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Evaluation of Antioxidant properties of Quercetin in DEN-induced Hepatocellular Carcinoma in BALB/c mice

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Abstract

This study aimed to determine the antioxidant properties of Quercetin in diethylnitrosamine (DEN)-induced Hepatocellular Carcinoma (HCC). HCC is one of the most common malignant tumors in the world.DEN was used to induce HCC, and mice were divided into three groups, each having five animals. Group I served as normal control, group II received DEN (20mg/kg BW), and group III received DEN along with Quercetin (50mg/kg BW), respectively, for 6 weeks. Antioxidant potential was evaluated by employing in vitro and invivo assays. In the invitro study, Quercetins howed high radical scavenging activity with IC_{50} *= 19.17 and 36.22 µg/ml for DPPH and* H_2O_2 *scavenging activity, respectively. In the ferric reducing assay, the absorbance was found to increase with an increase in the concentration of the extracts revealing reducing power. In-vivo studies revealed that Quercetin significantly (p ≤ 0.05) retained oxidative liver markers (CAT,SOD,GSH, and AChE) activity to about normal levels. These results indicate the excellent antioxidant and hepatoprotective properties of Quercetin against oxidative-stress in DEN treated mice.*

Keywords: Hepatocellular Carcinoma (HCC), DEN, Quercetin, DPPH, H_2O_2, liver oxidative markers.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common and a major form of liver cancer and it is the ninth leading cause of cancer deathsworldwide (Center for Disease Control and Prevention (CDC), 2010). Multiple factors including chronic liver disease, cirrhosis, chronic HBV (hepatitis B virus) and HCV (hepatitis C virus) infections, autoimmune hepatitis, chronic alcohol use, obesity, and diabetes mellitus, etc. (Yang and Roberts, 2010) are responsible for its development. HCC progression may differ depending on diverse factors and therefore, several mechanisms might be involved such as loss of cell cycle control, loss of senescence control, dysregulation of apoptosis etc. (Alotaibi *et al.,* 2016; Singh, 2018). Direct or indirect exposure to radiation as well

as exposure to chemicals such as aflatoxins, 2-acetylaminofluorene, DEN etc., leads to several genetic and epigenetic changes such as chromosomal deletions, rearrangements, aneuploidy, gene amplification, mutations, formation of DNA adducts, DNA strand-break, modulation of DNA methylation, and modulation of cell signaling pathways which ultimately leads to cancer (Villalta and Balbo, 2017; Basu, 2018).Several carcinogens are commonly used for inducing cancer in experimental animals (Biswajit *et al.,* 2012). In this study, Diethylnitrosamine (DEN) was used to induce Hepatocellular carcinoma (HCC) in mice. DEN is a Nitrosoalkyl compound that is commonly used as an initiating agent for inducing liver cancer in experimental animals. DEN, a potent hepatocarcinogen is present in cheese, soybean, fish, cured meat, alcoholic beverages, groundwater having a high level of nitrate, tobacco smoke, agricultural chemicals, cosmetics, and pharmaceutical agents (Yurchenko and Molder, 2006). It is also produced from the metabolic processing of drugs such as chlorpromazine, methadone, chloroquine, primaquine, and phenacetin (Gupta *et al.,* 2010; Ryo *et al.,* 2012). DEN induces liver cancer by forming DNA adducts where it causes methylation of the N^7-atom in the guanines of nucleic acids and inducing chromosomal aberrations and micronuclei in the liver. Due to the carcinogenic properties of nitrosamines, the application of these substances, and in particular DEN, has become highly attractive for inducing liver tumorigenesis in rodents as an experimental model of human hepato-carcinogenesis(Swann and Magee, 1968; Tolba *et al.,* 2015).Due to its asymptomatic nature, it is very difficult to diagnose HCC at early stages, and in most cases, it is detected at an advanced stage, which is incurable. Sorafenib, a multikinase inhibitor enzyme is the first targeted therapy approved for the treatment of advanced HCC (Gauthier and Ho, 2013). Other includes, radiation therapy, chemotherapy;however, a high number of recurrences have been reported, and also several side effects are associated with all of these above modalities (Daher *et al.,* 2010; Chun *et al.,* 2015).

Owing to several side effects, plant-based products received more attention for scientific researchers as they have fewer side effects and less expensive than current treatment methods. Herbal medicine has been used as a primary source of medical treatment by traditional practitioners since time immemorial (Maqsood *et al.,* 2010). Plants are used in medical treatment because of their natural therapeutic properties, which triggers researchers to developed techniques for investigation of their medicinal properties and their potential prevention or treatment of diseases including cancer. Several phytochemicals have been studied and also used as a cancer chemopreventive and treatment agents. Some examples include apigenin, curcumin, crocetin, cyanidins, epigallocatechingallate, fisetin, genistein, gingerol etc. (Hu *et al.,* 2012). The antioxidant/ free radical scavenging properties of many medicinal plants such as *Panax ginseng, Lagerstroemia* (Saumya and Mahaboob, 2011), *Carthamus tinctorius* (Mandate *et al.,* 2011) and *Trichodesma zeylanicumm*(Frank, 2013) have been studied. Since anticancer properties of plants and their products are due to their antioxidant/free radicals scavenging properties, therefore, evaluation of their antioxidant/free radicals scavenging properties will give an insight into their anticancer properties.

Quercetin (3,3',4',5,7–pentahydroxyflavone) is a flavonefrom the flavonoid group of polyphenol. It is abundantly found in apples, red grapes, onions, raspberry, honey, cherries, citrus fruit, and green leafy vegetables (Hashemzaei *et al.,* 2017). Many recent studies have found that Quercetin exerts various biological effects, including antioxidative, anti-inflammatory, antitumor, antiviral, and apoptosis−inducing effect (Mandate *et al.,* 2011; He, 2016). Quercetin inhibits cancer cell proliferation by causing cell cycle arrests like G2/M or G1 arrest in different cell types and also mediates apoptosis *(*Seufi *et al.,* 2009*)*. It also promotes intracellular ROS-scavenging enzymes such as SOD, CAT, glutathione peroxidase, etc., thereby reducing intracellular ROS level (Li *et al.,* 2014). Therefore, the present study was aimed to evaluate the antioxidant activity of Quercetin in both *in-vitro* and in DEN-induced HCC in mice.

Materials and methods

Chemicals

Quercetin, DPPH, 30% H2O2, ascorbic acid, ammonium molybdate, potassium ferricyanide, Sucrose, Triton X-100, 5-5' dithiobis - (2- nitrobenzoic acid) [DTNB] and others chemicals were purchased from Sigma-Aldrich and HiMedia, India. All chemicals used in the study were of analytical grade.

Sample preparation

About 1 mg/ml stock solutions of Ascorbic acid and Quercetin were prepared in 0.1% DMSO solution.

DPPH radical scavenging assay

2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radicals scavenging assay was determined by the method (Mathangi and Prabhakaran, 2013) with slight modifications. Different concentration of Quercetin (10-50 µg/ml) was mixed with 1.5 ml of DPPH (1 mM, prepared in methanol). The reaction mixture was incubated for 30 min in the dark after which absorbance was measured at 517 nm. The radical scavenging capacity of Quercetin was then compared with ascorbic acid which is the standard reference compound. The percentage DPPH scavenging effect of Quercetin was then calculated using the following equation.

$$\% \text{ scavenging effect} = \frac{A \text{ control} - A \text{ sample}}{A \text{ control}} \text{ X } 100$$

Where *A control*= absorbance of the control (which contains the entire reagent except the sample) and *A sample* = absorbance of the test sample.

Hydrogen peroxide radical scavenging assay

This assay was performed according to the method described (Pavithra and Vadivukkarasi, 2014) with slight modification. Different concentrations of Quercetin (10-

50 µg/ml) were mixed with 0.6 ml of hydrogen peroxide (40 mM) solution prepared in phosphate buffer (0.1 M pH 7.4). The reaction mixture was incubated for 10 min in dark and the absorbance was measured at 230 nm against a blank solution. Ascorbic acid was used as a standard reference compound. The percentage inhibition was then calculated using the following equation.

$$\% \text{ inhibition } = \frac{A \text{ control} - A \text{ sample}}{A \text{ control}} \text{ X } 100$$

Where *A control* = absorbance of the control (which contains all the reagents except the sample) A *sample* = absorbance of the test sample.

Reducing power assay (Ferric reducing activity)

Reducing power assay was performed according to the method(Hajaji *et al.,* 2010) with slight modification. Different concentrations of Quercetin (10-50 µg/ml) were mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide [$K_3Fe(CN)_6$] (1%). The reaction mixture was incubated at 50 ^{0}C for 20 min after which 2.5 ml of trichloroacetic acid (10%) was added. The reaction mixtures were then centrifuged at 3000 rpm for 10 min. 2.5 ml was then taken from the reaction mixture and 2.5 ml distilled water was added, followed by the addition of 0.5 ml $FeCl_3$ (0.1%). This was allowed to stand for 10 min and the reaction mixture turned greenish. The absorbance was then measured at 700 nm against a blank solution. As concentration increases, absorbance increases which indicated an increase of reduction capability and this was compared with ascorbic acid.

Experimental animals

Swiss albino adult mice weighing 20-30g were obtained from the Pasteur Institute, Shillong, Meghalaya, India. The animal was grouped and housed in polyacrylic cages of 5 mice per cage and maintained standard laboratory conditions (temperature 25-28ºC) with 12 h light and 12 h dark cycle. The experimental protocols were followed according to the Institutional Animal Ethical Committee (IAEC) regulations approved by the committee and conducted humanely.

Induction of Hepatocellular Carcinoma

The animals were divided into 3 groups of 5 each (I-III). The freshly prepared Diethylnitrosamine (DEN) (20 mg/kg BW) suspended in normal saline solution (0.1% DMSO) was administered by a single intravenously injection to induce hepatocellular cancer after overnight fasting for 18 h. Groups I served as a normal control received normal saline (0.1% DMSO) instead of Quercetin and DEN respectively, group II was treated with only DEN (20mg/kg BW) once a week for 6 weeks and group III was treated with DEN (20 mg/kg) once a week and Quercetin (50mg/kg) was administered intraperitoneally twice a week for6 weeks.

Collection of sample

After 6 weeks, the animal was anesthetized and sacrificed,target organ such as liver was removed and washed with ice-cold saline and, weighed and cut into separate portions for antioxidant estimations, and the remaining tissues were stored immediately at -80 ºC for future analysis.

Tissue analysis

Liver homogenate (1g) was prepared in a ratio of 1:10 (w/v) in ice-cold 0.25M sucrose (pH – 7.4) and homogenized by using a Teflon homogenizer. The homogenate was then centrifuged at 20,000g for 30 min at 4 ^{0}C. The supernatant was collected and stored at 4 ^{0}C,this supernatant was used for the estimation of catalase(CAT) (Aebi, 1984), Superoxide Dismutase (SOD) (Fridovich, 1986), Reduced Glutathione (GSH) (Owens and Belcher, 1964), and Acetylcholinesterase (AChE) (Oct *et al.,* 1975) and total proteinconcentration (Bradford, 1976).

Statistical analysis

All the determinations were conducted at least three times ($n = 3$); Linear regression analysis was used to calculate IC_{50} for both standard and Quercetin. The statistical analysis was carried out by Students't-test,where P value < 0.05 was considered as statistically significant. Data were processed with graph pad prism version 8.02 software.

Results and discussions

DPPH radical scavenging assay

Quercetin was found to show DPPH free radical scavenging activity and was compared with ascorbic acid. The % inhibition of the various concentration of Quercetin as well as of ascorbic acid was calculated and a graph of concentration vs. % inhibition was plotted (Figure 1 and 2). The IC_{50} of Quercetin and ascorbic acid was calculated and was found to be 0.74 and 9.53, respectively as shown in table 1.

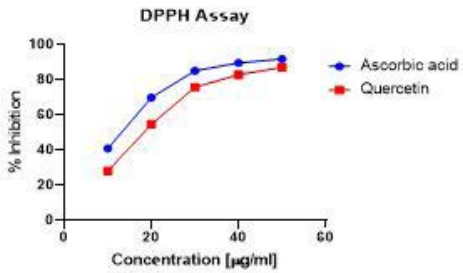

Figure 1. Percentage inhibition of Quercetin and Ascorbic acid at various concentrations on DPPH.

Table 1. DPPH radical scavenging activities of ascorbic acid and Quercetin at different concentrations. Each value in the table is represented as mean ± SD (n = 3).

Sample	Concentration (µg/ml)	% Inhibition (mean ± SD)	IC_{50} (µg/ml)
Ascorbic acid	10	41.04 ± 1.33	8.95
	20	70.02 ± 1.76	
	30	85.28 ± 0.55	
	40	89.83 ± 0.37	
	50	92.06 ± 0.14	
Quercetin	10	28.18 ± 0.21	19.17
	20	54.79 ± 0.16	
	30	75.96 ± 0.12	
	40	83.07 ± 0.25	
	50	87.28 ± 0.15	

Based on their IC_{50} values, it was found that ascorbic acid is a better radical scavenging compound than Quercetin. DPPH assay is the most commonly used method for screening antioxidant activity of various plant extracts or plant-based products as it is a very simple and sensitive method (Ilhami *et al.,* 2019). Using the assay, free radical scavenging properties of an antioxidant can be determined by measuring the decrease in absorbance of DPPH at 513 nm. The absorbance decreases when DPPH is being scavenged by an antioxidant through the donation of an electron or hydrogen. DPPH upon accepting an electron or hydrogen becomes a stable diamagnetic molecule with resulting changes in color from purple to slightly yellow.

Hydrogen peroxide radical scavenging assay

Quercetin was found to show H_2O_2 radical scavenging activity and was compared with ascorbic acid. The % inhibition of all the various concentrations of Quercetin as well as of ascorbic acid was calculated and a graph was plotted (Figure 3). The IC_{50} of Quercetin and ascorbic acid was calculated from the graph and was found to be 16.26 and 36.22, respectively as shown in table 2.

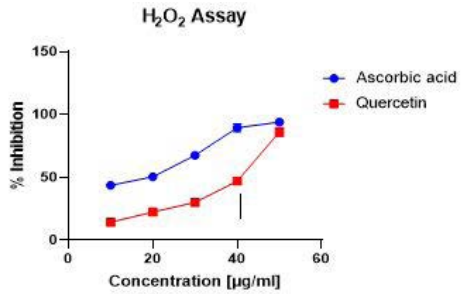

Figure 2. Percentage inhibition of Quercetin and Ascorbic acid at various concentrations on H_2O_2.

Table 2. H_2O_2 radical scavenging activities of ascorbic acid and Quercetin at various concentrations. Each value in the table is represented as mean ± SD (n = 3).

Sample	Concentration (µg/ml)	% Inhibition (mean ± SD)	IC_{50} (µg/ml)
Ascorbic acid	10	43.68 ± 1.43	16.26
	20	50.48 ± 2.38	
	30	67.55 ± 2.77	
	40	89.38 ± 3.02	
	50	91.97 ± 2.36	
Quercetin	10	14.36 ± 2.49	36.22
	20	19.49 ± 1.28	
	30	30.08 ± 0.77	
	40	46.92 ± 1.03	
	50	86.04 ± 3.54	

Hydrogen peroxide is a strong oxidizing agent which can oxidize several biomolecules and cause oxidative stress (Ilhami *et al.,* 2019). H_2O_2 itself is not very reactive, but it can sometimes be toxic to cells because it may give rise to hydroxyl radicals in the cells (Vadivukkarasi and Pavithra, 2014). In this assay, a decrease in the absorbance of H_2O_2, which absorbed maximally at 230 nm was observed as the concentration of antioxidants increases. This is because H_2O_2 is being scavenged by the compounds and higher the concentration of compounds, more is the scavenging effect and hence, lesser is

the amount of H_2O_2 in the reaction mixture which resulted in decreasing absorbance. The IC_{50} of ascorbic acid and Quercetin are 16.26 and 36.22, respectively which indicates that ascorbic acid has a higher H_2O_2 scavenging capacity than Quercetin.

Reducing power assay (Iron reducing activity)

Reducing power assay of Quercetin was evaluated and compared with ascorbic acid and from the graph; we can see that Quercetin has less Fe^{3+} reducing capability compared to Ascorbic acid. The reductive capabilities were found to increase with increasing concentration of both ascorbic acid and Quercetin as shown in figure 3.

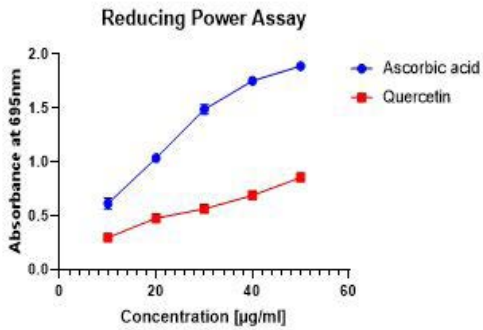

Figure 3. Reducing Capacity of Quercetin and Ascorbic acid at various concentrations.

This assay is based on the reduction of Fe^{3+} ions. The reducing capacity of ascorbic acid and Quercetin was compared and it was found that Ascorbic acid has higher reducing capability than Quercetin. This shows that Ascorbic acid can donate an electron very easily to Fe^{3+}. As we know that free radicals have free unpaired electron which makes it very reactive towards biomolecules, and therefore, the presence of a reductant, an electron donor, will stabilize the reactive radicals. Hence from this assay, we can predict the reducing power of antioxidant compounds or plant-based products based on their ability to reduced Fe^{3+}.

Liver oxidative stress markers

The antioxidant enzyme activities such as CAT, SOD, and GSH were found to be decreased significantly ($P \leq 0.05$) and AChE activity was found to be significant increases ($P \leq 0.05$) in DEN-treated mice when compared to normal control (Group: I) (Figure 4).

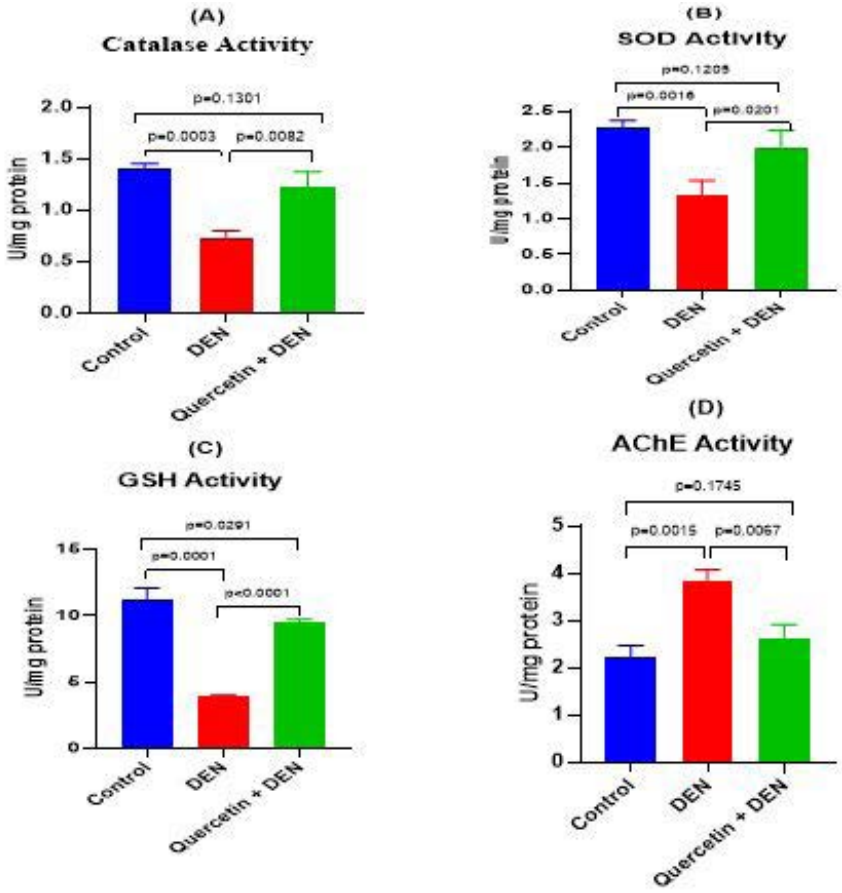

Figure 4: Effects of Quercetin on changes in liver enzyme levels of mice treated with DEN. (A) Catalase (CAT), (B) *Superoxide dismutase* (SOD), (C) Glutathione (GSH) (D) Acetylcholinesterase (AChE). I- Normal control, II –DEN treated (20mg/kg BW), III- Quercetin treated (50 mg/kg), + DEN (20mg/kg BW). Values are mean ± S.D, error bar indicating the standard deviation, n = 5 animals. *p-value* less than 0.05 ($p \leq 0.05$) is statistically significant.

Catalase is a heme-containing enzyme that can protect the cells against oxidative stress caused by reactive oxygen species, such as H_2O_2 by degrading it into oxygen and water. Catalase activity was decreased significantly (p=0.0003) in DEN- treated mice (Group II) compared to the control group (Group I). Treatment with Quercetin (20mg/kg) significantly elevated (p=0.0082) when compared with GroupII and showed no significant changes (p=0.1301) with the activity of the control group as shown in figure 4(A).The decrease in SOD activity (p=0.006) was observed in DEN-treated mice when compared with Group I, and significantly elevated (p=0.020) in activity compared with Group II, as close to the activity of Group I which show no significant changes (p=0.1205) as shown in figure 4 (B). Superoxide Dismutase (SOD) catalyzes the dismutation of superoxide to either ordinary molecular oxygen or to more stable compounds such as H_2O_2 which in turn degraded by catalase. Similarly, the reduced GSH decreased significantly (p=0.0001)

in DEN-treated mice (Group II). Treatment with Quercetin (50mg/kg) significantly elevated ($p<0.0001$) when compared with Group II, and the GSH activity is almost of the same level as that of control Group I as shown in figure 4 (C). GSH is required to maintain the normal reduced state and to counteract the deleterious effects of oxidative stress. During the reduction of hydrogen peroxide, GSH is oxidized to GSSG. When GSSG levels increased, the GSH-reductase activity was activated to convert GSSG in GSH (Cui *et al.,* 2011). In Group II, the low level was observed which indicates that GSH is being oxidized to GSSH as a result of increased radicals due to oxidative stress. Thus, GSH/GSSG homeostasis is disrupted. However, in Group III, even though the mice are exposed to DEN, its level is almost equal to that of the healthy mice. Thus, treatment with Quercetin showed a significantly elevated level of CAT, SOD, and CAT. This shows that Quercetin plays an important role in preventing oxidative stress by reducing the level of reactive oxygen speciesin a biological system.

AChE is an enzyme that catalyzes the breakdown of acetylcholine into choline and acetyl group and breakdown of many other choline esters that function as neurotransmitters. AChE is found mainly at neuromuscular junctions and in chemical synapses of the cholinergic type, where its activity serves to terminate synaptic transmission. The Acetylcholinesterase (AChE) activity was found to be significantly increased ($p=0.0015$) in Group II, whereas its activity in Group III showed no significant changes ($p=0.1745$) when compared with Group I as depicted in figure 4(D). Although AChE is well known for its function at cholinergic synapses, it also plays a non-catalytic role where it participates directly or indirectly in motility, proliferation, differentiation and cell-cell interaction. Studies have found that AChE function as a tumor growth suppressor in hepatocellular carcinoma and also in lung cancer(Perez-Aguilar *et al.,* 2015). The increase of cholinesterase activity in DEN-treated mice may enhance cholinergic signalling and contribute to tumor progression. In Group III, even though DEN was administered but treatment with Quercetin significantly reduced the activity of AChE as closed to that of normal mice. This showed that Quercetin has anticancer properties that prevent cancer growth in the liver by reducing the activity or level of AChE.

Conclusion

Despite many advances in cancer therapy, cancer is still one of the major causes of mortality worldwide. Natural products, such as Quercetin (3,3′,4′,5,7-pentahydroxyflavone), which is abundantly found in apples, red grapes, onions, raspberries, honey, cherries, citrus fruits, and green leafy vegetables, exerts various biological effects including antioxidant, anticancer, antiviral, etc. This current study demonstrated the antioxidant properties of Quercetin in both *in vitro* and *in vivo*. The *in vitro* analysis revealed that Quercetin is a good free radical scavenging species. Although ascorbic acid, a well-known antioxidant shows a much higher activity, Quercetin still shows a good free radical scavenging activity. To further confirm its antioxidant properties, an *in vivo* study was carried out and it was found that Quercetin significantly increases the activity of antioxidant enzymes (CAT & SOD) and GSH level and significantly reduces the liver marker enzyme (AChE) activity

when compared to DEN treated group. Thus, in each assay, Quercetin retained the activity of liver oxidative markers to about the normal level. From these observations, Quercetin shows excellent antioxidant and hepatoprotective properties, which might be useful for the treatment of oxidative-stress related diseases such as Hepatocellular carcinoma HCC.

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