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# Chemopreventive effect of quince (Cydonia oblonga Mill.) fruit extract on hepatocellular carcinoma induced by diethylnitrosamine in rats

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**ABSTRACT:**

**Introduction:** Hepatocellular carcinoma (HCC) or primary liver cancer is one of the most prevalent and deadliest cancers, which has been increasing greatly worldwide. Diethylnitrosamine (DEN) is a well-known environmental toxin and potent hepatocarcinogenic dialkylnitrosoamine present in air, water, and in a number of foodstuffs. In the present study, we evaluated preventive effect of aqueous extract of quince (Cydonia oblonga Mill.) fruit (ACO) against DEN-induced hepatocellular carcinoma (HCC) in rats. **Methods and Results:** The model of hepatocellular carcinoma was induced by a single intraperitoneal injection of DEN (200 mg/kg) as an initiator that after two weeks followed by daily oral administration of 2-acetylaminofluorene (30 mg/kg) as a promoter for two weeks. Quince-treated rats were pretreated with ACO intragastrically at three different doses two weeks prior to DEN injection. The marked reduction of serum biomarkers of liver damage and cancer, including alfa-fetoprotein (AFP), gamma glutamyl transpeptidase (GGT), alanine transaminase (ALT), and aspartate transaminase (AST) were observed in ACO supplemented animals as compared with HCC rats at the end of the experiment. Moreover, the quince extract exhibited in vivo antioxidant activity by elevating glutathione (GSH) contents as well as preventing lipid peroxidation in the liver tissues of DEN-treated rats. The relative weight of liver was also reduced in quince-treaded rats as a prognostic marker in HCC. **Conclusion:** Our results clearly demonstrated that quince has a chemopreventive effect against HCC in rats and can be proposed as a promising candidate for the prevention of DEN-induced hepatocarcinogenesis.

**Keywords:** *C. oblonga*; Chemoprevention; Hepatocarcinogenesis; Liver cancer; Quince.

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## 1. Introduction

Hepatocellular carcinoma (HCC) is an aggressive tumor with poor prognosis and is the second leading cause of cancer related mortality throughout the world [1, 2]. It is a primary liver cancer that originates in a background of liver inflammation and it is well-demonstrated that chronic alcohol abuse, viral infections [hepatitis B virus (HBV) or hepatitis C virus (HCV)], diabetes, non-alcoholic steatohepatitis (NASH) as well as a number of genetic diseases like hemochromatosis, and Wilson's disease are the most common causes of HCC.

Industrial chemicals, environmental pollutants, tobacco smoking, food additives, and aflatoxin-B1 are other risk factors that contribute in hepatocarcinogenesis [3-5].
Diethylnitrosamine (DEN) or N-Nitrosodiethylamine is a representative environmental carcinogen with the potential to induce tumors in diverse organs especially in the liver. DEN is widely reported to be found in drinking water, cheese, soybean, processed meats, alcoholic beverages, tobacco products, cosmetics, and a variety of workplaces [6-8]. It has been widely used as an experimental carcinogen in cancer research to induce HCC or different types of benign and malignant tumors in rodents. It is strongly believed that DEN is metabolized in the liver by cytochrome P450 enzymes and is converted to a biological reactive intermediate (BRI). Consequently, this free radical causes DNA alkylation and oxidative damage leading to the development and progression of HCC [9].
Currently, there is no proven effective chemotherapy or surgical intervention for HCC and a lot of focus has been put on cancer preventive strategies as an acceptable approach for controlling the HCC incidence. Natural products and medicinal plants play a crucial role in cancer therapeutics and recently there is a concentration on the prevention of major lethal cancers by natural substances [10].
Quince (Cydonia oblonga Mill.) fruit is one of such medicinal herbs, which has a wide variety of pharmacological effects such as antioxidant, anti-inflammatory and hepatoprotective effects [11-14]. Recent findings have shown the antiproliferative activity of quince fruit against human kidney and colon cancer cells and this valuable fruit has been suggested as a promising agent for cancer prevention and treatment [15, 16]. Hence, the current study aimed to investigate the preventive activity of aqueous extract of Cydonia oblonga Mill. fruit (ACO) against DEN-induced HCC in rats.

## 2. Materials & Methods

### 2.1. Chemicals

All chemicals were purchased from Sigma- Aldrich Co. (Taufkrichen, Germany) with the highest commercial grade available.

### 2.2. Plant and extract preparation

Cydonia oblonga Mill. fruits were collected from Shahriar, Alborz province, Iran and were authenticate by Mr. Kamalinejad, a qualified botanist at the Department of Botany, Shahid Beheshti University of Medical Sciences (8054, voucher specimen in Shahid Beheshti University of Medical Sciences Herbarium, Tehran, Iran). The fresh fruits were cleaned with their peels and the extraction was carried out by the maceration of 100 g fruit with 900 mL distilled water for 30 min. The resulting extract was evaporated by placing in water bath 90°C. Finally, the extract was filtered and kept at –20°C until use. The extract was dissolved in distilled water to receive desired concentrations just before use. The moisture level of the extract was determined by weight loss after placing 2 g of the final extract in an oven at 60–65 °C for 72 hr. The final extract contained 24% water [17].

### 2.3. Standardization of Extract

Total polyphenol content was determined by spectrophotometry, using gallic acid as the standard based on the Folin–Ciocalteu method [18]. Total phenolic content was 51.08 ± 1.86mg gallic acid equivalents (GAE) per gram of ACO (mg of GAE/g of plant extract). Total flavonoid content was measured with the aluminium chloride colorimetric assay [18]. Quercetin was used as the standard and flavonoid contents were expressed as mg of quercetin equivalent per gram of ACO. Flavonoid contents were 16.27 ± 0.86 mg quercetin equivalents (QE) per gram of ACO (mg of QE /g of plant extract).

### 2.4. Animals

Male Sprague-Dawley rats weighing 180 to 200 g were housed in ventilated plastic cages over PWI 8-16 hardwood bedding. There were 12 air changes per h, 12 h light photoperiods, an environmental temperature of 21–23 °C, and a relative humidity of 50–60%. The animals were fed a standard and normal chow diet and given tap water ad libitum. Principles of laboratory animal care (NIH publication No. 85-23, revised 1985) were followed.

### 2.5. Hepatocarcinogenesis model

The experimental model of HCC was induced in rats by DEN as the initiator and 2-acetylaminofluorene (2-AAF) as the promoter of hepatocarcinogenesis according to the protocol described previously [19]. Briefly, rats were fasted for 96 hours and then were refed for 24 hours as a proliferative stimulant. Afterward, rats were injected only a single intraperitoneally (i.p.) dose of DEN (200 mg/kg body weight) for initiating hepatocarcinogenesis. After two

weeks, liver cancer development was promoted with daily dose of oral 2-AAF (30 mg/kg body weight) for two weeks. HCC was confirmed by histopathological evaluations and the measurement of liver damage and cancer markers, including alfa-fetoprotein (AFP), gamma glutamyl transpeptidase (GGT), alanine transaminase (ALT), and aspartate transaminase (AST) [20].

### 2.6. Experimental design

As shown in Fig. 1, rats were randomly divided into six different groups of six rats each (n = 6). Group 1 (Control) animals fed with standard diet and served as a normal control, which injected with the single dose of saline. Group 2 were normal animals that treated only with daily oral dose of ACO (300 mg/kg body weight) from the beginning of the experiment. Group 3 (HCC) rats served as untreated HCC animals as described before. Test groups animals (Groups 4- 6) were pretreated with the increasing doses of ACO (75, 150, and 300 mg/kg body weight, respectively) two weeks prior to DEN injection that continued until the end of the study. The different concentrations of the extract were selected based on our previous study and were administered orally once daily using an intragastric tube for 8 weeks [14, 21].

### 2.7. Sample preparations

At the end of the experiment, animals were anaesthetized by ether inhalation and blood was collected from the orbital sinus and centrifuged at 1500Xg for 20 minutes at 4°C to obtain serum for biochemical analysis. In addition, body weights of the animals were recorded and rats were sacrificed. Then, the liver tissues were quickly taken, weighed, and frozen for the determination of hepatic oxidative stress markers. Frozen liver samples were homogenized in ice-cold Tris- HCL buffer (150 mM, pH 7.4). The relative liver weight was calculated as the percentage ratio of liver weight to the body weight.

### 2.8. Evaluation of biochemical parameters

AFP level has been widely used clinically as a tumor marker for HCC, which was measured by Calbiotech AFP ELISA Kit [22]. The activities of biochemical markers of liver damage, including GGT, ALT, and AST were determined by commercially available enzyme kits (Pars Azmoon, Tehran, Iran) [14]. The assays were performed according to the protocols supplied with the kits.

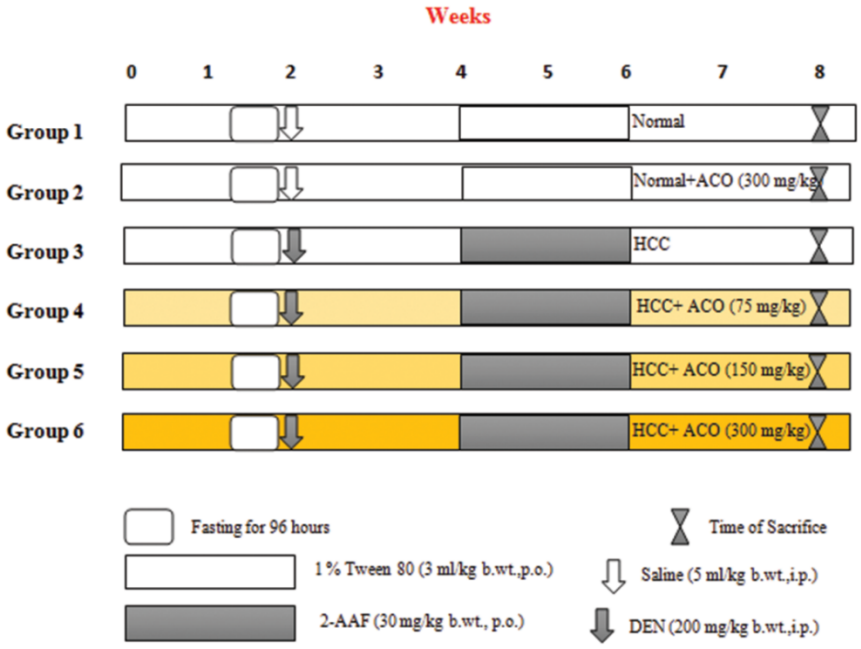


**Figure 1.** Experimental design. b.wt. body weight; i.p.: intraperitoneal; p.o.: per os (orally).

### 2.9. Determination of protein concentration

The concentration of liver protein was measured by the Coomassie blue protein-binding method using BSA as the standard [23].

### 2.10. Determination of hepatic oxidative stress markers

Hepatic glutathione (GSH) contents were estimated in liver homogenate by a spectrophotometric method using dithiobis-2-nitrobenzoic acid (DTNB) as the indicator of GSH and expressed as µg/mg protein. The intensity of the yellow color produced in the samples was recorded at 412 nm with a UV spectrophotometer (Infinite M200, TECAN) [24].

The level of malondialdehyde (MDA) as a reliable marker of lipid peroxidation was estimated in liver homogenate by reading the absorbance of the supernatant layer at 532 nm with an ELISA reader instrument (Infinite M200, TECAN). MDA levels were presented as µg/mg protein [25].

### 2.11. Statistical analysis

The homogeneity of variances was tested using Levene's test. The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD as the post hoc test. The data were presented as the mean±SD (n=6). The results with level of significance ($P$<0.05) were regarded as significant.

## 3.Results and discussion

### 3.1. Effects of ACO on serum biochemical parameters

Serum AFP is one of the most frequent diagnostic markers for HCC, which in this investigation was significantly enhanced in HCC rats as compared to normal rats ($P$ < 0.001). As can be seen in Fig. 2, the oral pretreatment of different concentrations of ACO to HCC rats caused a significant reduction in AFP ($P$ < 0.001). In addition, our results showed that the activities of GGT, ALT, and AST were clearly increased in DEN-treated rats compared with normal animals at the end of the experiment. As shown in Fig. 2, the elevations in the serum markers of liver injury, including GGT, ALT, and AST were effectively decreased in ACO-treated rats. The preventive effects of quince extract were dose dependent and the concentration of 300 mg/kg was statistically more effective than the other concentrations in decreasing AFP, GGT, ALT, and AST. It was also shown that the treatment of normal rats (Group 2) with ACO (300 mg/kg body weight daily) did not alter the biochemical markers (Fig. 2).

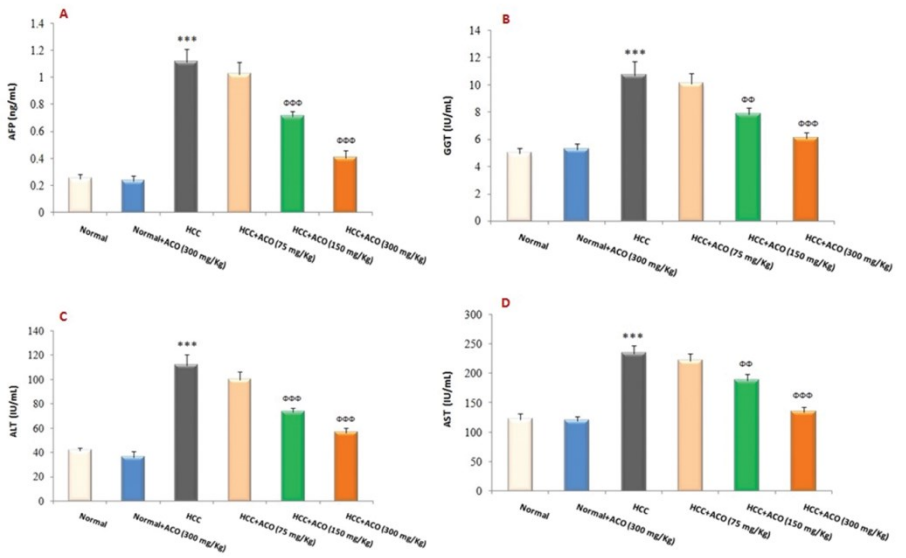


**Figure 2.** Effects of the quince extract (ACO) on serum biochemical parameters: (A) AFP, (B) GGT, (C) ALT, and (D) AST. Values are presented as mean±SD. *** $P$<0.001 compared to normal group; ΦΦ $P$<0.01, ΦΦΦ $P$<0.001 compared to HCC rats.

### 3.2. Effects of ACO on the hepatic markers of oxidative stress

GSH and its related antioxidant defense system are required to neutralize free radicals such as electrophilic carcinogens and to counteract oxidative stress. As indicated in Fig. 3, hepatic GSH levels were decreased in DEN-treated rats compared with normal rats ($P < 0.001$).

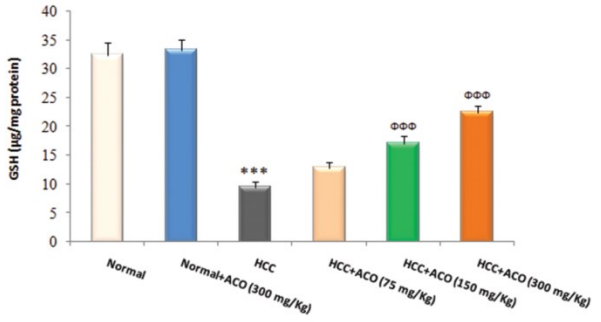


**Figure 3**. Effects of the quince extract (ACO) on hepatic GSH contents. Values are presented as mean±SD. *** $P<0.001$ compared to normal group; ΦΦΦ $P<0.001$ compared to HCC rats.

Furthermore, MDA levels as one of the important markers of oxidative stress were increased in HCC rats compared to normal animals ($P < 0.001$) (Fig. 4). In the current study, the pretreatment of quince extract to HCC rats caused an elevation in GSH levels as well as a reduction in lipid peroxidation marker, MDA (Figs. 3 and 4). Again, the fruit extract at the highest concentration (300 mg/kg) significantly acted more powerful than the other concentrations in attenuating hepatic oxidative stress markers. It can be also seen that the oral administration of ACO (300 mg/kg body weight/day) to normal rats (Group 2) did not change the oxidative stress markers (Figs. 3 and 4).

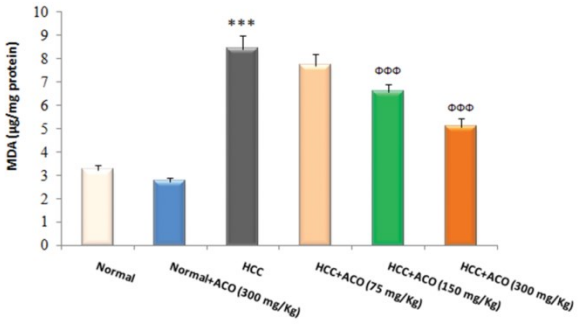


**Figure 4.** Effects of the quince extract (ACO) on liver lipid peroxidation. MDA formation as the marker of lipid peroxidation was expressed as µg/mg protein. Values are presented as mean±SD. *** $P<0.001$ compared to normal group; ΦΦΦ $P<0.001$ compared to HCC rats.

### 3.3. Effect of ACO on relative liver weight

As presented in Fig. 5, the relative liver weight of HCC rats was enhanced as compared to normal rats ($P < 0.01$). Our results indicated that the oral administration of ACO to DEN-treated rats caused a significant decline in relative liver weight ($P < 0.01$). Another time, the concentration of 300 mg/kg was more effective than the other concentrations in diminishing relative liver weight. Again, the pretreatment of normal rats (Group 2) with ACO (300 mg/kg body weight daily) did not affect the relative liver weight (Fig. 5).

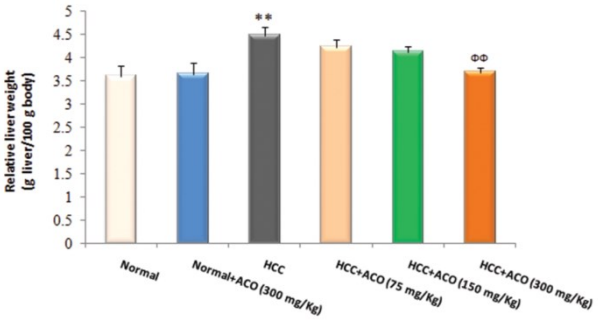


**Figure 5.** Effects of the quince extract (ACO) on relative liver weight. Values are presented as mean±SD.
** $P<0.01$ compared to normal group; ΦΦ $P<0.01$ compared to HCC rats.

## 4. Discussion and Conclusion

DEN is considered a potent chemical hepatocarcinogen from environmental point of view, which induces all the three stages of carcinogenesis i.e. initiation, promotion, and progression [26]. This environmental toxin can be found in all parts of our environment even in drinking water and foodstuffs [6]. The incidence and mortality of primary liver cancer or HCC are rising throughout of the world and unfortunately, it is often diagnosed at advanced stages. In the absence of proven effective chemotherapy and surgical intervention for HCC treatment, chemoprevention is needed for decreasing the risk or delaying the onset of this progressive cancer especially in high-risk individuals. Recently, much attention has been focused on medicinal plants and phytochemicals as the effective chemopreventive because of their preventive activity against common cancers like HCC [10].

Cydonia oblonga Mill. fruit commonly known as quince is considered as a promising agent for cancer prevention and treatment due to antiproliferative activity against

human kidney and colon cancer cells [15, 16]. We have recently done some experiments about the different pharmacological effects of the fruit and reported that quince has cardioprotective activity against doxorubicin-induced cardiotoxicity [27]. We have also shown that the fruit of quince has hepatoprotective, renoprotective, and hypolipidemic effects in a rat model of diabetes [14]. Subsequently, we focused on the hepatoprotective effects and demonstrated that quince has hepatoprotective activity against liver damage induced by carbon tetrachloride and it was suggested that the fruit may have potential hepatoprotective effects against xenobiotics-induced hepatotoxicity that share the same mechanism [28]. In this investigation, we evaluated the chemopreventive effect of quince fruit against DEN-induced HCC in rats as the end stage of hepatotoxicity. Our results clearly demonstrated that quince possesses a significant chemopreventive activity in the rat model of HCC induced by diethylnitrosamine as there is evident from reduction in the serum markers of liver damage and cancer.

Serum AFP level is the golden standard among diagnostic markers for HCC [29]. AFP is a glycoprotein that is synthesized during early foetal life and after birth its serum concentration falls rapidly. It has been suggested that AFP acts as a transport molecule for many ligands such as heavy metals, bilirubin, and many xenobiotics. In addition, AFP has a role in regulation of cell proliferation and an immunosuppressive activity [30]. Elevated AFP serum levels are only seen in certain tumors (e.g. HCC), non-tumoral conditions (e.g. cirrhosis), and maternal serum during pregnancy. As presented in Fig. 2, the elevated level of AFP was efficiently reduced by the quince extract.

GGT, ALT, and AST are also the most widely used HCC tumor markers. GGT is a cell surface enzyme that involves in glutathione metabolism. Its main function is to sustain cysteine levels in the body to conserve intracellular homeostasis of oxidative stress. GGT expression in tumor cells provides a selective advantage to the cells during tumor promotion and enables them to preserve elevated levels of intracellular GSH. Therefore, it has been suggested that GGT is an independent prognostic indicator in patients with HCC [31, 32]. As shown in Fig. 2, the activities of GGT, ALT, and AST were excellently decreased by the quince extract.

Glutathione and glutathione-related enzymes play a pivotal role in the metabolism and detoxification of free radicals and carcinogenic compounds. They are also involved in the regulation of carcinogenic mechanisms and cancer cell death [33]. It has been reported that GSH levels and the activities of GSH-dependent enzymes in the liver tissue of HCC patients were decreased due to the impairment of antioxidant defense [34, 35]. Our results clearly indicated that the quince extract counteracted DEN-induced oxidative stress and restored GSH concentrations in the liver of rats treated with quince.

Lipid peroxidation, as an important marker of oxidative stress, is a well-defined mechanism of cellular damage in different pathological conditions. MDA is the end product of lipid peroxidation, which is implicated in the promotion and progression stages of carcinogenesis [36, 37]. In this study, the oral administration of quince to DEN-treated rats decreased MDA levels and liver lipid peroxidation. Finally, our results indicated that the fruit extract prevented increase of relative liver weight caused by DEN treatment.

It is strongly believed that inflammation-mediated processes and oxidative stress contribute to the development and progression of hepatocarcinogenesis through different mechanisms [38-40]. Regarding the antioxidant and anti-inflammatory as well as hepatoprotective and anti-cancer activities of quince fruit, it can be suggested that these biochemical properties are responsible for the chemopreventive of the fruit.

Natural polyphenols are secondary metabolites of plants found in different kinds of fruits and vegetables that have shown various biological activities such as potent antioxidant and anti-inflammation effects. It has been demonstrated that polyphenols have numerous benefits for human health and a protective role against various diseases [41]. In recent years, there has been a considerable interest in their potential therapeutic use as chemopreventive agents. Several *in vitro* and *in vivo* studies have shown that plant polyphenols possess protective effects against the development and growth of liver cancer suggesting that these natural compounds are promising candidate agents for HCC chemoprevention. They reverse, suppress or prevent the different steps of hepatocarcinogenesis through various mechanisms [10].

The different kinds of phenolic acids and flavonoids are present in quince fruit, including quercetin 3-galactoside, rutin, caffeoylquinic acids, kaempferol-3-rutinoside, and kaempferol 3-glucoside [42]. It has been also reported that this fruit contains minerals such as calcium, sodium, potassium, phosphorus as well as pectin, amino acids, tannins, and vitamin C [11, 43]. In the present investigation, the quince extract was prepared from whole fruit and about 5% of the total weight related to polyphenolic compounds. So, it can be suggested that the preventive activity of quince against liver cancer may be mediated through the antioxidant, anti-inflammation, and anti-carcinogenic effects of polyphenols.

However, further studies to elucidate mechanisms underlying the effect would be complementary to this study. In addition, the authors believe that additional experiments are necessary to explain what active constituents are responsible for the chemopreventive effect of quince against liver cancer and to detect synergy among different compounds of this valuable fruit.

To conclude, our findings indicated that quince has a preventive effect against DEN-induced liver cancer by decreasing serum biomarkers of liver damage and cancer, including AFP, GGT, ALT, and AST. Moreover, quince exhibited *in vivo* antioxidant activity by elevating GSH contents as well as preventing lipid peroxidation in the liver tissues of HCC rats. The relative weight of liver was also reduced in quince-treaded rats.

It can be suggested that the chemopreventive activity of quince against DEN-induced hepatocarcinigenesis, at least partly, is mediated through polyphenols.

## Acknowledgment

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## Conflict of interest

There is no conflict of interest.

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