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REGULAR ARTICLE

Can methanolic extract of *Nigella sativa* seed affect glyco-regulatory enzymes in experimental hepatocellular carcinoma?

N. M. Abdel-Hamid · M. I. Abdel-Ghany · M. H. Nazmy · S. W. Amgad

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Abstract

Background and aim To investigate the possible modulating role of "*Nigella sativa*" (NS), a plant commonly used in Egyptian traditional medicine, on premalignant perturbations in three glycol-regulatory enzymes in an experimental rat model of hepatocellular carcinoma (HCC).

Methods Thirty-six (36) male albino rats were divided into four groups (n = 9). Group 1 served as a normal control, group 2 was treated with methanolic extract of *Nigella sativa* (MENS) (1 g/kg/day, orally) for 14 weeks, group 3 received a single intraperitoneal dose of diethyl nitrosamine (DENA) (200 mg/kg), followed 2 weeks later by a subcutaneous injection of carbon tetrachloride (CCl_4, 3 ml/kg/week/6 weeks) and group IV was treated with MENS for 2 weeks prior to administration of the carcinogenic combination (DENA + CCl_4, as in group 3) until the end of the experiment. The total period of the experiment was 14 weeks.

Results In the DENA + CCl_4-treated group, there was a significant increase in the relative liver weight, serum alpha fetoprotein level and the activities of hexokinase, glyceraldehyde phosphate dehydrogenase and glucose 6 phosphate dehydrogenase in both the serum and liver homogenate; this was accompanied by a subsequent decrease in body weight. Pre-treatment with MENS significantly maintained these parameters close to the normal condition.

Conclusion Based on these results, we conclude that MENS has a chemo-preventive effect against the progression into liver malignancy through its modulation of the energy metabolic pathways (i.e. glycolysis) that may be involved in hepatocarcinogenesis.

Keywords Premalignant hepatocellular changes · Glyco-regulatory enzymes · *Nigella sativa* · Glucose metabolism · Chemoprevention

N. M. Abdel-Hamid · M. H. Nazmy · S. W. Amgad
Biochemistry Department, Faculty of Pharmacy,
Minia University, Minia, Egypt

N. M. Abdel-Hamid (✉)
Diagnostic Laboratory, Abtal El-Faluga Street,
Mit-Gomre, Dakahlia, Egypt
e-mail: nabilmohie@yahoo.com

M. I. Abdel-Ghany
Pathology Department, Faculty of Medicine,
Minia University, El Minia, Egypt

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and the most common primary cancer of hepatocytes [1, 2]. It is responsible for approximately one million deaths each year. Variability in the incidence rates correspond closely to the prevalence and pattern of the primary etiologic factors. More than 80 % of HCC cases occur in developing countries. Areas of particularly high incidence are Eastern and South-eastern Asia and Sub-Saharan Africa [3]. According to recent reports, the incidence of HCC has increased sharply in the last decade, especially in Egypt, where there has been a doubling of the incidence rate during the last 10 years. This sharp rise has been attributed to several biological (e.g. hepatitis B and C virus infection) and environmental factors (e.g. aflatoxin), but many other factors, such as cigarette smoking, occupational exposure to chemicals (e.g. pesticides) and endemic infections in the community (e.g.

Schistosomiasis) may also contribute to the etiology or progression of the disease [4]. Liver carcinogenesis may also develop through the progressive accumulation of different mutations (genetic) and/or gene products (protein), which eventually lead to malignant transformation [5, 6].

Due to the increasing awareness of the side effects of conventional medicine, the use of natural products as an alternative to conventional treatment in the healing and treatment of various diseases, including cancer, has been increasing in the last few decades [7]. Several herbal drugs have been evaluated for their potential to protect the liver against diethylnitrosamine (DENA)-induced hepatotoxicity in rats [8]. *Nigella sativa* (NS), commonly known as black seed or black cumin 'Al-Habba Al-Sauda' or 'Habbet Al-Barakah'(in Arabic), is a seed of a capsulated plant that belongs to the Ranunculaceae family. NS has been employed for thousands of years as a spice and food preservative, as well as a protective and curative remedy for numerous disorders [9]. The seed contains 36–38 % fixed oils, proteins, alkaloids, saponin and 0.4–2.5 % essential oil as unsaturated fatty acids, including C20:2 arachidic and eicosadienoic acids [10]. The major active constituents are thymoquinone (TQ; 27.8–57.0 %), *ρ*-cymene (7.1–15.5 %), carvacrol (5.8–11.6 %), *t*-anethole (0.25–2.3 %), 4-terpineol (2.0–6.6 %) and longifoline (1.0–8.0 %) [11]. TQ readily dimerizes to form dithymoquinone (DTQ), which is believed to be nigellone [12, 13], and is the main bioactive constituent of the volatile oil of NS seeds. It has been shown to exert several pharmacological activities, including antioxidant, anti-inflammatory, chemotherapeutic and anti-tumor activities [14], as well as hepatoprotective activity [15].

Cancer cells are characterized by an abnormal pattern of energy metabolism that is manifested by an increase in glucose, fatty acid and amino acid metabolism and a decrease in oxidative phosphorylation [16]. Glucose is the primary energy source, and a high rate of glycolysis, which is one of the earliest discovered hallmarks of cancer cells, provides the tumor with metabolic and survival advantages [17, 18]. The early changes in carbohydrate metabolism are of particular interest, since anomalies of the glycolytic pathway are well-known biochemical disturbances in hepatomas [19, 20]. Elevated glucose catabolism is important for the production of the energy required by rapidly growing tumors. Early studies established the presence of abnormalities in the glucose-metabolizing enzymes with the transformation of normal liver cells into high glucose-utilizing hepatoma cell lines [21]. The high rate of aerobic glycolysis exhibited by some cancer cells is called the Warburg effect, in recognition of Otto Warburg's discovery some 80 years ago, who determined that tumor cells produce lactate in the presence of oxygen. Based on this observation, Warburg championed the notion that aerobic glycolysis is necessary during carcinogenesis [22–24]. A definite correlation exists between tumor progression and the activities of glycolytic enzymes [19, 20]. Therefore, in the study reported here, we have focused on the assessment of alterations in the activity of hexokinase (HK), glyceraldehyde-3-phosphate dehydrogenase (GAP-DH) and glucose-6-phosphate dehydrogenase (G6PD) enzymes in the serum and in the liver homogenate to investigate whether these enzymes are potential candidates as diagnostic and prognostic markers for HCC.

Materials and methods

Animals

Thirty-six adult male Wister Albino rats weighing 150–200 g were purchased from the Experimental Breeding House of Assiut University, Egypt. The animals were housed in plastic cage with wood chips for bedding and maintained at a temperature of 22 ± 2 °C, with 45 ± 4 % relative humidity under a 12/12-h (light/dark) cycle. The experiment commenced after acclimatization for 1 week to the animal house conditions. Both food and water were available for all animals ad libitum.

Chemicals

Diethylnitrosamine was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of analytical grade.

Preparation of *Nigella sativa* extract

Nigella sativa seeds, identified and harvested during the summer season, were purchased from the specialized Agricultural Institute in Dokki, Giza, Egypt. They were cleaned, dried, mechanically powdered, and extracted with 95 % aqueous methanol (1:10, w/v) for 24 h. The extract was filtered through a Buchner funnel, and the plant residue re-extracted with 50 % methanol for additional 2 h. The two extracts were combined and concentrated under reduced pressure on a rotatory evaporator at <40 °C until most of the methanol had been removed. The brownish-black crude extract was coded as methanolic extract of NS (MENS) and kept in a refrigerator at 4 °C. TQ is the main bioactive constituent of the aqueous alcoholic extract.

Experimental design

The research protocol, including treatment of the animals and the experimental design, was approved by the Research Ethics Committee of the College of Pharmacy, Minia University, Egypt and was performed in accordance to the

Guidelines for Care and Use of Laboratory Animals of Minia University.

The rats were classified into four experimental groups of nine rats each:

Group 1: untreated control (designated IP); the rats were given saline only

Group 2: *Nigella sativa* control group (designated NS). The rats received orally 1 g/kg/day of methanolic extract of *N. sativa* (MENS) by gavage for 14 weeks [25].

Group 3: [DENA + carbon tetrachloride (CCl_4) group]. The rats received a single intraperitoneal injection of DENA (200 mg/kg body weight), freshly dissolved in sterile 0.9 % saline [26]. Two weeks later, they received a subcutaneous injection of CCl_4 (3 ml/kg/week) for 6 weeks to promote the carcinogenic effect of DENA [27–29].

Group 4. The rats were treated with MENS for 2 weeks prior to receiving the carcinogenic combination DENA + CCl_4 (as in group 3) until the end of the experiment (14 weeks).

Preparation of serum and liver homogenate

At the end of the experimental period (14 weeks), the rats were fasted overnight, sacrificed and decapitated. Blood was collected and allowed to clot, then centrifuged; serum aliquots were kept frozen at −80 °C. The livers were immediately excised, rinsed with ice-cold saline, blotted dry and accurately weighed. The relative liver weight for each rat was calculated as the percentage ratio of absolute liver weight to the total body weight. A small portion of liver was kept in formalin for histopathological studies after hematoxylin and eosin (H&E) staining, while the remaining liver tissue was used for preparation of the homogenate. For the latter, 10 % of the homogenate was placed in 0.1 M Tris–HCl buffer (pH 7.4) using a Potter–Elvehjem homogenizer with a Teflon pestle. The liver homogenates were centrifuged at 4,000 rpm for 15 min and the supernatant stored in aliquots in Eppendorf tubes at −80 °C until enzyme activity was determined.

Biochemical analysis

The serum alpha fetoprotein (AFP) level was measured quantitatively by a solid phase enzyme-linked immunosorbent assay using a Calbiotech kit (Spring Valley, CA) following the instructions of the manufacturer. Enzyme activities of HK, GAPDH and G6PD were assayed spectrophotometrically in the serum and liver tissue homogenate. HK was measured according to Bergmeyer et al. [30], GAPDH was measured according to the procedures described by Velick [31] and G6PD was determined according to the method of Kornberg [32] using kits from Biodiagnostic (Dokki, Giza, Egypt). Total protein concentration in the supernatants of the tissue homogenates was determined according to the method adopted by Gornall et al. [33, 34] using a local kit (Spectrum).

Definition of units and specific activity

One unit of HK activity was taken to reduce 1 μmol of $NADP^+$ per minute at pH 7.6 at 25 °C; 1 U of GAPDH activity was taken to reduce 1 μmol of NAD per minute at 25 °C and pH 8.5 and 1 U of G6PD activity was considered to reduce 1 μmol of $NADP^+$ per minute at pH 7.6 at 25 °C. The specific activities of HK, GAPDH and G6PD were expressed as units per milligram of protein.

Statistical analysis

The results are presented as the mean ± standard deviation for nine rats in each group, and differences between mean values were determined by one-way analysis of variance, followed by Tukey's test. For multiple comparisons, values of $P > 0.05$ were considered to be statistically significant. The GraphPad Prism ver. 5 software program (GraphPad, San Diego, CA)was used for this purpose.

Results

Body weight and relative liver weight

Table 1 shows the final body weight, liver weight and the relative liver weight of the four groups. There was significant decrease in the final body weight of rats subjected to DENA and CCl_4, compared to control group. The body weights of the group 4 animals increased, indicating clearly that MENS had no toxic effects on the growth responses of the rats and was fairly well tolerated; in contrast, the change in relative liver weight of these animals was significant ($P < 0.001$) compared to the normal control (group I).

Biochemical investigations

Treatment with DENA + CCl_4 caused a significant increase in the serum AFP level. However, the administration of MENS significantly decreased the serum AFP level in the cancer group in comparison to premalignant group (group 3) (Table 2). The activities of HK, GAPDH and G6PD in the serum and liver tissue homogenate were significantly increased among the premalignant group. However, relative to the premalignant group (group 3), the administration of MENS significantly decreased the activities of these three enzymes in both the serum and liver

Table 1 Effect of methanolic extract of *Nigella sativa* on body weight and relative liver weight after 2 weeks of treatment

Groups	Final body weight (g)	Liver weight (g)	Relative liver weight (liver/100 g body weight)
1 (normal control)	228.5 ± 8.8	5.8 ± 0.4	2.51 ± 0.14
2 (MENS control)	260 ± 21.3^{++}	6.8 ± 0.41	2.63 ± 0.14
3 (premalignant group)	203.1 ± 5.7$^{\#}$	6.9 ± 0.46	3.40 ± 0.13$^{\#\#\#}$
4 (premalignant treated group)	243.4 ± 18.2***	6.7 ± 0.76	2.73 ± 0.15 ***

MENS, Methanolic extract of *Nigella sativa*

Data are presented as the mean ± standard deviation (SD) ($n = 9$ in each group)

$^{+}$ MENS control group is compared to normal control, $^{\#}$premalignant group is compared to normal control, *premalignant treated group is compared to premalignant group (1 symbol = $p < 0.05$, 2 symbols = $p < 0.01$, 3 symbols = $p < 0.001$)

Table 2 Serum level of alpha fetoprotein and activities of serum HK, GAPDH and G6PD in the experimental rat groups

Parameter	Groups			
	1 (normal control)	2 (MENS control)	3 (premalignant group)	4 (premalignant treated group)
AFP (ng/ml)	4.04 ± 0.61	5.13 ± 0.33	61.06 ± 2.71$^{\#\#\#}$	17.9 ± 0.99***
HK (mU/ml)	5.7 ± 2.0	5.20 ± 2.6	9.84 ± 1. 65$^{\#\#}$	6.56 ± 2.61*
GAPDH (mU/ml)	25.3 ± 3.41	22.31 ± 5.12	33.16 ± 5.43$^{\#\#}$	24.12 ± 3.7**
G6PD (mU/ml)	0.50 ± 0.38	0.56 ± 0.32	2.4 ± 0.44$^{\#\#\#}$	1.75 ± 0.46*

AFP, Alpha fetoprotein; HK, hexokinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G6PD, glucose-6-phosphate dehydrogenase

Data are presented as the mean ± SD ($n = 9$ in each group)

$^{+}$ MENS control group is compared to normal control, $^{\#}$premalignant group is compared to normal control, *premalignant treated group is compared to premalignant group (1 symbol = $p < 0.05$, 2 symbols = $p < 0.01$, 3 symbols = $p < 0.001$)

homogenate [serum: G6PD ($P < 0.05$), GAPDH ($P < 0.01$); liver homogenate: G6PD ($P < 0.015$), GAPDH ($P < 0.001$)] (Tables 2, 3 respectively).

Histopathological examination

Representative samples were taken from the liver tissue of each animal and prepared for histopathological examination. Study of the liver tissue sections from rats in the normal and *Nigella* control groups revealed a normal hepatic lobular architecture and the presence of normal hepatocytes with granulated cytoplasm and small uniform nuclei and nucleolus, indicating the non-toxic nature of MENS (Fig. 1a, b). In contrast, the study of sections obtained from rats subjected to DENA + CCl_4 treatment revealed hydropic degeneration, with most liver cells showing a cloudy swelling due to the presence of foamy cytoplasm that resulted from the intracellular accumulation of water. Changes in fat content, variable areas of necrosis, congestion of portal tracts, bile stasis and inflammatory infiltration were prominent (Fig. 1c–e). Animals pre-treated with MENS showed minimal changes in hepatocyte morphology and histology. Most of the histopathological changes seen in the cells of the premalignant HCC group were greatly reduced (Fig. 1f).

Discussion

The results of this study collectively and clearly demonstrate the efficacious effect of MENS on the activities of three glycol-regulatory enzymes (HK, GAPDH, G6PD) in experimentally induced hepatocarcinogenesis. Animals treated with DENA and CCl_4 showed significant histological and biochemical variations, reflecting the instability of liver cell metabolism and leading to distinctive changes in serum enzyme activities and AFP (the relevant tumor marker). DENA is known to cause perturbations in the nuclear enzymes involved in DNA repair/replication [35] and is normally used to induce liver cancer in animal models [36, 37]; [38, 39]. Treatment with DENA and CCl_4 has been shown to induce extensive necrosis and inflammatory infiltration, clusters of hepatocytes, necrosis, bile duct proliferation and marked atypia [38, 40].

In this study, the activities of three glyco-regulatory enzymes were up-regulated in group 3 rats (premalignant

Table 3 Activities of HK, GAPDH and G6PD in liver tissue homogenate of the experimental rat

Parameters	Groups			
	1 (normal control)	2 (MENS control)	3 (premalignant group)	4 (premalignant treated group)
HK (mU/mg protein)	7.926 ± 2.319	8.473 ± 2.167	15.58 ± 2.725###	10.66 ± 3.395**
GAPDH (mU/mg protein)	28.34 ± 4.025	27.73 ± 4.275	46.42 ± 8.907###	29.54 ± 4.025***
G6PD (mU/mg protein)	3.425 ± 1.316	4.438 ± 1.178	6.750 ± 0.707###	4.625 ± 1.061**

Data are presented as the mean ± SD (n = 9 in each group)

+ MENS control group is compared to normal control, #premalignant group is compared to normal control, *premalignant treated group is compared to premalignant group (1 symbol = $p < 0.05$, 2 symbols = $p < 0.01$, 3 symbols = $p < 0.001$)

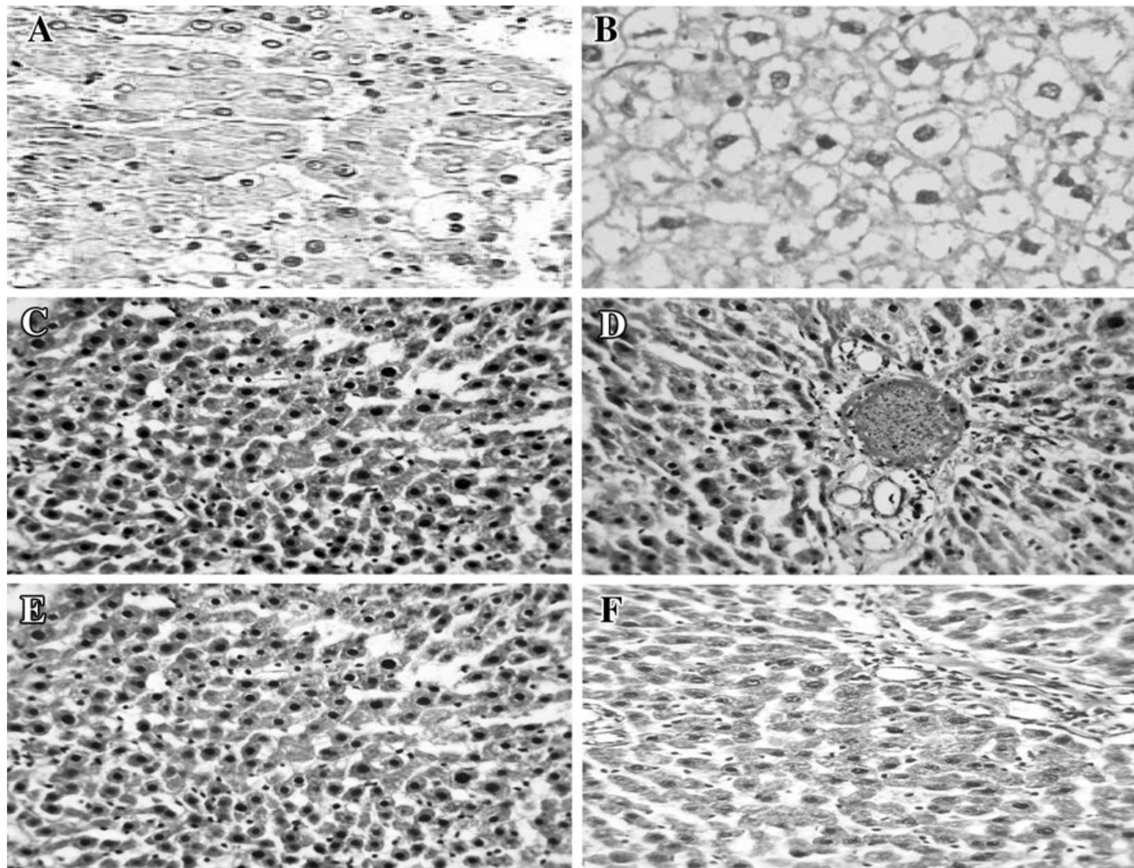

Fig. 1 Representative photomicrographs of liver sections of the studied groups. **a** Normal control, **b** methanolic extract of *Nigella sativa* (MENS) control. Cells of both groups showed normal hepatic lobular architecture and hepatocytes with granulated cytoplasm and small uniform nucleus and nucleolus [hematoxylin and eosin (H&E) staining, ×200]. **c–e** Group 3 (premalignant group): **c** focal areas of dysplasia marked with large prominent hyperchromatic nuclei, atypia and eosinophilic cytoplasm, **d** markedly congested portal tract, proliferated bile ductules, bile stasis and infiltration with some inflammatory cells; adjacent liver cells show dysplasia, hydropic degeneration and focal areas of necrosis, **e** changes in fat (both micro- and macro-vesicular) (H&E, ×400). **f** Group 4 [animals pretreated with MENS, then DENA (diethyl nitrosamine) and CCl_4 (carbon tetrachloride)]; cells showed more or less normal histology and no inflammation, as evidenced by lesser leukocyte infiltration. Collectively, most of the histological disturbances observed in the premalignant group were greatly reduced (H&E, ×400)

group). It is well-known that cancer cells generally need increased levels of aerobic glycolysis and therefore produce lactate even in the presence of oxygen (the Warburg effect) for ATP generation [22, 41]. Thus, increased glycolysis or pentose phosphate metabolism may confer adaptive advantages if excess pyruvate is synthesized for lipid synthesis, thereby providing essential anabolic substrates, such as ribose, for nucleic acid synthesis [42]. Glucose consumption through the pentose pathway may also provide essential reducing equivalents (NADPH) to reduce the toxicity of reactive oxygen species, conferring resistance to senescence [43, 44]. Consequently, cancer

cells have a high glycolytic rate, which is advantageous in terms of tumor growth [45]. Rapidly growing, highly malignant tumor cells can obtain up to 60 % of their total ATP production from glycolysis. An elevated rate of glycolysis in tumor cells results in an increase in the intracellular concentration of glucose-6-phosphate, a key precursor in the de novo synthesis of nucleic acids, phospholipids and other macromolecules. This increased glucose-6-phosphate level is likely to be essential to keep pace with rapid cell division and membrane biosynthesis during tumor growth. Thus, rapidly proliferating tumors have an excess demand for nuclear energy, which is demonstrated by the nuclei having elevated activities of glycolytic enzymes [20].

The increased activity of rate-limiting glycolytic enzymes, such as HK, in tumor cells is one of the factors responsible for this increased aerobic glycolysis [46] in cancer cells and plays an important role in determining the glycolytic capacity of these cells [20]. We found that GAPDH, also a regulatory enzyme in the glycolytic pathway, was increased in our experimental hepatocarcinogenesis rat model. GAPDH has been reported to be overexpressed in cancers, including lung [47], colorectal-, prostate-, bladder-cancer [48] and several transformed tumor cell lines [49], and to be strongly up-regulated in advanced stages of HCC [50]. This enzyme is an also indicator of metastatic growth and increases specifically after metastasis. There have been reports of the increased activity of G6PD in hepatic carcinoma being a very useful diagnostic and prognostic tool to detect early pre-neoplastic lesions [51].

There is increasing evidence that G6PD activity is of major importance in NADPH production for defense against oxidative stress rather than for ribose production during proliferation [52]. Several key genetic alterations associated with tumor development have recently been shown to affect glycolysis directly, such as p53 mutation and the activation of hypoxia inducible factor [53, 54]. It is conceivable that the metabolic alterations in malignant cells may be exploited to develop therapeutic strategies to target this metabolic abnormality. One possibility is to inhibit glycolysis and preferentially kill the cancer cells that are dependent on glycolytic pathway for ATP generation [22, 41].

Our results indicate that the administration of MENS significantly curtailed liver tumor development and protected the cells against the histological and biochemical effects induced by the carcinogen. Earlier observations also support this proposal [55]. In an earlier study, a decoction of NS seed and some indigenous herbs was shown to have the potential to suppress hepatic tumor in rats induced by DENA [56]. It has also been shown to possess cytotoxic activity, even at low concentrations, against the human hepatoma HepG2 cell line through its inhibitory effects on DNA synthesis [57–60]. In this respect, the possible modes of action of thymoquinon (TQ) include antioxidant activity, interference with DNA synthesis and enhancement of the detoxification processes. Moreover, the aqueous extract of NS seeds was found to have a strong inhibitory effect on NO production by murine macrophages [61].

It can be concluded that the administration of MENS was highly successful in revert DENA-induced alterations in glyco-regulatory enzyme activities in liver tissue and the circulation back to the normal condition. Collectively, these data highlight one of the mechanisms by which *N. sativa* may exert its chemo-preventive potential, i.e. by modulation of energy metabolism in both the target organ and circulation, possibly through its potency in normalizing abnormal cell behavior. The lack of toxicity associated with this natural agent combined with its cost effectiveness are additional advantages for its use as a chemo-preventive agent. Based on our results, we recommend the use of NS either as a decoction or its dried methanolic extract for individuals at risk for HCC in order to prevent hepatocarcinogenesis or to break the link between the risk and carcinogenesis.

Conflict of interest The authors declare that they have no competing interests.

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