Original Article

Chemoprotection of MNNG-initiated Gastric Cancer in Rats Using Iranian Propolis

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Abstract

Background: Iranian propolis is a natural product of honeybees that has significant and varied anti-cancer benefits. The present study was designed to investigate the protective effects of Iranian propolis on gastric tissue carcinogenesis in an animal model.

Method: Propolis samples were collected from Hamadan and Taleghan districts of Iran, followed by ultra performance liquid chromatography mass spectrometry analysis. Fifty-five rats were divided into three groups; control, Taleghan propolis and Hamadan propolis. All the animals received N-methyl-N-nitro-N-nitrosoguanidine (MNNG, 100 µg/ml) in drinking water *ad libitum* for 34 weeks. In the treated groups, nutrition with propolis was started two weeks before MNNG administration. At the end of the study, the entire gastrointestinal tract was scrutinized for tumors, and the rest of the body was assessed for metastatic deposits.

Results: Results indicated that the incidence and number of tumors were significantly decreased by propolis in comparison with the control group ($P < 0.05$). The nuclear/cytoplasmic ratio, epithelial stratification, nuclear dispolarity, structural abnormality, and Beta-catenin and Bcl-2 proteins expression were significantly reduced in the propolis group compared to the control group ($P < 0.05$). In addition, Bax protein expression was significantly increased in the propolis group in comparison with the control group ($P < 0.05$).

Conclusion: The present study demonstrated the potential chemoprotective effects of the Iranian propolis against gastric cancer in a typical animal model. The results provide evidence for the hypothesis that Iranian propolis may exert a chemoprotective effect on MNNG-initiated gastric cancer through inhibition of cell proliferation and apoptosis induction.

Keywords: Apoptosis, gastric cancer, Iranian propolis, rat

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Introduction

Gastric cancer is the second cause of cancer related death worldwide, and its incidence and mortality is still rising in Asian countries. The high incidence of gastric cancer is a major public health problem in Iran and other countries.[1] Yet, there is no effective measure to prevent gastric cancer development. Hence, new chemoprotective and chemotherapeutic approaches are required to reduce complications and mortality.

Propolis is a natural product derived from various plant resins collected by honeybees. It contains a wide variety of chemical compounds with potent biological effects, including anti-cancer activity, and has been used as a folk medicine for centuries in Iran, China, Japan and South America.[2] Propolis differs in composition due to differences in local vegetations.[3] Identification of the effective components and their mechanism of action are important to assessment of their potential for clinical use and possible side effects. Polyphenol compounds and their derivatives have been identified in propolis. Among these, caffeic acid phenethyl ester (CAPE) is a structural relative of flavonoids, and one of the important components of propolis which is largely used in folk medicine.[4] CAPE and similar flavonoids have several biological and pharmacological properties including immunomodulatory and anti-inflammatory effects.[5] Xiang, et al. (2007) reported that CAPE is an excellent inhibitor of beta-catenin and T-cell factor in colon cancer.[6] Although the above-described findings are noteworthy, there are only limited studies focusing on possible chemoprotective properties of propolis in gastric cancer.[7] Thus, the present study was designed to investigate the protective effects of Iranian propolis on gastric carcinogenesis in a typical animal model.

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Materials and methods

Materials

N-methyl-N-nitro-N-nitrosoguanidine (MNNG, Corporation, Germany), and monoclonal mouse antiRat/Rabbit Beta-catenin, polyclonal mouse antiRat/Rabbit Bax and polyclonal mouse antiRat/Rabbit Bcl-2 antibodies were purchased from Dako Co. (DAKO Corporation, USA). Propolis samples were collected from two selected areas of Taleghan and Hamadan (in Iran) by

field staff of the Honey-bee Department, Animal Sciences Research Institute Karaj, Iran. Extraction was carried out in ethanol. The extracted fluid was filtered and evaporated under reduced pressure to remove ethanol. The remaining crude extracts were dissolved in dimethyl-sulfoxide (Sigma Chemical Co., St. Louis, MO) with a final concentration of 100 mg/ml and stored at –20 °C until the treatment day.

Propolis origin

Propolis samples were collected in the fall of 2010 from the distribution areas of *Poplar* and *Ferula ovina* plants in Hamadan (Moradbeig) and Taleghan (Dehdar) districts of Iran, respectively.

Preparation of ethanol extracts of propolis

The propolis samples were cut into small pieces and dissolved in ethanol with a ratio of 3:10 (w/v) (30 g of propolis in 100 ml of 96% ethanol). After 7 days of shaking at room temperature, the ethanolic extract of propolis (EEP) was filtered and kept in a dark place for the preparation of 20% EEP (w/w).[8,9] Then, EEP was evaporated on a rotary evaporator to remove ethanol. The remaining crude extracts were dissolved in dimethyl-sulfoxide (Sigma Chemical Co., St. Louis, MO) with a final propolis concentration of approximately 500 mg/ml and stored at –20°C until the treatment day.

UPLC-MS analysis

The Ultra Performance Liquid Chromatography - Mass Spectrometry (UPLC-MS) system was used for bio-compounds analysis in the current study. Briefly, the samples were extracted with ethanol and filtered, and then ethanol was removed by evaporation under a stream of nitrogen gas at 40 ˚C. The dry extracts were then diluted to a concentration of 10 mg/ml in ethanol. A second dilution in water was made to a final concentration of 5 mg/ml. Therefore, the concentrations above represent the amount in 5 mg of dry resin. The quantification was based on a calibration curve using quercetin standard. Two μL of each solution was injected in Acuity Waters Micro Mass UPLC-MS system.[10] The compounds were identified by comparing with the outcomes of the study by Gardana, et al. (2007).[11]

Animals

All the animal studies have been conducted according to relevant national and international guidelines of the Weatherall report, and Institutional Animal Care and Use Committee (IACUC) of Tehran University of Medical Sciences. Administration of MNNG in drinking water is a well established animal model for studying the differentiated type of human stomach cancer.[12] Male Wistar rats (purchased from the Iran Pasteur Institute) were kept in a temperature-controlled environment on a 12 : 12 h light/dark cycle with free access to food and water. Animals were housed in pens exceeding the stipulated size requirements.

Study design

Fifty-five rats were used to study the protective effects of Iranian propolis on MNNG-induced tumor in gastric cancer model. According to the study protocol, animals were divided into 3 groups: **i)** control (n = 15), **ii)** Taleghan propolis (n = 20) and **iii)** Hamadan propolis (n = 20). All animals received MNNG (100 μg/ml) in drinking water *ad libitum* for 34 weeks. In the treated groups, propolis was added to the food two weeks before MNNG administration. MNNG solution was prepared three times per week in drinking water at a concentration of 100 μg/ml. It was protected from light and given *ad libitum* to animals in their drinking water. In addition to MNNG, all animals were given 10% sodium chloride weekly in the initial six weeks to enhance the development of gastric cancer.[12]

Characterization of gastric tumors

Rats were weighed on a weekly basis and observed for evidence of rectal bleeding. They were killed moribund or at the end of 36th week. Then a thorough necropsy was made, and the entire gastrointestinal tract was scrutinized for lesions. The rest of the body was also scrutinized for metastatic deposits. All animals were monitored closely for general health during the study period. At the end of the 36th week, all rats were sacrificed. Autopsy was performed on animals that died before the end of the experiment to determine the cause of death and the presence of gastric tumors. On laparotomy, the organs were examined for possible metastasis. The stomach was also opened along the greater curvature and carefully examined. All dissections were performed by investigators blinded to the different treatment groups. The number, size and location of lesions were documented.

Histological assay

The gastric lesions and adjacent mucosal tissues from the specimens were fixed in 10% buffered formaldehyde, passaged and embedded in paraffin. The paraffin blocks were then sectioned by 3 – 5 μm thickness for hematoxylin and eosin (H & E) staining. In each case, 9 serial sections were used for H & E and immunohistochemical stains.[13]

Hematoxylin and eosin examinations

Histological evaluations were performed by routine H & E staining. The grade of histological abnormality was semi-quantitatively scored using the following four parameters:[13] **a)** nuclear/cytoplasmic ratio (< 25%: 0, 25% – 50%: 1, > 50%: 2); **b)** epithelial stratification (none: 0; mild: 1, severe: 2); **c)** nuclear dispolarity (none: 0, mild: 1, severe: 2); and **d)** structural abnormality (none: 0, mild: 1, severe: 2). At least five sections were examined for grading. Two evaluators without access to the cases studied the slides independently. The agreement between their results was greater than 90%, and the discordant cases were jointly re-evaluated by the pathologists to reach a decision through consensus. The total score of each parameter was regarded as the score of histological abnormality. Almost all gastric cancers were scored as 6 – 8 points while normal samples earned 0.

Immunohistochemistry examinations

Immunohistochemistry was carried out in 5 μm tissue sections from formalin-fixed paraffin blocks using the avidin-biotin immunoperoxidase method.[14] Sections were stained by monoclonal mouse antiRat/Rabbit Beta-catenin, polyclonal mouse antiRat/Rabbit Bax and polyclonal mouse antiRat/Rabbit Bcl-2 antibodies (DAKO Corporation, USA) according to the manufacturer's instructions. Briefly, the paraffin sections were deparaffinized with xylene and rehydrated through a series of descending graded ethanol solutions. Endogenous peroxidase activity was blocked by incubation for 15 min in 0.3% H_2O_2 buffer. Biotinylated secondary antibody and avidin-biotin complex with horseradish peroxidase were applied, followed by addition of the chromogen 3,

3'-diaminobenzidine (DAB) (Sigma Chemical Co.). Finally, slides were counterstained with hematoxylin and observed under a light microscope. Slides of histological sections, previously confirmed to be positive for these markers, were used as positive controls. The same slide was used as negative control by subtracting the primary antibody from the reaction. The two evaluators read the same slides as previously described for the H & E study.

The criteria was used to evaluate the beta-catenin, Bax and Bcl-2 markers based on the estimated proportion of positive cells and average staining intensity of positive cells in cytoplasm (for Bax) and membrane, cytoplasm or nucleus (for Beta-catenin and Bcl-2). The semi-quantitative score was adopted as follows: no staining: 0; faint/barely staining in at least ⅓ of cells: 1; moderate staining in at least ½ of cells: 2; and strong staining in almost all cells: 3.

Statistical analysis

Analysis of variance and Tukey test were used for comparison among groups after normality tests. Differences in lesions' incidence (percent of animals with gastric adenocarcinoma) were analyzed by Fisher's exact test. Ratio comparison was determined by Chi-square test. Values are represented as mean ± S.E.M. Also, $P < 0.05$ was considered statistically significant.

Results

UPLC-MS data

UPLC-MS analysis of propolis samples collected from Hamadan and Taleghan are presented in Tables 1 and 2. The chemical components of propolis from two study areas differed considerably in terms of their aromatic acids and phenolic groups, as well as geographical origins. The amount of aromatic acids and phenolic compounds in Hamadan propolis were 2270 and 1238 µg/ml, while in Taleghan propolis, these figures were 489 and 1568 µg/ml, respectively (Tables 1 and 2). The amount of aromatic acids in the two study areas differed significantly (2270 in Hamadan versus 485 in Taleghan) while the phenolic group compound was almost the same in the two areas (1238 and 1568 µg/ml, respectively) (Tables 1 and 2). However, some of the compounds could not be identified and were not quantified in this analysis.

General observations

The animals' weight in the control and propolis-treated groups increased with no significant difference. Body weight increased steadily to reach a plateau after about 18 weeks in all rats, but showed a slight fall in the MNNG group as tumors developed. There were no significant differences in food intake (g/day) in the control and treatment groups, except for a slight fall in the control group. No behavioral changes were observed in the rats during the course of administration, or in the ensuing follow-up period.

Tumor type and metastases

The lesions varied from mild to severe dysplasia and gastric adenocarcinoma (Figure 1). The lesions were more common in the stomach than the intestines or colon. Metastatic lesions were most frequently seen in para-cecal and para-aortic lymph nodes.

Tumor incidence

The incidence of lesions in rats was higher in the stomach compared to the colon. Rats in the control group had higher lesion induction in the stomach compared to the propolis groups. Among the treatment groups, reductions in the incidence of lesions in propolis fed rats ranged from 35% to 40% compared to controls.

Tumor number

In vivo application of Iranian propolis inhibited gastric cancer growth in animal model. The control rats had the highest lesion numbers in both gastric and colon specimens. Results showed that the incidence of gastric lesions was 70% in the control group. Administration of Iranian propolis significantly inhibited the incidence of gastric lesions by about 38% compared with the control group ($P < 0.05$).

Tumor size

Compared to the control rats, rats treated with Iranian propolis

Table 1. Chemical compositions of Taleghan propolis ethanol extract analyzed by UPLC-MS

Compound name	µg/ml	Percentage
Aromatic acids (489 µg/ml)		
Caffeic acid	33	0.66
Caffeic acid isoprenyl esther (isomer1)	409	8.18
Caffeic acid isoprenyl esther (isomer 2)	44	0.88
Ferrulic acid	< 1	0.02
Isoferrulic acid	< 1	0.02
P-coumaric acid	< 1	0.02
Phenolic compounds (1568 µg/ml)		
Quercetin	< 1	0.02
Quercetin -3 methyl ether	< 1	0.02
Quercetin -7 methyl ether	1	0.02
Kaempferol	1	0.02
Pinobanksin	826	16.52
Pinobanksin 5,7- dimethyl ether	1	0.02
Pinobanksin 3 methyl ether	< 1	0.02
Pinobanksin -3-O- acetate	716	14.32
Pinobanksin-3-O- proprionate	Not Qualified	–
Pinobanksin--3-O- butyrate	< 1	0.02
Pinobanksin-3-O- pentanoate	15	0.3
Luteolin -5-methyl ether	< 1	0.02
Other compounds	2947	58

Table 2. Chemical compositions of Hamadan propolis ethanol extract analyzed by UPLC-MS

Compound name	µg/ml	Percentage
Aromatic acids (2270 µg/ml)		
Caffeic acid	62	1.24
Caffeic acid isoprenyl esther (isomer 1)	580	11.6
Caffeic acid isoprenyl esther (isomer 2)	1626	32.52
Ferrulic acid	< 1	0.02
Isoferrulic acid	< 1	0.02
P-coumaric acid	305	6.1
Phenolic compounds (1238 µg/ml)		
Quercetin	< 1	0.02
Quercetin -3 methyl ether	1	0.02
Quercetin -7 methyl ether	1	0.02
Kaempferol	2	0.04
Pinobanksin	305	6.1
Pinobanksin 5,7- dimethyl ether	1	0.02
Pinobanksin 3 methyl ether	1	0.02
Pinobanksin -3-O- acetate	889	17.78
Pinobanksin-3-O- proprionate	1	0.02
Pinobanksin--3-O- butyrate	< 1	0.02
Pinobanksin-3-O- pentanoate	33	0.66
Luteolin -5-methyl ether	2	0.04
Other compounds	1491	29.82

Table 3. Effects of Iranian propolis on histological abnormality grading in MNNG-initiated gastric cancer

Groups	Parameters					
	N	Number of lesions	Nuclear/ cytoplasmic ratio	Epithelial stratification	Nuclear dispolarity	Structural abnormality
Control	15	2.14 ± 0.42	1.5 ± 0.17	0.80 ± 0.20	0.8 ± 0.15	1.87 ± 0.20
Hamadan Propolis	20	*0.15 ± 0.08	*0.25 ± 0.1	*0.15 ± 0.08	*0.1 ± 0.29	*0.15 ± 0.08
Taleghan Propolis	20	*0.25 ± 0.1	*0.35 ± 0.1	*0.20 ± 0.09	*0.2 ± 0.19	*0.20 ± 0.09

The grade of histological abnormality was semi-quantitatively scored using the following four parameters: **(a)** nuclear/cytoplasmic ratio (<25% :0, 25–50% :1, >50% :2); **(b)** epithelial stratification (none: 0, mild: 1, severe: 2); **(c)** nuclear dispolarity (none: 0, mild: 1, severe: 2) and **(d)** structural abnormality (none: 0, mild: 1, severe: 2). The number of lesions per animal is shown. Values represented mean ± S.E.M. *P < 0.05 compared to control group.

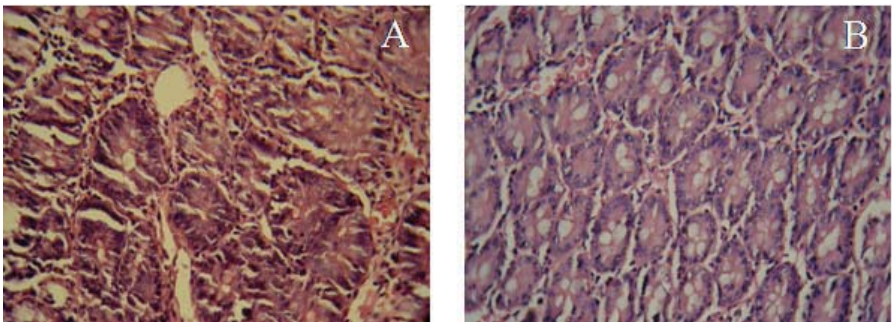

Figure 1. The histological features of lesions in gastric tissue. The histological abnormality scores assessed by semi-quantitative analyses were: 6 points in A (Control group): (nuclear/cytoplasmic ratio =1; epithelial stratification = 1; nuclear dispolarity = 2 and structural abnormality = 2); H & E (×400). Two points in B (propolis group): (nuclear/cytoplasmic ratio =0; epithelial stratification = 0; nuclear dispolarity =1 and structural abnormality =1); H & E (×400).

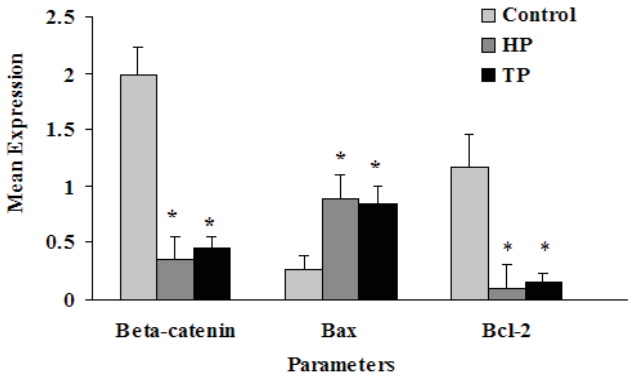

Figure 2. Effects of Iranian propolis on immunohistochemical markers in MNNG-initiated gastric cancer. The semi-quantitatively scoring uses the following guidelines (no staining: 0, faint/barely staining in at least 1/3 of cells: 1, moderate staining in at least ½ of cells: 2 and strong staining in almost all cells: 3). Values are represented as mean ± S.E.M. *P < 0.5 compared to their respective control group. HP = Hamadan Propolis, TP = Taleghan propolis.

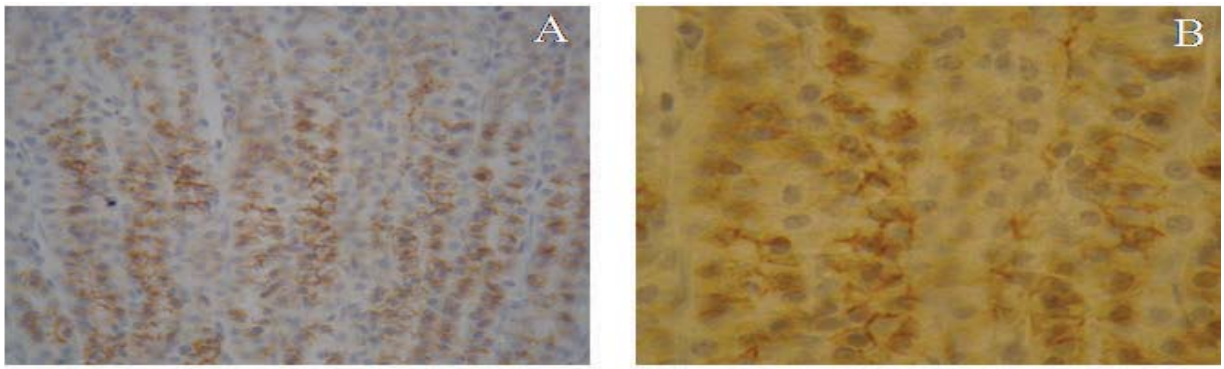

Figure 3. The immunohistochemical features of Bcl-2 expression in representative gastric tissue **A)** Control group, **B)** Propolis group (×400).

had smaller lesions (mm) in the gastric specimens. Reduction in lesions size (mm) in rats fed with propolis was 33% compared with the control group ($P < 0.05$).

Tumor histology

On hematoxylin and eosin examination, the mean number of lesions, nuclear/cytoplasmic ratio, epithelial stratification, nuclear dispolarity and structural abnormality were declined in the propolis groups compared to the control group ($P < 0.05$) (Table 3, Figure 1).

On immunohistochemistry examination, MNNG reduced the expression of Bax protein, whereas it increased the expression of Beta-catenin in the gastric tissue in the control group ($P < 0.05$) (Figures 2 and 3). Proapoptotic Bax protein expression was increased by Iranian propolis in comparison with their respective control group ($P < 0.05$) (Figures 2 and 3). Beta-catenin and antiapoptotic Bcl-2 protein expression in gastric mucosa expectedly found to be overexpressed after MNNG therapy (Figure 2). Furthermore, Beta-catenin and Bcl-2 protein expression and Bcl-2/Bax ratio were dramatically decreased in the propolis groups in comparison with their respective control group ($P < 0.05$) (Figure 2).

Discussion

The major goal of this study was to develop novel strategies for preventing gastric cancer by means of potential chemoprotective agents of natural products, such as propolis, in an animal model. The outcomes of the present study showed that tumor incidence, the number of lesions, structural abnormalities and Beta-catenin of propolis groups were significantly declined in comparison with their control group. Propolis also induced the expression of pro-apoptotic Bax and reduced anti-apoptotic Bcl-2 expression. The present study provides evidence that propolis exerts a significant chemoprotective effect on MNNG-initiated gastric cancer through inhibition of cell proliferation and apoptosis induction.

In this study, the effects of different geographical and botanical origins of propolis extracts were investigated on the growth, cytotoxicity and apoptosis, implying that Iranian propolis may be an anti-tumor agent. Many investigations have shown that diverse types of propolis extracts appreciably inhibit cell growth and reduce the differentiation or proliferation of tumor cells.[15,16] Polyphenols are strong antioxidants, so the UPLC-MS analysis was used to determine the chemical composition of propolis.[17] In the present study, the UPLC-MS analysis of the EEP revealed the presence of common metabolites for the Iranian type of propolis: aromatic acids (489 μg/ml) and phenolic compounds (1568 μg/ml) in Taleghan area, and aromatic acids (2270 μg/ml) and phenolic compounds (1238 μg/ml) in Hamadan area. Furthermore, the UPLC-MS analysis also demonstrated the high amounts of flavonoids, including caffeic acid and pinobanksins in the Iranian propolis. The percentage of CAPE in propolis samples that collected from our two study areas were quantitatively sufficient to guarantee for their applications. Interestingly, although the difference in aromatic acids compounds of our two study areas is significant (489 versus 2270), the high presence of CAPE is promising due to its anticancer effects. It is worth mentioning that CAPE, even at low doses, prevents cellular mistakes in healthy cells and induces apoptosis.[18] CAPE possesses potent inhibitory effects on cell proliferation, and shows powerful antioxidant activity. It has been shown to be a safe pharmacologic compound with known anti-inflammatory, immunomodulatory, anti-carcinogenic, and antioxidant properties.[19] It was concluded that CAPE has a cytotoxic effect, and can also block the invasive metastasis noted in these tumors.[20] In addition, CAPE inhibits the formation of aberrant crypts induced by azoxymethane *in vivo*, which are related to colon carcinogenesis.[21]

Previous studies showed that the Beta-catenin is a key intracellular messenger in gastrointestinal tract malignancies such as stomach and colon cancers.[22,23] They suggested that activation of the Beta-catenin transcription could play a role not only in initiation of gastric carcinogenesis but also in its promotion in mice.[22] In our study, propolis was more active in suppressing Beta-catenin activation and other abnormalities. Propolis induced the expression of pro-apoptotic protein Bax and reduced anti-apoptotic Bcl-2 expression and Bcl-2/Bax ratio compared to the control group. It also inhibited the growth of a wide variety of tumor cells in the rat gastric cancer model. One of the mechanisms of the anti-tumor activity of propolis has been shown to be through the induction of apoptosis.[23,24] In recent studies, attempts have been made to induce apoptosis directly by triggering core components of the cell death machinery such as Bax and Bcl-2 family proteins. Apoptosis inducers are currently being used in cancer therapy.[21] Our data demonstrated that treatment with Iranian propolis was associated with a strong inhibition of growth and apoptosis in gastric tissue.

Propolis provides an opportunity to expand the clinical repertoire of this efficacious agent. Future studies utilizing propolis are warranted in pre-clinical and *in vivo* models of cancer to show its potential benefit and elucidate the signaling pathways and possible roles of these natural products. This can be a reliable method in taking advantage of natural and inexpensive products like propolis in the prevention and treatment of cancers. The efficacy

of propolis *in vitro* and *in vivo* protocols suggests its therapeutic properties, but before establishing a strategy to use this bee product, it is necessary to study the chemical nature of the propolis samples. This will allow us to introduce further applications of propolis with respect to its safe use.

In summary, the present study demonstrated the probable potential chemoprotective effects of the Iranian propolis against gastric cancer in a typical animal model. The results provide evidence for the hypothesis that propolis may exert a chemoprotective effect on MNNG-initiated gastric cancer through inhibition of cell proliferation and apoptosis induction. We suggest that further study should be done about Iranian propolis anti-cancer effects.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content of the paper.

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