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Assessing the Efficacy of Fenugreek Saponin Nanoparticles in Attenuating Nicotine-Induced Hepatotoxicity in Male Rats

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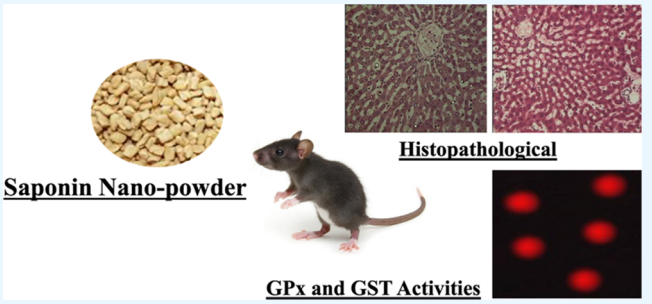
ABSTRACT: During smoking, nicotine, the most bountiful compound in cigarettes, is absorbed into the body by the lungs and quickly metabolized in the liver, causing three major adverse impacts such as toxic, neoplastic, and immunomodulatory effects. Saponins extracted from several plants are reported to exhibit various biological actions, such as anticancer effects. So, the potential protective effect of fenugreek saponin and nanofenugreek saponin against toxicity induced by nicotine in male rats was investigated in this study. Animals were exposed to nicotine (1.5 mg/kg/day) and/or treated with fenugreek saponin (25, 50, and 100 mg/kg/day) and nanofenugreek saponin (20, 40, and 80 mg/kg/day). Comet assays, histopathological examination, and analyses for the expression levels of glutamate aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1) genes in liver tissues as well as the activity of glutathione peroxidase (GPx) and glutathione-S-transferase (GST) were conducted. The results revealed that nicotine treatment induced a significant increase in DNA damage, decrease in the expression levels of (GLAST) and (GLT-1) genes, and increase in histopathological alterations in liver tissues. Moreover, nicotine treatment induced a significant reduction in the activity of antioxidant enzymes GPx and GST. On the other hand, administration of fenugreek saponin or nanofenugreek saponin with nicotine significantly decreased the DNA damage, increased the expression levels of (GLAST) and (GLT-1) genes, and decreased histopathological alterations in liver tissues. Additionally, a significant increase in the activities of GPx and GST was observed. The results suggested that DNA damage and histological injuries induced by nicotine were decreased by the administration of fenugreek saponin or nanofenugreek saponin; thus, fenugreek saponin and nanofenugreek saponin can be used as ameliorative agents against nicotine toxicity.



1. INTRODUCTION

Nicotine is actually a colorless liquid and does not turn brown until it is burned.[1] As a result of nicotine's ability to enhance attention by its effect on the nervous system, it is considered the main reason why smokers continue to smoke and enter the process of tobacco addiction,[2,3] and it was also found that nicotine affects cognitive performance.[4] Nicotine is absorbed through the whole respiratory tract, throughout the oral and nasal mucosa, to the gastrointestinal tract including liver tissues.[5] It is found that nicotine is accounted for many psychopharmacological effects.[6] It increases inflammation in lung epithelial cells and airway hyper-reactivity in lipopolysaccharide-challenged mice.[5,7,8] Nicotine also suppresses apoptosis in lung tumors.[9] Moreover, it is known to produce epigenetic alteration of DNA in germ and somatic cells.[10] Additionally, cigarette smoke contains more than 1000 chemical ingredients, many of which can produce changes in DNA methylation.[11] Despite of many of natural compounds are presently known,[12] the significant incident of nature always put up as golden spot for achieving the herbal drug finding.[13−15] Antioxidants are considered as reducing agents that inhibit oxidative reactions, frequently by scavenging the reactive oxygen collections before they can damage cells.[6,16]

Fenugreek is an annual plant that belongs to the Leguminosae family.[1717] Its seeds and leaves are generally used as a flavoring and condiment, as well as a maize and wheat flour supplement for preparing bread.[1818] The whole parts of this plant have shown antidiabetic, antimicrobial, anticancer, anti-inflammation, and antioxidant activities.[19−22] Saponins and phenolics from fenugreek are considered as natural antioxidants which have the ability to scavenge the free radicals produced in human body induced by several environmental pollution issues.[23−25] Nanomedicine makes a huge influence in healthcare field in dealing with many types of chronic diseases.[26−30] Hence, biodegradable synthesis of

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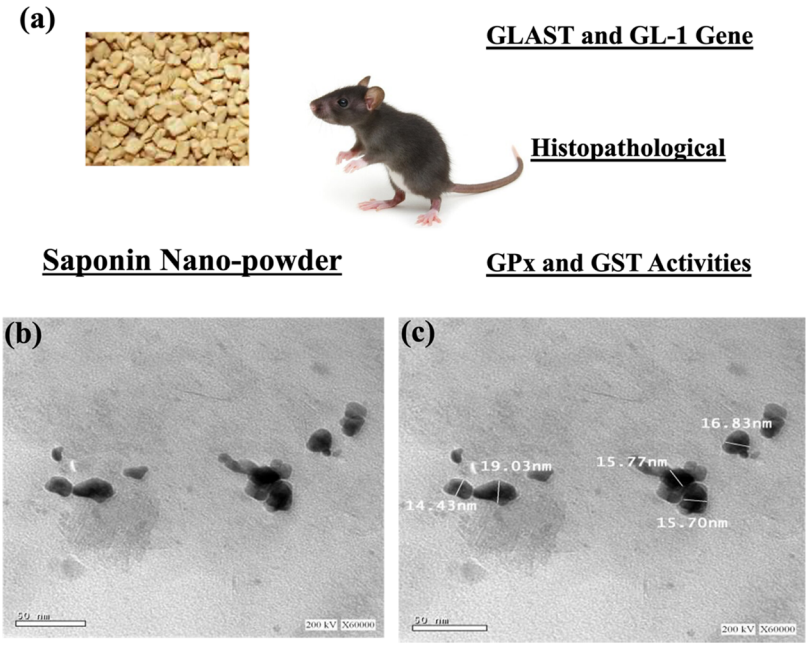

Figure 1. (a) Schematic illustration of fenugreek saponin and nanofenugreek saponin and their application in nicotine-induced hepatotoxicity in male rats. (**b,c**) Transmission electron microscopic (TEM) images showing the amorphous-shaped saponin nanoparticle.

Table 1. Degrees of DNA Damage Observed by Comet Assay in Rat Genomic DNA Treated with Nicotine and/or Fenugreek Saponin or Nanofenugreek Saponin[a]

group	dose[b]	% DNA	tail length	tail intensity	tail moment
control		95.72 ± 2.60^{ab}	4.28 ± 0.84^{b}	234.50 ± 622.68^{e}	0.25 ± 0.15^{c}
nicotine		89.56 ± 4.71^{b}	13.21 ± 1.63^{a}	2856.29 ± 59.32^{a}	1.01 ± 0.01^{a}
saponin	25	93.15 ± 2.24^{ab}	2.72 ± 0.12^{c}	563.07 ± 52.29^{d}	0.14 ± 0.03^{c}
	50	95.72 ± 2.60^{ab}	4.28 ± 0.84^{b}	743.61 ± 486.45^{c}	0.25 ± 0.01^{c}
	100	94.14 ± 0.92^{ab}	2.50 ± 0.24^{c}	436.56 ± 159.57^{c}	0.61 ± 0.06^{b}
nanosaponin	20	85.85 ± 2.62^{ab}	2.99 ± 0.14^{c}	644.04 ± 574.11^{cd}	0.21 ± 0.05^{c}
	40	98.16 ± 0.71^{a}	11.76 ± 1.44^{a}	255.56 ± 64.62^{e}	0.30 ± 0.04^{c}
	80	97.98 ± 1.21^{a}	4.88 ± 0.89^{b}	240.07 ± 175.39^{e}	0.27 ± 0.02^{c}
nicotine + saponin	25	93.49 ± 2.90^{ab}	5.53 ± 0.89^{b}	1502.75 ± 47.44^{b}	0.32 ± 0.04^{c}
	50	94.58 ± 2.72 ^{ab}	4.76 ± 1.10^{b}	898.90 ± 317.48^{c}	0.28 ± 0.04^{c}
	100	98.87 ± 0.61^{a}	2.22 ± 0.24^{c}	874.31 ± 70.98^{c}	0.60 ± 0.18^{b}
nicotine + nanosaponin	20	96.88 ± 0.95^{a}	4.72 ± 1.58^{b}	808.25 ± 78.18^{c}	0.08 ± 0.03^{c}
	40	96.96 ± 1.72^{a}	5.77 ± 1.71^{b}	549.53 ± 151.67^{d}	0.29 ± 0.06 ^{c}
	80	96.27 ± 2.21^{a}	2.92 ± 1.06^{c}	479.73 ± 74.16^{d}	0.19 ± 0.08^{c}

[a]In the same column, means marked with the same superscript letter are insignificantly different ($P > 0.05$), whereas those marked with different ones are significantly different ($P < 0.05$). Data are presented as mean ± SEM. [b]Dose in mg/kg b.wt.

nanoparticles is considered as building blocks of the forthcoming generations to treat many diseases.[31−34] Great efforts have been made in manufacturing nanoparticulate carriers, which function as proficient diagnostic or therapeutic agents for cancer.[35−37]

The green synthesis of nanoparticles is very effective as it is ecofriendly[27,38−41] and less expensive and is a time-saving method for preparation of nanoemulsions and nanometallic synthesis. Keeping in mind the emerging function of nanomedicine and nanoparticles prepared by the green chemistry process, nicotine is a dangerous and cancer-causing agent in liver and lungs. Therefore, the present study was aimed to evaluate the nanofenugreek saponin in comparison with fenugreek saponin against nicotine-induced hepatotoxicity in male rats.

2. RESULTS AND DISCUSSION

2.1. Characterization of Fenugreek Saponin Nanoparticles. In the present work, saponin nanoparticles were mixed as fenugreek saponin and nanofenugreek saponin and their characterization and biological evaluation were carried out in vivo as demonstrated in Figure 1a. The TEM images were taken to verify the primary particle sizes and morphologies of saponin nanoparticles. The diameter of ultrasonicated nanosaponin was measured in a random view field. The ultrasonicated saponin nanoparticles were amorphous with an average diameter of 14.36 ± 0.723 nm. Transmission electron microscope imaging and analysis (TIA) software was used for spectrum acquisition and analysis of EDX peaks in Figure 1b,c. The role of the given doses of fenugreek saponin and nanofenugreek saponin against the genotoxicity of nicotine represented in the comet parameters,

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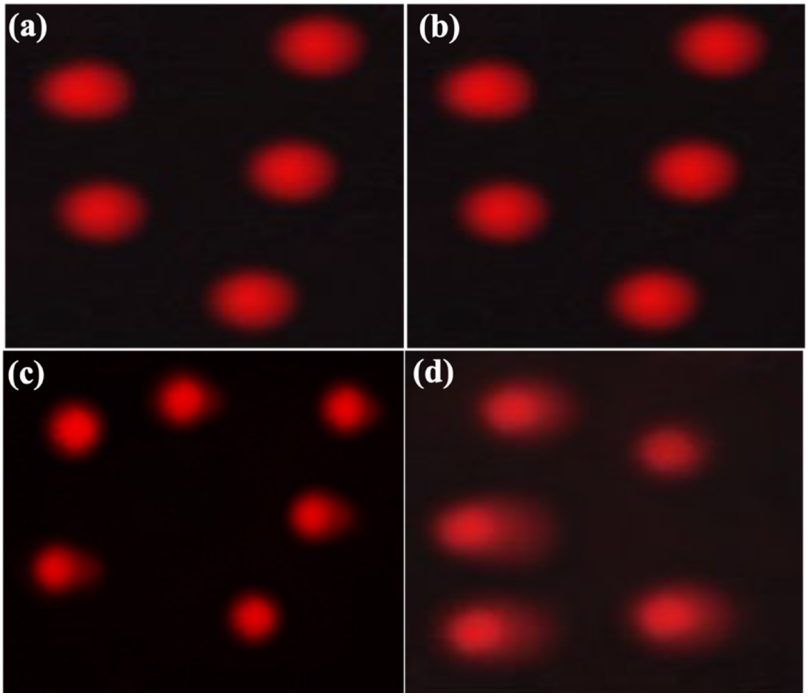

Figure 2. DNA damaging effect of fenugreek saponin and nanofenugreek in male rats (magnification ×400). (a, c) Undamaged cells; (b, d) heavily damaged cells.

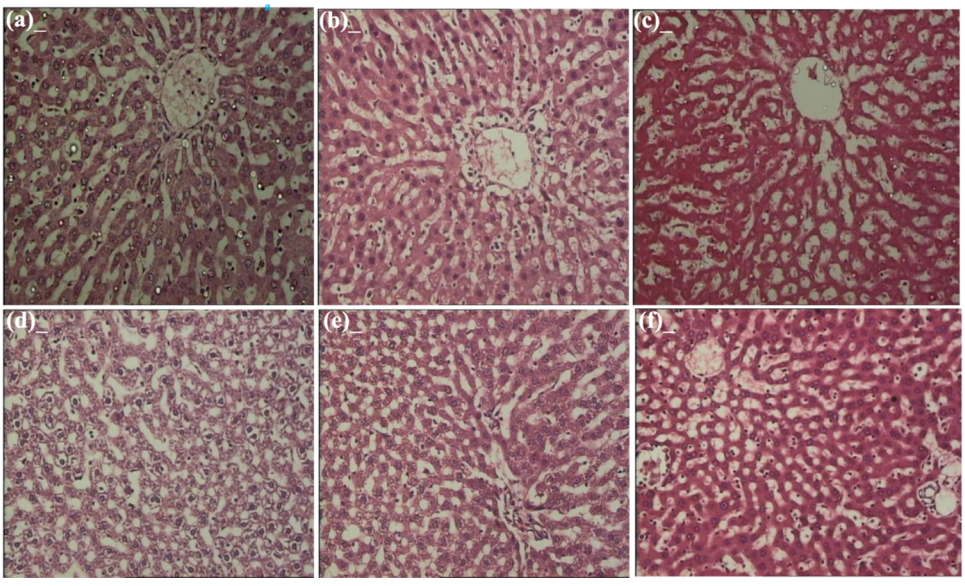

Figure 3. Light photomicrographs of several sections of liver tissues: (a) liver tissues of control rats, (b) liver tissues of rats treated with high dose of fenugreek saponin, (c) liver tissues of rats treated with high dose of nanofenugreek saponin, (d) liver tissues of rats treated with nicotine alone, (e) liver tissues of rats treated with nicotine in combination with high dose of fenugreek saponin, and (f) liver tissues of rats treated with nicotine in combination with high dose of nanofenugreek saponin (H&E x 400).

DNA damage, tail length (TL), tail intensity (TI), and tail moment (TM), is summarized in Table 1.

The results revealed that the administered doses of fenugreek saponin 25 mg (GIII), 50 mg (GIV), 100 mg (GV) and nanofenugreek saponin 20 mg (GVI), 40 mg (GVII), 80 mg (GVIII) did not induce any effect on the studied comet parameters compared with the control group. Thus, there are no significant differences between each of the levels of DNA, TL, TI, and TM among all groups of rats given fenugreek saponin and nanofenugreek saponin (Table 1). On the other hand, the hepatic DNA content of rats given 1.5 mg of nicotine alone (GII) significantly decreased when compared with the control group (GI) and the groups treated with several doses of nanosaponin combined with nicotine (GXII, GXIII, and GIVX). Moreover, the rates of TI, TL, and TM were significantly higher in the nicotine group than those in the control group and the groups treated with several doses of nanosaponin combined with nicotine.

From Table 1, administration of nanofenugreek saponin (20, 40, and 80 mg) with nicotine (1.5 mg) caused a significant severe reduction of most comet parameters, especially the TL and TM did not differ from group I.

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From Figure 2a−d, all types of comets are seen in all studied groups. Photomicrographs showing typical nuclei with various degrees of DNA damage are observed as comet. There was no damage or aberration shown in the nucleus of the control group (Figure 3a,c). However, we can see in Figure 2b,d that it was heavily damaged. Actually, the frequency of each type differed between normal and groups treated with nicotine alone and in combination with fenugreek saponin and nanofenugreek saponin (magnification ×400).

2.2. Expression Profile of GLAST and GL-1 Genes. The expression levels of the hepatic GLAST & GLT-1 genes in rats treated with nicotine and/or fenugreek saponin or nanofenugreek saponin are summarized in Table 2. The results

Table 2. Expression Levels of Hepatic GLAST and Hepatic GLT-1 Genes in Rats Treated with Nicotine and/or Fenugreek Saponin or Nanofenugreek Saponin[a]

group	dose[b]	GLAST	GLT-1
control		1.00 ± 0.00[a]	1.00 ± 0.00[a]
nicotine		0.27 ± 0.01[d]	0.28 ± 0.02[d]
saponin	25	0.98 ± 0.02[a]	0.97 ± 0.02[a]
	50	0.95 ± 0.01[a]	0.96 ± 0.003[a]
	100	0.96 ± 0.01[a]	0.93 ± 0.01[a]
nanosaponin	20	0.94 ± 0.02[a]	0.98 ± 0.02[a]
	40	0.95 ± 0.01[a]	0.99 ± 0.02[a]
	80	1.13 ± 0.003[a]	1.08 ± 0.02[a]
nicotine + saponin	25	0.37 ± 0.02[d]	0.40 ± 0.02[d]
	50	0.54 ± 0.01[c]	0.59 ± 0.02[bc]
	100	0.72 ± 0.02[b]	0.77 ± 0.02[b]
nicotine + nanosaponin	20	0.56 ± 0.01[c]	0.62 ± 0.02[b]
	40	0.85 ± 0.03[b]	0.97 ± 0.003[a]
	80	0.93 ± 0.03[a]	0.99 ± 0.01[a]

[a]In the same column, means marked with the same superscript letter are insignificantly different ($P > 0.05$), whereas those marked with different ones are significantly different ($P < 0.05$). Data are presented as mean ± SEM. [b]Dose in mg/kg b.wt.

demonstrate that rats treated with nicotine showed a significant reduction in the expression levels of GLAST & GLT-1 genes compared with those in the control group and groups of rats treated with fenugreek saponin or nanofenugreek saponin (Table 2). In contrast, treatment of nicotine-exposed rats with fenugreek saponin increased significantly the expression levels of GLAST & GLT-1 genes at medium and high doses compared with those in rats exposed to nicotine alone. Moreover, treatment of nicotine-exposed rats with nanofenugreek saponin increased significantly the expression levels of GLAST & GLT-1 genes at the low, medium, and high doses compared with those in rats exposed to nicotine alone (Table 2).

2.3. Histopathological Lesions in Liver. The liver sections of the control group showed a normal central vein and endothelial lining without any bleeding, normal hepatocytes radiating arrangement from central vein, and blood sinusoids appearing between the hepatocytes. Hepatocytes were granular and acidophilic cytoplasm, with normal arrangement of hepatic cords, normal sinusoids between the hepatocytes, and containing normal number of Kupffer cells. In the portal area, a typical portal canal contained branches of portal vein, hepatic artery, and bile duct; the triad was clearly bordered by the surrounding normal hepatocytes and interlobular septa. The normal structure of functional metabolic zones between the central vein and portal tract, zone, normal hepatic sinusoids connecting the zones is observed in Figure 3a.

The liver sections of rats treated with fenugreek saponin and nanofenugreek saponin at different doses showed mild histopathological morphology of the liver tissue (cell enlargement, some multi-nucleated cells, and few necrotic hepatocytes). The mild changes observed in the liver tissue showed the dose-dependent activity of fenugreek saponin and nanofenugreek saponin (Figure 3b,c).

The liver sections of rats treated with nicotine showed severe histopathological changes in the central vein area such as bleeding, damage in the lining endothelium, congestion in sinusoids around central vein, disturbance of hepatic lobules, some aggregation of inflammatory cells around the portal triad, congestion in portal blood vessels, fibrosis of portal tract, and distraction in hepatocytes surrounding the portal tract. The sections also exhibited pyknosis, Karyorrhexis and karyolysis, apoptotic hepatocytes, prominent Kupffer cells, and vacuolar degeneration along with apoptosis, focal necrosis, and inflammation characterized by a focal group of contiguous cells with cell swelling and loss of cellular detail when compared with control sections, as shown in Figure 3d.

In the groups treated with nicotine in combination with fenugreek saponin and nanofenugreek saponin, respectively, the liver sections exhibited a decrease in the histopathological changes and became slightly close to the control group when compared with nicotine-exposed rats in Figure 3e,f.

The activities of the antioxidant enzymes GPx and GST were determined and are shown in Figures 4 and 5, respectively. The results exhibited that nicotine treatment decreased the activities of GPx and GST in liver tissues of male rats compared to those in control rats. On the other hand, treatment of male rats with fenugreek saponin or nanofenugreek saponin did not alter the activities of GPx and GST in liver tissues of male rats compared to those in control rats. Additionally, treatment of nicotine-exposed rats with fenugreek saponin increased significantly the mean values of GPx and GST activities in liver tissues at only a high dose compared with those in rats exposed to nicotine alone. Moreover, treatment of nicotine-exposed rats with nanofenugreek saponin increased significantly the mean values of GPx and GST activities in liver tissues at the medium and high doses compared with those in rats exposed to nicotine alone, Figures 4 and 5.

This study reported that the DNA damage assessed by the comet method in the hepatocyte in rats treated with nicotine only showed a significant increase when compared with those of rats treated with distilled water, which agreed with the results of Tsuda et al.,[42] who illustrated that single inhalation of cigarette smoke caused DNA damage in the lung, stomach, and liver, but not in the kidney, brain, or bone marrow of mice. Also, smokers identified with lung cancer were compared to cases with no cancer history including smokers and nonsmokers.[43] Tobacco usage has been inducing a multitude of genetic abnormalities, including gene mutations, CA, MN, and DNA strand breaks.[44]

The saponin fraction was found to reduce the viability of the human tumor cell line in a dose-dependent manner. Previous studies have reported that saponins and phenolics, as natural antioxidants, have the ability to scavenge the free radicals produced in human body as a result of environmental pollution or the normal metabolism in the body.[45−47] These works can

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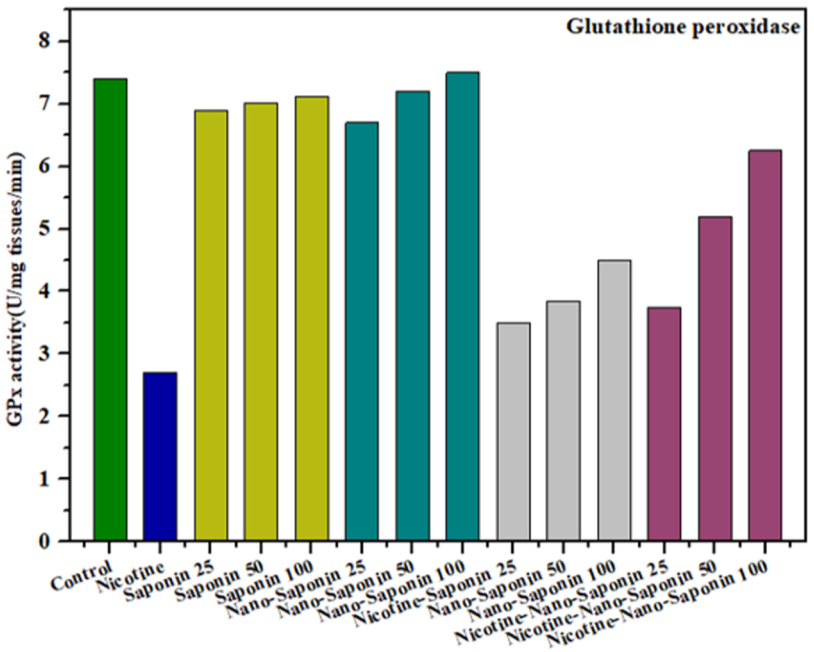

Figure 4. Glutathione peroxidase (GPx) activities in male rats treated with nicotine and/or fenugreek saponin or nanofenugreek saponin. In different treatments (columns), means marked with the same superscript letters are insignificantly different ($P > 0.05$), whereas those marked with different ones are significantly different ($P < 0.05$). Data are presented as mean ± SEM.

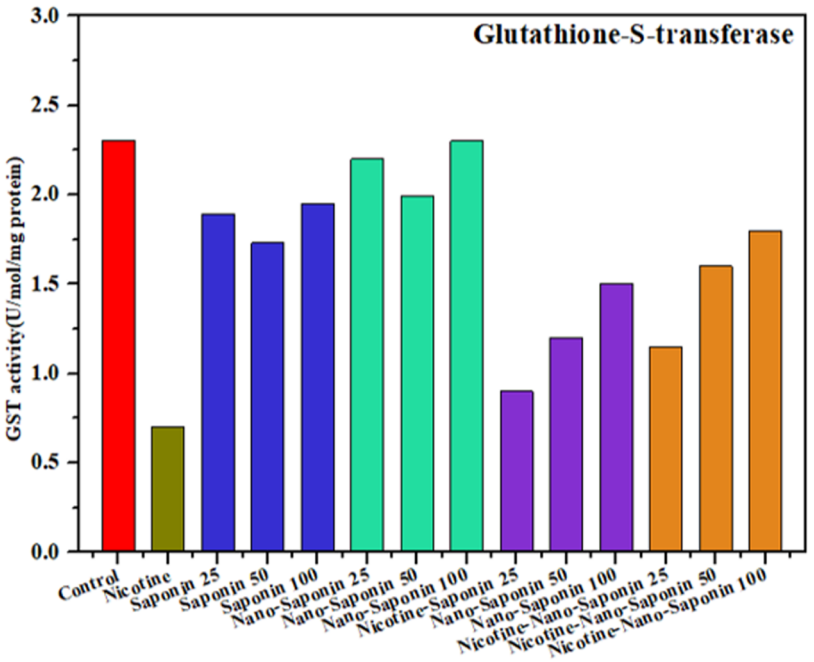

Figure 5. Glutathione-S-transferase (GST) activities in male rats treated with nicotine and/or fenugreek saponin or nanofenugreek saponin. In different treatments (columns), means marked with the same superscript letters are insignificantly different ($P > 0.05$), whereas those marked with different ones are significantly different ($P < 0.05$). Data are presented as mean ± SEM.

explain this study that fenugreek saponin and nanofenugreek saponin significantly reduced the DNA damage caused by nicotine when both were administrated in combination with nicotine. A group of rats administrated fenugreek saponin in combination with nicotine showed a significant depletion in the hepatocytic DNA damage and became slightly close to the control group. Moreover, administration of nanofenugreek saponin in combination with nicotine caused a significant severe reduction of most comet parameters in a dose-dependent manner. These results are in line with those of Das et al.,[48,4948,49] who reported that plant-derived nanoparticles have the potential to control tumor cell growth. Moreover, Melek et al. reported that the saponin fraction was found to reduce the viability of the human tumor cell line in a dose-dependent manner.[45] Numerous studies found that administration of drugs induced a marked increase in extracellular glutamate concentration in the mesocorticolimbic regions.[50−53] It has been reported that this effect can be

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coupled with downregulation of glutamate transporters (GL-1 and GLAST).[54,5554,55] Several glutamate transporters regulate glutamate uptake in astrocytes.[56] GLT-1 is responsible for the removal of the majority of extracellular glutamate concentration in astrocytes.[57] Glutamate/aspartate transporter (GLAST) is an additional glutamate transporter, colocalized with GLT-1 in astrocytes, and is chiefly expressed in the cerebellum and retina.

The present study found that rats treated with nicotine showed a significant reduction in the expression levels of GLAST & GLT-1 genes compared with those in the control group and the groups treated with fenugreek saponin or nanofenugreek saponin. A significant reduction in the expression levels of GLT-1 and GLAST was also observed in rats treated with nicotine compared with those of the control group treated with distilled water. The study also reported that the usage of e-cigs for six months induced downregulation of GLT-1 in the striatum and downregulation of cystine/glutamate antiporter (xCT) in the striatum and hippocampus of female mice. In general, underneath physiological conditions, the prohibition of glutamate in the synapse is operated by astrocytic glutamate transporters (GL-1 and GLAST), the representation of which might be significantly weakened under pathological conditions.

As fenugreek comprises several phytochemicals, carbohydrates, alkaloids, steroidal saponins, minerals, and amino acids, it can be used for nutritional, nutraceutical, medicinal, and therapeutic purposes. Previous studies have also confirmed the nutraceutical and physiological properties of fenugreek, resulting in its probable applications increasingly in functional food products and pharmaceutical products. Our study reported that co-administration of fenugreek saponin and nanofenugreek saponin in combination with nicotine showed a significant increase of GL-1 and GLAST in the rat liver compared with that of the group treated with nicotine alone. Also, it has been reported that fenugreek seeds have been exhibited to lower blood glucose levels and partially repair the actions of key enzymes of carbohydrate and lipid metabolism similar to normal values in numerous animal model systems. Additionally, supplementation of fenugreek seed residue in the food leads to a reduction in biomarkers of oxidative damage in alloxan diabetic rats. Seeds and leaves are the most commonly used parts of the plant containing saponin, which exhibited many pharmacological properties such as antidiabetic, antinociceptive, antioxidant, anticarcinogenic, anti-inflammatory, and hypo-cholesterolemic effects. Furthermore, the administration of the fenugreek seed extract has also shown promising results in ameliorating cerebral shortages associated with diabetes.

Recently, nanoencapsulated therapeutic agents have been used to selectively target disordered organs much better than traditional drugs. This fact may explain the results of this study which demonstrate a significant increase in the expression of GL-1 and GLAST genes in the groups treated with nanofenugreek saponin in combination with nicotine and became considerably close to normal levels. This finding is in line with previous studies that nanomedicine generates a huge influence in the healthcare field in treating many types of chronic diseases. Hence, the environmental synthesis of nanoparticles is believed as building blocks of the communicative generations to control various diseases. As shown in the present study, nicotine administration resulted in degeneration of the hepatocytes and expansion of portal tracts associated with chronic infiltrating inflammatory cells. The previous alterations were dose dependent. This resulting hepatic toxicity following nicotine treatment was previously reported, which provided evidence that smoking cigarettes causes oxidative stress and apoptosis in the liver tissue. Fenugreek seeds have high antioxidant activity in the way of inhibiting hydrogen peroxide and scavenging lipid peroxidation. The aqueous isolation of fenugreek contains gallic acid, which is known to acquire antioxidant activity. Thus, fenugreek seeds were identified as the main source of steroidal saponins (protodioscin, diosgenin, yamogenin). In the present study, fenugreek saponin ameliorated the histopathological changes in the liver section when administrated in combination with nicotine and when compared with rats treated with nicotine alone. Bin-Hafeez et al. reported that the immunostimulatory property of fenugreek is caused by the saponins, fibers, and flavonoids it contains.

The distinct behavior of nanoparticles from larger particles is correlated to their smaller size and large surface area. In a while, nanotechnology plays a vital role in the management of numerous chronic diseases such as cancer, diabetes, and tuberculosis. The current results exhibit that nicotine treatment decreased the activities of GPx and GST in liver tissues of male rats compared to those in control rats. The antioxidative enzyme activities GPx and catalase (CAT) have significant associations with changes in the period of tobacco use. Tobacco consumption causes the formation and stabilization of free radicals as a result of increased heat (generated during smoking) and pH (change during chewing) during smoking. Antioxidants play a shielding role by scavenging free radicals, and GPx and GST form the first-line defense antioxidants, as explained in previous reports. It has been reported that cigarette smoke enhanced peroxidation in the brain. Exposure to nicotine resulted in a depletion of glutathione content often causing oxidative tissue injuries in mouse and a diminution in the viability of some oxygen free radical scavengers, such as superoxide dismutase and catalase.

3. MATERIALS AND METHODS

3.1. Experimental Animals. Adult male rats, *Rattus norvegicus*, weighing 180 ± 15 g were used as an experimental animal model. These rats were purchased from the NRC (National Research Center), Dokki, The Arab Republic of Egypt (ARE). The rats were acclimatized under laboratory conditions for 14 days prior to the experiments at a temperature 25−30 °C and relative humidity 40−45% in a 12 h light/dark cycle. Rats were allowed free access to a commercial pellet diet and water. The food debris and feces were removed from cages and were cleaned daily to keep sawdust dry throughout the course of experiments. All animals received humane care in line with the guidelines of the Animal Care and Use Committee of the National Research Center, Egypt, with registration number 16456.

3.2. Chemicals and Reagents. The chemically independent variable (IV) used to induce toxicity was the nicotine drug. The fenugreek saponin and nanofenugreek saponin IV drugs play an ameliorative role against the nicotine toxicity in the present experimental design. Both drugs were purchased from the Faculty of Pharmacy, Cairo University, ARE.

3.3. Ultrasonication of Saponin Nanopowder. In order to prepare the dry saponin nanopowder (with a primary particle size ≤50 nm) for the process of characterization and administration to the experimental rats, saponin was ultra-

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sonicated and suspended in deionized water by the aid of the Biologics Ultrasonic Homogenizer, Model 150VT, with vibration at 20 kHz. The ultrasonicated nanoparticle products were dispersed and appeared as a homogeneous suspension of nanoparticles suitable for characterization and administration.

3.3.1. Characterization of Saponin Nanopowder. The physical morphology of both saponin nanopowders was examined by transmission electron microscopy (TEM) using a Tecnai G20 (FEI, The Netherlands). Briefly, a drop of nanoparticle suspension was placed on a parafilm, and a carbon-coated grid (Agar Scientific, UK) was allowed to float on the nanoparticle drop and kept for 5 min and then fixed in 2% phosphor-tungstic acid (PTA) (Sigma) for another 5 min. The grid was removed to blot excess liquid and dropped over a Whatman paper (GE Healthcare, UK) in a Petri dish. The grid was further dried in a desiccator. Two different modes of imaging were employed. The first was the bright field at an electron accelerating voltage of 200 kV using a lanthanum hexaboride (LaB6) electron source gun. The second was diffraction pattern imaging. An Eagle CCD camera with a 4k image resolution was used to acquire and collect transmitted electron images.

3.4. Experimental Design. Seventy rats (N = 70) were randomly divided into 14 experimental groups with an equal sample size (n = 5). The 14 groups were designed as shown in Table 1. Group I: Rats were daily given distilled water throughout 60 days. Group II: Rats were daily injected with 1.5 mg of nicotine (1.5 mg/kg b.wt.) throughout 60 days (this dose is equivalent to a human who smokes about 15−30 cigarettes per day, Zimmerman and Mcgeachie, 1985). Groups III, IV, and V: Rats were daily given orally 25, 50, and 100 mg of fenugreek saponin/kg b.wt. throughout 60 days, respectively (the doses were selected according to Hamden et al., 2010). Groups VI, VII, and VIII: Rats were daily given orally 20, 40, and 80 mg of nanofenugreek saponin/kg b.wt. throughout 60 days, respectively (the doses were selected as a result of our preliminary nanofenugreek saponin toxicity test that affirmed the absence of toxicity and/or lethality throughout the applied experimental periods). Groups IX, X, and XI: Rats were daily given orally 1.5 mg of nicotine/kg b.wt. in combination with 25, 50, and 100 mg of fenugreek saponin/kg b.wt. throughout 60 days, respectively. Groups XII, XIII, and IV: Rats were daily given orally 1.5 mg of nicotine/kg b.wt. in combination with 20, 40, and 80 mg of nanosaponin/kg b.wt. daily throughout 60 days, respectively.

3.5. Sampling. At the end of the experimental time (60 days), all animals were euthanized by cervical dislocation, dissected, and the desired tissues (liver) were collected for further analyses.

3.6. Comet Assay. The alkaline comet assay (pH > 13) of the hepatic tissue was performed. A small section of the liver was minced and placed in 1 mL of cold Hanks' Balanced Salt Solution (HBSS) containing 20 mM ethylenediaminetetraacetic acid (EDTA) and 10% of dimethyl sulfoxide (DMSO) to obtain an aliquot of hepatic cell suspension. 10 μL aliquot of hepatic suspension was mixed with 80 μL of 0.5% low melting point agarose (Sigma) and distributed on a fully frosted slide pre-dipped in normal melting agarose (1%). The slides were then allowed to harden on a cold surface. Subsequently, the slides were placed in cold lysis buffer for 24 h at 4 °C in the dark. Consequently, the slides were incubated in fresh alkaline buffer for 20 min. The unwinding DNA was electrophoresed for 20 min at 300 mA and 25 V (0.90 V/cm). After electrophoresis, slides were immersed in excessive amounts of 0.4 M Trizma base (pH 7.5) to neutralize the alkali medium. Afterward, the neutralized slides of the hepatic cells were fixed in cold absolute ethanol, dried by air, and finally stored at room temperature in desiccators to preserve them until the time of scoring. Prior to scoring, the slides were stained with ethidium bromide (2 μg/mL). The level of DNA migration in each sample was determined by the simultaneous image capturing and scoring of 100 cells at 400× magnification using Komet 5 image analysis software developed by Kinetic Imaging, Ltd. (Liverpool, UK). The degree of DNA damage for all samples in this study was estimated according to the following endpoint determinations. *Tail Length*: It is the horizontal distance of DNA migration from the body of the nuclear core. It was used to evaluate the extent of DNA damage away from the nucleus and expressed in μm. *Percentage of DNA in Tail:* It is the intensity of all tail pixels divided by the total intensity of all pixels in the Comet and expressed as a percentage. *Tail Moment*: It is the product of the tail length and the fraction of total DNA in the tail and incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed/broken pieces (represented by the intensity of DNA in the tail). Tail moment = Tail length × % of DNA in Tail/100

3.7. Expression Levels of GLAST and GL-1 Gene Using qRT-PCR. *3.7.1. Isolation of RNA.* Total RNA was isolated from the liver tissues of male rats using the method based on extraction by a standard Reagent (Invitrogen, Germany). The total RNA was handled with 1 U of RQ1 RNase-free DNase (Invitrogen, Germany) to break down DNA residues, which were resuspended in DEPC-used water. Purity of the total RNA was measured by the 260/280 nm ratio (between 1.8 and 2.1). Furthermore, integrity was ensured with the ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis. Aliquots were used directly for reverse transcription (RT), otherwise stored at −80 °C.

3.7.2. Reverse Transcription Reaction. The complete Poly(A)$^+$ RNA isolated from liver tissues of male rats was reverse-transcribed into commentary DNA (cDNA) in a total volume of 20 μL using the Revert Aid First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). The retort tubes containing cDNA preparations were used for DNA amplification through the real-time polymerase chain reaction (RT-PCR).

3.7.3. Real-Time Polymerase Chain Reaction. Real-time PCR scheme from Applied Biosystems (Thermo Fisher Scientific, Waltham, MA) was used to establish the rat's cDNA copy number. PCR was organized in 25 mL reaction mixture containing 12.5 mL of 1 x SYBR Premix (TaKaRa, Biotech. Co. Ltd.), 0.5 mL of 0.2 M sense primer, 0.5 mL of 0.2 M antisense primer, 6.5 mL of distilled water, and 5 mL of cDNA template. The sequences of the specific primer of the genes consumed are listed in Table 2. At the end of each qPCR, a melting curve analysis was performed at 95.0 °C to check the quality of the used primers. The relative quantification of the target to the reference was determined by using the two methods.

3.8. Histopathological Determination. The liver was preserved in 10% formaldehyde fixative for 48 h, washed, dehydrated in ascending grades of alcohol, cleared in xylene, and embedded in paraffin wax. Then, 5 μm thick paraffin sections were prepared, mounted on clean slides, and stained

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with Ehrlich's hematoxylin eosin (Bancroft & Gamble, 2002). Stained slides were examined under an Olympus microscope (BX41, Hamburg, Germany) and the images were captured using a Nikon camera for histopathological and histomorphometrical investigation.

3.9. Determination of GPx and GST Activities. Glutathione peroxidase and glutathione-S-transferase activities were determined in liver tissues. The resulting mixture contained 8 mM H_2O_2, 40 mM guaiacol, 50 mM sodium acetate buffer, pH 5.5, and an appropriate quantity of the enzyme preparation. The change in absorbance at 470 nm due to guaiacol oxidation was followed at 30 s intervals. One unit of GPx and GST activity was well defined as the amount of enzyme that increases the optical density (O.D.) 1.0/min under standard assay conditions.

3.10. Statistical Analysis. Data were evaluated using Statistical Package of the Social Sciences (SPSS) software version 22. According to the Kolmogorov–Smirnov and Shapiro–Wilk tests, data were normally distributed within groups. Accordingly, parametric analysis was applied for the statistical analysis of data. One-way analysis of variance (ANOVA) was utilized to study the result of the treatment on the studied factors. Duncan's test was utilized to study the similarity among the studied groups. Data were presented as mean ± standard error of mean.

4. CONCLUSIONS

In conclusion, the results suggested that DNA damage, alteration in the gene expression profile, and antioxidant enzyme suppression, as well as histological injuries induced by nicotine, were decreased by administration of fenugreek saponin and nanofenugreek saponin. These protective actions caused by fenugreek saponin and nanofenugreek saponin could be attributed to the presence of polyphenols and saponin preventing oxidative stress induced by nicotine.

■ ASSOCIATED CONTENT

Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article and its Supporting Information

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c05526.

Experimental design; primers sequence used for *RT-qPCR* (PDF)

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Notes

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