneficial for HCC patients [14] possibly through phosphorylation of proteins that are destined for caspase-mediated degradation [71,94]. Caspase-8 is an initiator caspase in the extrinsic apoptosis cascade and suppressed caspase-8 activity has been reported in murine and human HCC [58]. However, we found that caspase-8 mRNA levels were elevated in our model. Caspase-8 was found to possess a non-apoptotic role through sensing DNA damage and promoting replication errors contributing to cancer development [45]. Quercetin in both protective and curative groups lowered caspase-8 mRNA levels indicating that quercetin may not be directly involved in the extrinsic apoptotic pathway. These results are in agreement with an earlier study in human hepatoma cell lines [28]. Quercetin treatment also induced the expression of p53 in protective and curative groups. p53 has been found to trigger the release of mitochondrial cytochrome C which in turn activates caspase-3 [74].

Quercetin significantly inhibited proliferation and reduced gene expression of cyclin D1 *in vivo* and *in vitro* [112]. Cyclin D1 is a major regulator of cell cycle during the proliferative stage [76]. Cyclin D1 protein overexpression was reported in various human tumors including hepatocellular carcinoma [21,39]. Moreover, different studies showed that Hh signalling is regulated by cyclin D1 gene [42,53]. Recent evidence has also shown that cyclin D1 is an important Notch target in breast cancer [11,68]. Transgenic mice that overexpressed Notch1 in mammary glands develop neoplasms with upregulated cyclin D1 [43]. Cyclin D1 gene is a direct transcription gene for Notch1 and Notch3 [16]. Inhibition of CK2α activity interfered with cyclin D1 expression [73]8). Additionally, inhibition of CK2α markedly down-regulated cyclin D1 in multiple myeloma and mantle cell lymphoma cells [54]. Our results showed a significant increase in cyclin D1 activity in HCC group in comparison to normal control animals. Quercetin suppressed cyclin D1 activity potentially through inhibiting CK2α and Notch1 subsequently. We also investigated the expression of Ki-67 which is strongly linked to tissue proliferation and is expressed at all stages of cell cycle (G1, S, G2 and M) but absent in the resting stage G0 [48]. Ki-67 was strongly expressed in HCC group and quercetin suppressed its expression in the curative group and protective group as it has been shown previously in a xenograft murine model [30].

The role of oxidative stress in the progression of HCC has been well-established for decades [87]. Oxidative stress has also been linked to the increase in CK2α activity *in vitro* [86]. TAA is considered a powerful hepatotoxin that causes fibrosis and cirrhosis through a reactive oxygen species (ROS)-mediated pathway [32]. Treatment with TAA depleted GSH, reduced SOD levels and elevated NO and MDA in serum. Quercetin inhibits lipid peroxidation through capturing free radicals and binding to transition metal ions [34,72,75]. The antioxidant activity of quercetin has been thoroughly investigated *in vivo* [65] and *in vitro* [10,111]. Indeed, quercetin enhanced the antioxidant machinery in both protective and curative quercetin groups. Increased ROS production may stimulate regenerative pathways as Notch and Hedgehog signalling. This has been previously observed in colon epithelial cells [15,67]. Inhibition of CK2α decreased ROS production and promoted the antioxidant potentials [40]. We postulate that quercetin may act in a similar manner through its direct inhibition of CK2α.

In conclusion, our results demonstrate that the anticancer activity of quercetin in HCC is not only attributed to its antioxidant power. Quercetin may act by downregulating Notch and Hh signalling, inhibiting apoptosis and cell proliferation and suppressing CK2α activity. Quercetin has also induced apoptosis and inhibited cell proliferation through suppressing cyclin D1.

Author contribution

Y.A.S performed the experiments. Y.A.S, N.A, A.M.A designed the experiments, analysed data and wrote the manuscript. A.E performed histological and immunohistochemical experiments.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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