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The Antioxidative, Anticancer and Hepatoprotective of Quercetin Nanoparticles *In-vitro* and *In-vivo*

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Abstract

Quercetin is a flavonoid found in many plants, such as berries and green tea. It has several therapeutic properties, including anticancer, antioxidative, and hepatoprotective. Although quercetin has numerous health benefits and is a valuable phytomedicine, it does have some drawbacks, including low solubility and bioavailability. In the present study, to overcome these drawbacks, quercetin nanoparticles were synthesized and studied for *in-vitro* and *in-vivo* antioxidative, anticancer, and hepatoprotective properties. The results indicated that the quercetin nanoparticles show significantly improved activity in both *in-vitro* and *in-vivo* studies.

Keywords: Quercetin; quercetin nanoparticles; antioxidant activity; cytotoxic activity

1. Introduction

In addition to providing us with food, the plants have several ethnomedicinal benefits without causing harm. When it comes to nutrition and disease prevention, these fruits and vegetables are indispensable (Manna K *et al*., 2016, Prakash D *et al*., 2012). These polyphenols, flavonoids, and isoflavonoids present in fruits, seeds, herbs, and vegetables also contain phytochemicals that have a variety of health benefits at different stages of their life cycles (Shikov AN *et al*., 2017). One of the phytomolecules, quercetin, a flavonoid found in fruits and vegetables, has antioxidative, anticancer, anti-inflammatory, and other therapeutic properties (Figure 1). Quercetin's hepatoprotective and anti-inflammatory properties have been extensively studied (Inal ME & Kahraman A, 2000)(Kanadaswami C *et al*., 2005). Although, quercetin contains a variety of health benefits, it still has disadvantages over other flavonoids as it has very low water solubility and it is a very easily degradable molecule, which limits its uses as a drug (Frenzel M & Steffen-Heins A. 2015). One of the solutions to the solubility and bioavailability problems of quercetin could be nanoparticles. Less than 1000 nm in size, nanoparticles can enhance the bioavailability and absorption of medicines (Dai J *et al*., 2004) (Salatin S *et al*., 2017) (Hsu CH *et al*., 2003). In the current study, the antioxidative, anticancer, and hepatoprotective properties of QNPs in contrast to pure quercetin were examined after the QNPs were produced and *in-vitro* and *in-vivo* testing were conducted.

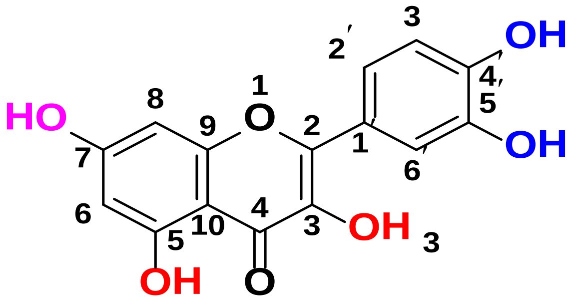

Figure1. Chemical structure of Quercetin

2. Materials and Methods

2.1. Chemicals

The chemicals utilized in this study were purchased from the American company Sigma Aldrich Chemical Co.

2.2. Synthesis and Characterization of QNPs:

QNPs were synthesized by an improved anti-solvation precipitation method by Bilati *et al*., (Bilati U *et al*., 2005) Briefly, PVA solution was infused with 300 mg of quercetin that was dissolved in 200 ml of ethyl alcohol. The solution was homogenized for 1 hour at 20,000 rpm, and the sample was then freeze dried after removal to

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remove ethyl alcohol. Furthermore, the lyophilized powder was stored in a moisture-proof container until needed. The morphology of quercetin nanoparticles was observed using a 20 kV SEM (Quanta 3D FEG/FEI).

The QNPs were initially analyzed by using FTIR to identify functional groups, phytochemical constituents, and other factors involved in the reduction and stabilization of the synthesized nanoparticles by using the Jasco FTIR 4100's attenuated total reflectance mode (Japan). The data was recorded between 4000 cm^1 and 400 cm^1. CuK1-X Ray diffractometer radiation (= 1.5406 A°) was used to confirm the presence of ZnO in the powdered sample as well as to analyze the crystallite structure and size. An NMR spectrometer (BRUKER) was used to measure the 1H NMR 400MHz spectra using DMSO.

2.3. Cell Lines:

The cell lines used in the study were obtained from the ATCC, U.S.A. and maintained in cell culture as per ATCC guidelines. For long-term usage, the cell lines were cryopreserved in liquid N2 containers. A total of two cell lines, Human Tumor Cell Line (Huh-7) (ATCC, U.S.A.) and Human Epithelial Amnion Cell Line (WISH) were used in the present study.

2.4. Antioxidant activity

The method outlined by Braca *et al*. was used to measure the free radical scavenging activity of quercetin and quercetin nanoparticles based on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical (Braca A *et al*., 2002). Three milliliters of a 0.004% methanol solution of DPPH were mixed with various amounts of quercetin and QNPs. After 30 minutes, the absorbance at 517 nm was measured. To determine the percentage inhibitory activity, the formula [(A0-A1)/A0] x 100 was used, where A0 represents the absorbance of the control and A1 represents the absorbance of the quercetin/QSRs or ascorbic acid (Standard). Inhibition curves were created in order to get IC50 values.

2.5. Sub culturing of cell lines:

The monolayer culture was transferred to a second vessel by diluting the cells with trypsin. After the cell reached 80% confluency, the culture flask was removed from the CO2 incubator. The old medium was completely removed from the flask and washed with 3ml of 1X PBS. Trypsin (3 ml, 0.25%) was added to that flask for 60 seconds and removed, leaving a few drops in the flask. The culture flasks were kept at 37^0 C for 20 minutes until all the cells were released from the surface in the residual trypsin solution. Furthermore, the required amount of growth medium (10 ml) was added to suspend the cells with the help of a Pasteur pipette, and the cells were distributed equally in two flasks. Finally, the culture flasks were incubated at 37^0C with 5% CO2.

2.6. In-vitro cytotoxic activity using human cancer and normal cell lines using SRB assay:

SRB assays were used to test the in-vitro cytotoxic activity of quercetin and QNPs (Houghton PJ *et al*., 1994]. In order for the 3x103 cells per well to adhere to the 96-well microtiter plates, 150 l of fresh media were incubated with the cells for 24 hours. After 24 hours, quercetin and quercetin nanoparticles were added in triplicate along with 0, 12, 5, 25, 50, and 100 g/ml. The plates were subsequently incubated once more for 24 hours with quercetin and quercetin nanoparticles present. Cell growth was examined in the last stage (Vichai V & Kirtikara K. 2006). Immediately after the operation, the cells were fixed by adding 50 l of cool, 40% (w/v) trichloroacetic acid, and they were once more kept at 4 °C for an hour. The plates with wells were thoroughly cleaned with distilled water after the supernatant was removed from them. Following the procedure, the cells were fixed by adding 50 l of cool, 40% (w/v) trichloroacetic acid, and they were once more maintained for an hour at 4 °C. After removing the supernatant from the plates containing wells, they were thoroughly cleaned with distilled water. 50 l of SRB solution was added to each well after the plate had dried, and it was left to incubate for 30 min at room temperature. The unconnected SRB was eliminated by repeatedly washing the plate in 1% acetic acid. To solubilize the dye, 100 l of 10 mM Tris base was then added to each well. The plates were placed on a plate and gently shaken for 20 minutes. After that, the absorbance (OD) was measured at 570 nm using an ELISA reader. The formula below was used to compute the cell's survival percentage.

Percentage cell survival = O.D. (treated cells)/ O.D. (control cells).

2.7. In-vivo toxicity assay:

Thirty adult male albino rats were used in this study. The animals were divided into four groups: the control group (A), animals treated with diethylnitrosamine and acrylamide to induce oxidative stress and hepatocarcinoma (B, C, and D), and animals not treated. The group B mice were considered as positive controls. The rest of the C and D group mice were treated with the different concentrations of quercetin and QNPs via intraperitoneal injection, after the four weeks of diethylnitrosamine and acrylamide administration. The liver function test was performed after the treatment of quercetin and QNR.

Decapitation was used to sacrifice animals by cervical dislocation, and the cardiac puncture technique was used to collect five milliliters of blood in gel tubes. The blood samples were then centrifuged for 15 minutes at 3000rpm. Human Germany provided standard reagents for the Humalyser 3000, a semi-automatic chemistry analyzer. Before and after the experiment, blood (5 mL) was taken from the vein. Lver function tests were carried out by analysing the AST, ALP, and ALP enzymes (Exarchou V *et al*., 2002).

2.8. Histopathological examination:

The liver tissues of the respective animals were fixed using 10% formaldehyde for 24 hours and embedded into paraffin after 16 h. Five to five m thick sections of each sample were obtained from the paraffin blocks. Each sample was stained using hematoxylin and eosin. The results were analyzed using a light microscope (Babu BH *et al*., 2002).

2.9. Statistical analysis

SPSS v16 was used to analyze the results of our study, with all values expressed as a mean standard error of mean. For ANOVA, P values of 0.05 and 0.01 were measured as statistically significant.

3. Results and Discussion

3.1. Characterization of synthesized QNPs:

3.1.1. FTIR spectroscopic analysis:

Figure 2 displays the quercetin IR spectrum. The bands of absorption at 3325 cm 1 correspond to the hydroxyl group. The C-H bond peak can be found at 3060 cm 1. Stretching of the aromatic C=C bond at 1604 cm-1, where hydroxyl group intensity reduced following QNPS synthesis, indicates the presence of an aromatic nucleus. The findings made it abundantly evident that intermolecular hydrogen bonding took place in the QNPs. **(Figure 3)**.

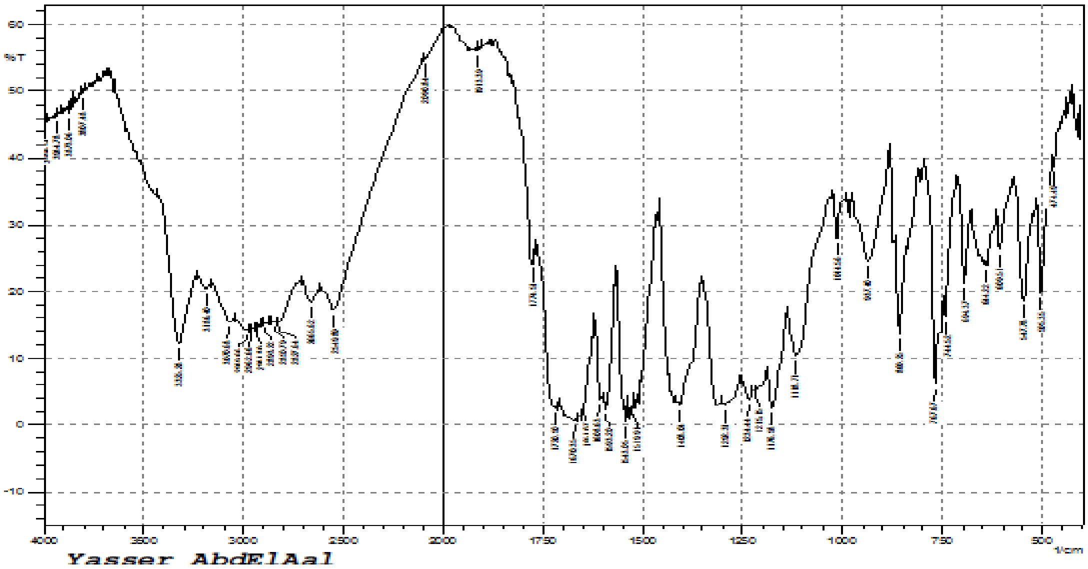

Figure2. IR spectrum of Quercetin

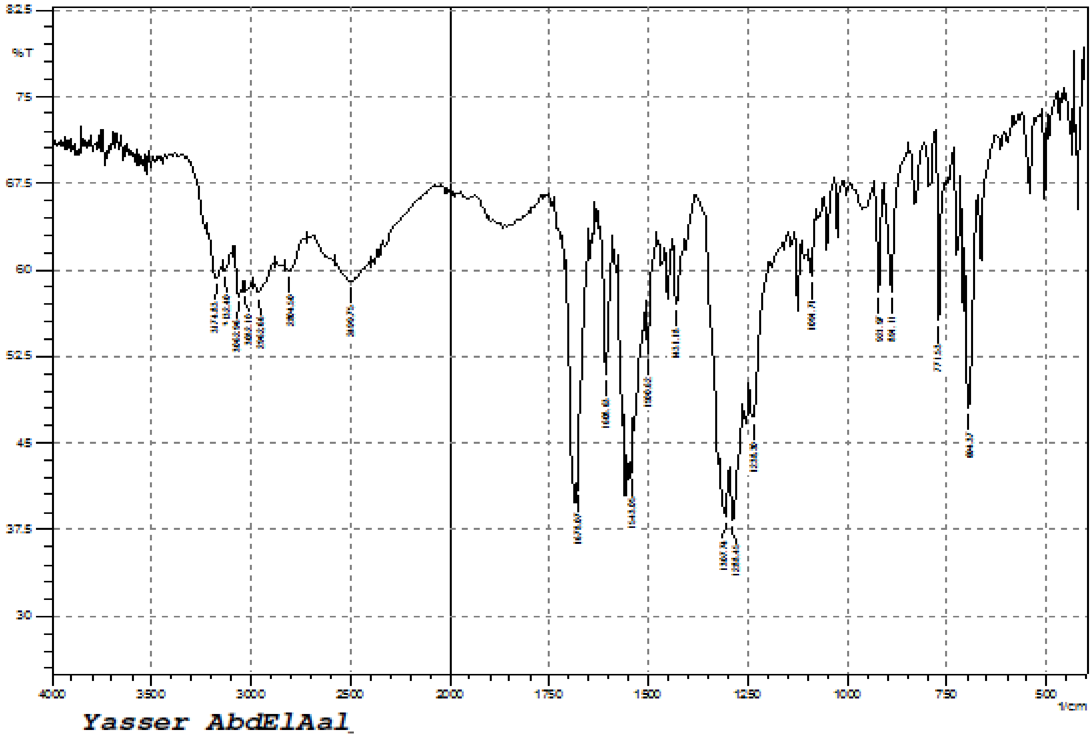

Figure3. IR spectrum of Quercetin nanoparticles

3.1.2. [1] H NMR spectrum of Quercetin and Quercetin nanoparticles:

The following values are displayed in the 1H-NMR spectra of quercetin in DMSO-d6 at 400 MHz: 11.24 (1H, s, C7-OH), 11.56 (1H, s, C4' -OH); 7.92 (1H, d, H-6), 7.94 (1H, d, H-8), 7.98 (1H, d, H-5'), 9.57 (1H, d, H-6'), and 9.87 (1H, s, H-2'). Furthermore, the aromatic proton had peaks in the 1H-NMR spectrum at C-6, C-8, C-5, C-6, and C-2, respectively (Figure 4). The spectra showed protons on aromatic groups ranging from 6 to 8 ppm, strong intramolecular hydrogen bonding at 12.62 ppm, a characteristic shift for intramolecular six-membered ring hydrogen bonding of the C5-OH and C4 O moiety. The QNP's results showed that the H6 and H8 aromatic protons had shifted upfield (Figure 5).

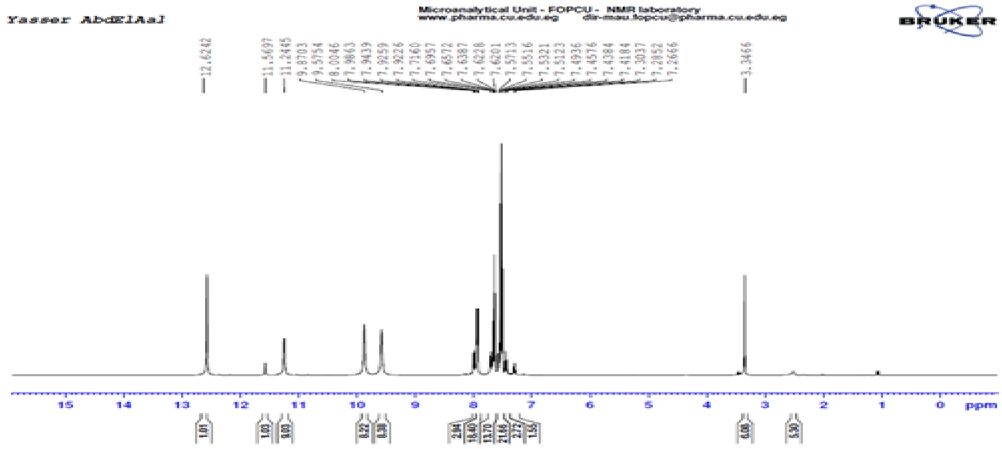

Figure4. [1] H NMR spectrum of Quercetin

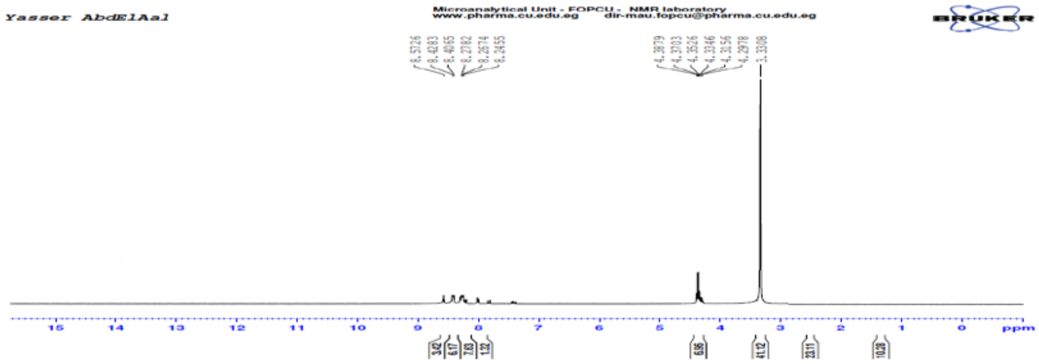

Figure5. [1] H NMR spectrum of Quercetin nanoparticles

3.1.3. XRD analysis:

By using X-ray diffraction, the dried crystalline quercetin nanoparticles were evaluated. The Quercetin nanocrystals' X-ray diffraction patterns are displayed in Figure 6. Numerous distinct peaks of 12.61°, 14.5168°, 16.09°, 17.1714°, 18.62°, 20.19°, 21.17°, 23.78°, 26.10°, 27.91°, and 29.1808° were visible in the X-ray patterns of the QNPs (Figure 6).

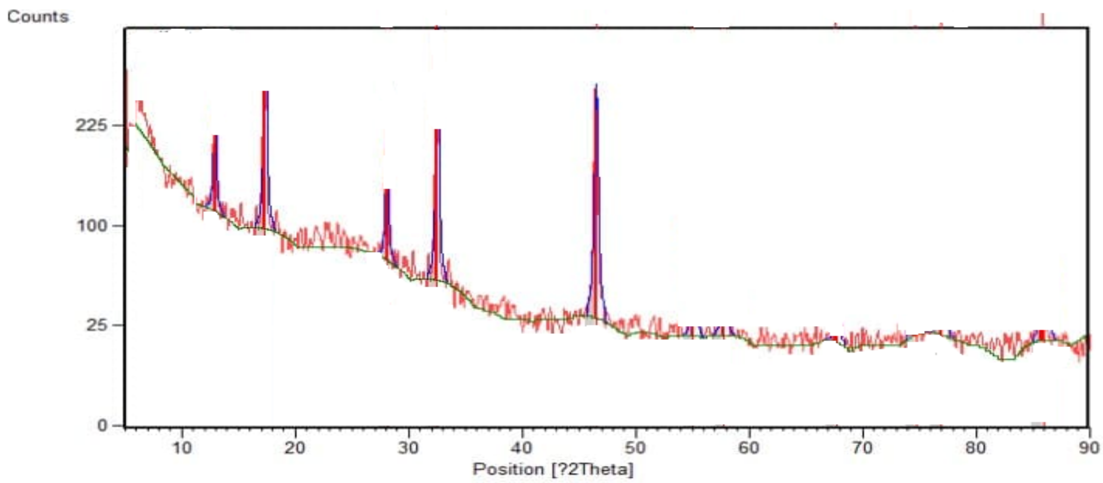

Figure6. X-ray diffraction of Quercetin nanoparticl

3.1.4. SEM analysis:

The size and crystalline properties of the produced NPs were studied by SEM examination. The QNPs' particle diameter was 17.25 nm at a flow rate of 8 ml/min. (Figure7).

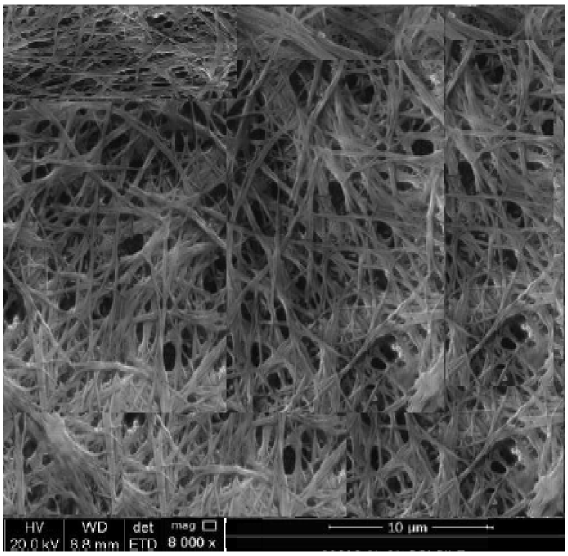

Figure7. SEM photographs of Quercetin nanoparticles at flow rate (8ml/ min)

3.2. Biological activities:

3.2.1. Antioxidant activity:

A Quantitative analysis of quercetin and quercetin nanoparticles using the DPPH radical-scavenging method was then performed. Antioxidant activity using DPPH increased in a focus-dependent manner. In comparison to Ascorbic acid, Quercetin nanoparticles had a lower IC50 value of 19.21 micrograms/ml (Table 1).

Table 1. Antioxidant activity of Quercetin and Quercetin nanoparticles, using DPPH free radical scavenging method

Conc.(µg/ml)	Quercetin	Quercetin nanoparticles	Ascorbic Acid
20	25.3 ±0.23	45.12 ± 1.21	42.12 ± 0.22
40	27.11 ± 1.12	56.11 ± 0.21	47.13 ± 1.10
60	33.54 ± 2.12	62.3 ± 0.13	50.4 ± 1.11
80	35.7 ± 1.11	64.1 ± 1.53	55.1 ± 0.03
100	40.31 ± 0.19	69.32 ± 2.44	56.312 ± 0.11
IC_{50}	55.05	19.21	31.16

3.3. Cytotoxic activity:

3.3.1. In-Vitro cytotoxicity assay:

Human hepatocellular liver carcinoma (Huh-7) and a normal liver cell (WISH) were used to test the cytotoxicity of quercetin and quercetin nanoparticles. For each cell line, IC50 value was used to determine the toxicity. On the whole, quercetin nanoparticles were cytotoxic to human hepatocellular liver carcinoma (Huh-7, IC50 8.35 g/mL) but showed significantly less toxicity towards normal cells (Table 2).

Table 2. Percentage cell survival of Quercetin and Quercetin nanoparticles different cell lines

Conc.(µg/ml)	Quercetin	Quercetin nanoparticles	WISH
	Huh-7		
0.0	2.000	1.068	1.160
12.5	1.868	0.600	1.891
25	1.659	0.415	1.531
50	1.222	0.325	1.375
100	1.232	0.222	1.291
IC_{50}	29.23	8.35	37.52

3.4. In-Vivo assay:

3.4.1. Biochemical analysis:

Earlier studies found that quercetin nanoparticles were highly effective against a liver cancer cell line (Huh-7). It was, therefore, decided to test the same cell line with quercetin nanoparticles *in-vivo*. The levels of AST (87.00 percent at 200 mg/kg.b.wt), ALT (50.00 percent at 200 mg/kg.b.wt), and albumin (317.11 at 200 mg/kg.b.wt) all showed dose-dependent anticancer activity (Table 3). The anticancer activity of quercetin nanoparticles (Figure 8) was confirmed by histopathology.

Table 3. In-vivo result of Quercetin and Quercetin nanoparticles

Parameter / Groups	AST	ALT	ALP
Control group (A)	83.50±1.500	50.67±0.27	312.23±1.182
Untreated group (B)	302.50±0.51	88.51±1.59	424.01±1.11
Treated group (C) Quercetin	91.00±1.01	67.01±1.01	397.21±1.02
Treated group (D) with Quercetin nanoparticles	87.00±1.21	50.00±1.05	317.11±1.11

(Mean±SD)

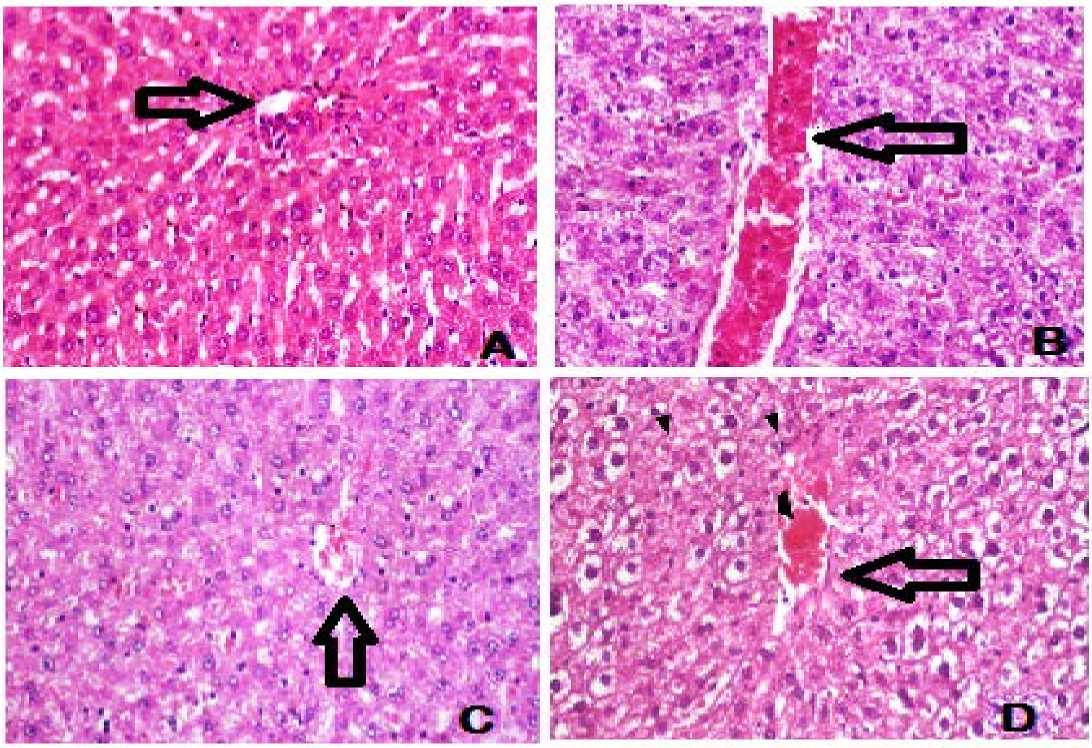

Figure 8: effect of Quercetin and Quercetin nanoparticles on livers section of different groups: **A)** of control rats revealed normal histological**; B)** induced control group (acrylamide) untreated with new compound; **C);** Experimental group treated with Quercetin and **D);** Experimental group treated with Quercetin nanoparticles.

3.5. Histopathological examination:

The hepatic lobules in the livers of rats in group 1 were found to be normal histologically (Figure 8A). Hepatocyte necrosis and fibroplasia were found in the portal triad in rats from group 2 (Figure 8B). Features include anaplastic carcinoma cells that are characterized by large hyperchromatic nuclei (Figure 8B). The quercetin treatment caused the acrylamide-induced rats to exhibit some nuclear pyknosis, granular and vacuolar degeneration, necrosis, and activation of Kupffer cells in the liver (Figure 8C).

Quercetin nanoparticle treated mice showed good restoration of the hepatic parenchymal cells with mild hepatocellular vacuolar degeneration and a few scattered necrotic cells (Figure 8D).

4. Conclusions

Quercetin is considered as one of the best naturally occurring flavonoids, consisting of several therapeutic properties, including anti-oxidative and anticancer (Butler MS. 2004) (Cho EJ *et al*., 2003). Despite having several therapeutic properties, quercetin shows low bioavailability. According to Ren K *et al*., (2017) QNPs displayed significant anticancer activity against, Hep3B, HCCLM3 and Bel7402MHCC97H (Ren K *et al*., 2017). To overcome the problem of bioavailability and solubility, we also synthesized QNPs and then compared the *in-vitro* and *in-vivo* anticancer and antioxidative properties of quercetin and its nanoparticle QNP. The results indicated that the QNPs have an advantage over quercetin molecules as the QNPs showed better antioxidative and anticancer activity in *in-vitro* and *in-vivo* studies in comparison with quercetin molecules.

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