Original Article

Study of the Apoptotic Impacts of Hazelnut Oil on the Colorectal Cancer Cell Line

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Abstract

Background: Hazelnut oil has a unique structure with a high oleic acid content, tocopherol, tocotrienols, and other bioactive compounds, such as phytosterols. These biochemical compounds have been widely studied because of their potential health properties. Understanding the process of apoptosis is the basis of new therapies contributing to cancer cells' death. Recently, the potential role of the evolutionary-reserved *bcl-2* protein family in tumor progression and prognosis of some malignancies has been addressed in several studies. The present study is aimed at evaluating the effect of apoptotic properties of hazelnut oil on colorectal cancer cells through the major members of this family (*bax* and *bcl-2*).

Materials and Methods: MTT assay, apoptotic cell staining (using Annexin V and propidium iodide), flow cytometry, and real-time PCR were used to evaluate the toxicity, percentage of apoptotic cells, and *bax* and *bcl-2* genes' expression after exposing HT29 cells to hazelnut oil.

Results: After hazelnut treatment, significant decreases in cell viability, and the gene expression of *bax* and *bcl-2* were observed compared to the control group ($P < 0.05$). In addition, the total percentage of apoptotic cells after hazelnut oil treatment showed a significant increase in comparison with the negative control group ($P < 0.05$).

Conclusion: Hazelnut oil appears to cause the death of cancerous cells through an apoptotic mechanism.

Keywords: Apoptosis, colorectal neoplasms, Corylus, oil, signal transduction

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Introduction

Understanding the process of apoptosis is the basis of new therapies that involve the deaths of cancer cells or their susceptibility to lethal agents and radiation therapy.[1] As a programmed mechanism of cell death, apoptosis may occur through either intrinsic or extrinsic pathways. The intrinsic pathway is usually mediated by the mitochondria and involves BL- family. This family contains several proteins that are categorized into two main groups. The first one contains proapoptotic proteins (e.g., BAX and BAK) while the second one includes antiapoptotic proteins (e.g., BCL-2 and BCL-XL). Apoptosis occurs as a result of an imbalance between these groups, and exceeding proapoptotic proteins expression over that of antiapoptotic proteins leads to an increased apoptotic index.

The hazel (*Corylus avellana* L.) is a tree that produces edible nuts. Each year, about 700,000 tons of hazelnuts are produced worldwide. About 70% of the world's total production comes from Turkey, the rest being produced in Italy, France, the United States, Spain, and Iran. Iran is the fifth-largest producer of hazelnuts.[2]

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The hazelnut kernel is about 60% oil. The essential fatty acid in hazelnuts includes oleic acid, palmitic acid, linolenic acid, and linoleic acid. Studies have shown that hazelnut oil contains a large amount of monounsaturated fatty acid (MUFA) and some polyunsaturated fatty acid (PUFA), vitamin E, and triacylglycerol compounds, tocopherol, tocotrienol, and other bioactive compounds, such as plant sterols (phytosterols).[3]

Alpha, beta, gamma, and delta tocopherols, which are types of vitamin E, are found in hazelnut oil. These compounds are antioxidants with a high shelf-life and maintain the qualitative properties of this oil. Alpha-tocopherol is the main tocopherol constituting 24.7 mg/100 g oil.[4] Hazelnut extract also inhibits the oxidation of low-density lipoprotein (LDL) in the body. In addition, its extract is capable to prevent the DNA in cancer cells from being destroyed.[5,6] Hazelnut is often reported to exert inhibitory effects on the proliferation of different human cancer cells such as Hep G2 (liver) and Caco-2 (colon) in the laboratory.[7] Although the anticancer properties of hazelnut oil have been confirmed, there is no study on the molecular mechanisms underlying these properties. Here, the aim was to evaluate the fat component profile of Iranian hazelnuts, and then, investigate its cell death effects on colorectal cancer using annexin V/PI staining. In addition, the expression of two important genes involved in the intrinsic apoptosis pathway, *bax* and *bcl-2*, was analyzed by evaluating their mRNA levels in the cell.

Materials and Methods

Oil extraction

The hazelnut kernels were taken out of shells and cleaned manually. Then, they were dehydrated for 3 days in a drying oven at 105°C. After drying, 20 g of kernels were ground and mixed with 50 mL petroleum using a homogenizer (Heidolph Silent Crusher M) and transferred to a thimble which was then capped with cotton. Next, oil extraction was performed using petroleum ether (40–60°C) in a Soxhlet apparatus (Gerhardt, model 173200, EV, Germany) at boiling temperature for 4 h. A rotary flash evaporator removed the solvents under reduced pressure (Heidolph, model Laborota 4000, Germany).[8] The "oil content" and "oil yield" were measured using the following equations:

Oil content (%) = 100 × (Weight of oil extracted (g)/Weight of the seed sample) (Eq. 1)

Oil yield (kg.da^{-1}) = Seed yield (kg.da^{-1}) × Oil content (%) (Eq. 2)

Evaluating the oil physicochemical properties

The AOAC method (921.08) was used for determining the oils' refractive index by triplicate assessments at 25°C using a refractometer (32-G 110e, Carl Zeiss Abbe, Germany). However, the peroxide value of all samples was evaluated using the AOAC method (965.33) and reported in milliequivalents of peroxide number per kilogram of oil. Anisidine (AnV) and totox (TxV) values of the samples were assessed according to Hosseini *et al*., 2020.[8-10]

Additionally, the free fatty acids content of samples (acid value) was evaluated according to the AOAC method (940.28) as the indicator of the hydrolysis reaction progress in the oil.[11] The fatty acid methyl esters were directly calculated according to Eq. 3 to quantitatively determine the iodine value of the extracted oils.

De ftn=(oo ode (March ftin)+4;%o eree (March fpnan)+6;%o ean) (Eq. 3)

Considering the obtained composition of fatty acids, the fatty acid methyl ester derivatives were prepared by the AOCS method (Ce 1–62) and evaluated using Gas Chromatography/Mass Spectrometry (GC/MS). Also, the oxidative stability of extracted hazelnut oils was determined using a Rancimat apparatus (model 679, Metrohm Herisau, Switzerland).[12]

Cell culture

The medium required for cell culture and propagation HT29 was purchased from Pasteur Institute (Iran) and was prepared as follows: 89% RPMI-1640 (GIBCO; USA) + 1% penicillin (10000 units/mL) and streptomycin (10000 units/mL) + 10% fetal bovine serum (FBS). The culture media were incubated at 5% CO_2 and 37°C and replaced every 2 days.

Cell viability test

The cell proliferation level was evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay as described in our previous study. Briefly, 96-well plates were cultured with 8000 hormone-treated cells per well in RPMI1640 medium containing 10% FBS and incubated at 5% CO_2 and 37°C for 24 h.

On the next day, the culture medium was replaced with a fresh one (100 µl) and cells were mixed with hazelnut oil prepared in lecithin as a buffer (Behshahr, Iran) at different dilutions of 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, and 1/256. Phosphate buffered saline (PBS) was used as the negative control. After 24 h, all culture media were again replaced with 100 µl of fresh medium without FBS. Then, 20 µl of MTT color (5 mg/mL) per well was added to the culture media and the plate was incubated at 37°C for a further 4 h.

After this time, the supernatant was discarded and 100 µl dimethyl sulfoxide (DMSO) (Merck; Germany) was added to wells and the optical absorption was read at 570 nm using an ELISA reader (Stat fax 2100; USA). Cells treated with PBS were tested as negative controls. All tests were performed in triplicate.[13]

Annexin/PI staining

To perform the PI coloring test, 500,000 cells/well were cultured in a 24-well plate which was then incubated in RPMI1640 medium containing 10% FBS at 37°C and 5% CO_2 for 24 h. Next, cells were treated with hazelnut oil at different dilutions of (1/2, 1/4, 1/8, and 1/16). After 24 h of treatment, the cells were isolated by trypsin from the plate and transferred to a microtube in 200 µl culture media without FBS. The float test was administered, and the results were analyzed using Flow Max software.

Gene expression evaluation

The apoptotic effect of hazelnut oil by altering the expression of *bax*, *bcl-2*, and β-*actin* genes was assessed by real-time PCR. For this purpose, HT29 colorectal cancer cell (500,000 cells per well) was cultured in a 24-well plate. After 24 h, the cells were placed in dilutions of 1/2, 1/4, 1/8, and 1/16 hazelnut oil and PBS as the negative controls at 37°C and 5% CO_2 for another 24 h. All trials were performed in triplicate.

Then, cells were collected by trypsin and the RNA was extracted using the RNX Plus kit (CinnaGen; Iran) according to the guidelines provided by the manufacturer. The degree of purity and concentration of the obtained RNA was evaluated using a NanoDrop spectrophotometer (USA), and the optical absorption of each sample at 260 nm was calculated. After determining the amount and concentration of RNA, extracted mRNA was used for cDNA synthesis by cDNA synthesis kit (Pishgam Biotech). One microgram of mRNA was converted to cDNA in 20 µl of master mix reaction containing M-MULV reverse transcriptase, water treated with DEPC, 10 pmol Oligo dT, and 10 pmol Random Hexamer primers. The thermal reaction was done using a PCR machine (Qiagen; USA) as 5 min at 94°C for initial denaturation, 1 h at 42°C for conversion of mRNA into cDNA, and 5 min at 85°C for enzyme inactivation. Then, the relative expression of *bcl-2* and *bax* genes was measured by real-time PCR using β-actin as the housekeeping gene. Briefly, 1 µl cDNA was amplified using 20 µl of a mixed reaction solution that contained 2 × Syber Green solution and 10 pmol of each primer [Table 1] using a Corbett Rotor-Gene 6000 real-time PCR cycler (Qiagen; Corbett; USA). The polymerization cycles included an initial denaturation step of 94°C for 3 min, 40 cycles of 94°C for 30 s, and the annealing step of 72°C for 45 s for each gene. Then, the genes' relative expression was calculated using the $2^{-\Delta\Delta CT}$ formula.[13]

Statistical analysis

The results were analyzed by SPSS software using the non-parametric Mann-Whitney method. The results of real-time PCR were statistically analyzed using REST2009 software (Qiagen; USA). A P value < 0.05 was considered significant.

Results

Hazelnut oil analysis

The fatty acid composition of the oil extracted from 1 kg hazelnut is presented in Table 2. According to the composition, oleic acid (C18:1) was assessed to be the predominant fatty acid present in the hazelnut kernels, followed by linolenic (C18:2) and palmitic (C16:0) acids. 91.25% of fatty acids were unsaturated [Table 2]. Acidity, peroxide value, anisidine value, and totox value [totox value = 2 (PV) + (AnV)] were 0.61 (%), 2.9 meq O_2/Kg oil, 0.82, and 6.62, respectively. Moreover, moisture, refractive index (25°C), iodine value, saponification value, and induction period are 0.18%, 1.469, and 84.10 (g I_2/100 g oil), 195.52 (mg KOH/g oil), and 21.50 h, respectively [Table 3].

Percentage of live HT29 cells for different dilutions of hazelnut oil by MTT method

Figure 1 shows the percentage of live HT29 cells in different dilutions of hazelnut oil (1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, and 1/256). In the dilutions of 1/2, 1/4, and 1/8 hazelnut oil, the percentage of live cells displayed a statistically significant reduction compared to the control group ($P < 0.05$). In dilutions of 1/16, 1/32, 1/64, 1/128, and 1/256 hazelnut oil, the percentage of live cells showed no significant difference with the negative control.

Gene expression analysis

Evaluation of mRNA expression of *bcl-2* gene compared to the β-*actin* gene as a control in the presence of 1/2, 1/4, 1/8, and 1/16 hazelnut oil showed a significant decrease compared to the negative control group ($P < 0.05$). As illustrated in Figure 2a, the expression declined in a concentration-dependent manner. Figure 2b shows that the expression of *bax* mRNA at 1/2, 1/4, 1/8, and 1/16 hazelnut oil had also a statistically significant reduction in the hazelnut oil-treated cells compared to the untreated group ($P < 0.05$). Furthermore, the apoptotic index for *bax* and *bcl-2* genes was the highest of all studied

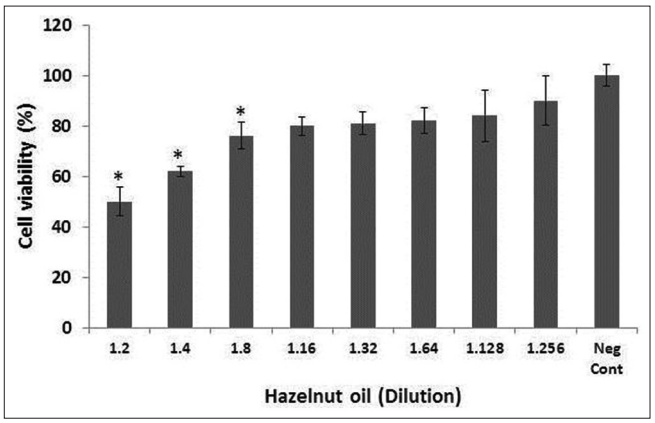

Figure 1: Percentage of live HT29 cells exposed to different dilutions of hazelnut oil. In the dilutions of 1/2, 1/4, and 1/8 hazelnut oil, the cell viability significantly decreases compared to the control group. * indicates the *P* value lower than 0.05

Table 1: Sequence of primers and the length of products derived from them

Gene	Forward (5'-3')	Reverse (5'-3')	Length (bp)
bax	TGGAGCTGCAGAGGATGATTG	GAAGTTGCCGTCAGAAAACATG	95
bcl2	CTGCACCTGACGCCCTTCACC	CACATGACCCCACCGAACTCAAAGA	119
β-actin	TCATGAAGATCCTCACCGAG	TTGCCAATGGTGATGACCTG	118

dilutions [Figure 2c]. At the 1/2 dilution, the index was greater than 3, which shows an increase compared to the negative control group.

Annexin-V/PI staining results

Evaluating the hazelnut oil-treated cells stained with Annexin-V/PI by flow cytometry showed the probability of obtaining a population of cells in four cases. The mechanically degraded necrotic cells located in the upper left quarter were only PI-positive (Q1). Cells involved in late apoptosis located in the top quarter were positive for both PI and Annexin-V (Q2). The cell population experiencing apoptosis (PI negative, Annexin-V positive) were located in the right-hand quarter (Q3). And finally, the living cell population located in the lower-left quarter were the controls being negative for Annexin-V and PI (Q4). Figure 3 shows that the total percentage of apoptotic cells in dilutions of 1/2, 1/4, and 1/8 hazelnut oil compared to the negative control group increased significantly ($P < 0.05$).

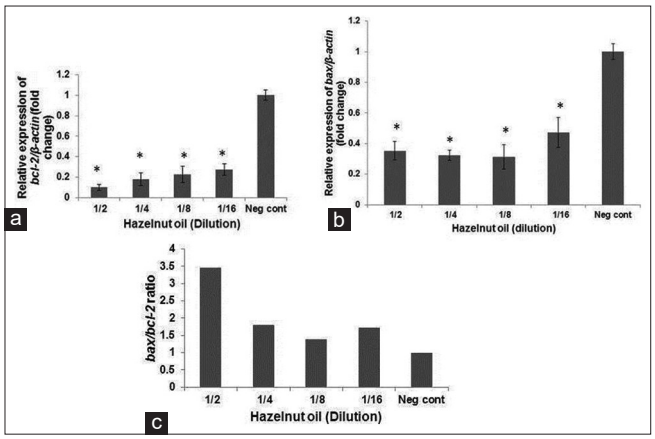

Figure 2: Relative mRNA expression of *bcl-2* gene (a) and *bax* gene (b) to the expression of the *β-actin* gene as a housekeeping gene. The ratio of mRNA expression of *bax* gene relative to the *bcl-2* gene as an apoptotic index (c). * indicates the *P* value lower than 0.05

Discussion

The most common treatment for patients with advanced colorectal cancer is surgery, followed by chemotherapy, for which patients are exposed to side effects and resistance to chemotherapy drugs. The current research and introduction of functional nutritional compounds, nutritional supplements, and nutrients anticancer drugs can be useful in improving clinical conditions in cancer. Studies have shown that hazelnut oil percentages vary from 46.7% to 76.8%. For example, Turkish hazelnut contains 65.7% oil, which is 20% more than Oregon hazelnuts. In general, it has been reported that roughly 60% of each hazelnut kernel is oil. The hazelnut oil fatty acid profile showed a very high concentration of oleic acid,

Table 2: Fatty acid composition of hazelnut oil

Fatty acids	(%)
Myristic acid (C14:0)	0.04
Palmitic acid (C16:0)	5.70
Palmitoleic acid (C16:1)	0.30
Stearic acid (C18:0)	2.70
Oleic acid (C18:1)	82.00
Linoleic acid (C18:2)	8.75
α-Linolenic acid (C18:3)	0.20
Arachidic acid (C20:0)	0.16
Saturated fatty acids (SFA)	8.56
Mono Saturated fatty acids (MUFA)	82.30
Poly Saturated fatty acids (PUFA)	8.95
unsaturated fatty acids (UFA)	91.25
Acidity (%)	0.61

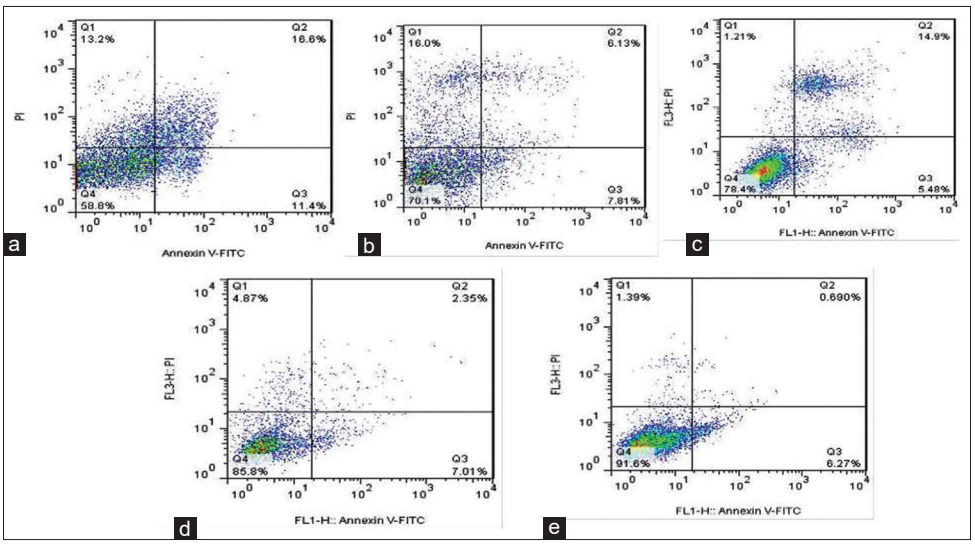

Figure 3: Results of coloring the AnnexinV/PI staining diluted at: (a) 1/2, (b) 1/4, (c) 1/8, (d) 1/16 hazelnut oil, and (e) negative control. (Q1) is related to PI-positive cells. (Q2) shows that the cells involved in late apoptosis are positive for both stains. (Q3) is related to PI negative and Annexin-V positive cells involved in early apoptosis, and (Q4) shows the population of living cells that provide negative controls for Annexin-V and PI

Table 3: Physicochemical properties of hazelnut oil

Trait	Value (unit)
Acidity	0.61 (%)
Peroxide value	2.9 (meq g O_2/Kg oil)
Anisidine value	0.82
Totox value	6.62
Moisture	0.18 (%)
Refractive index (25°C)	1.469
Iodine value	84.10 (g/g 100 oil)
Saponification value	195.52 (mg KOH/g oil)
Induction period	21.50 (h)

followed by linoleic acid, but its concentration was inversely related to the oleic acid concentration. Studies have shown that the oleic acid concentration in hazelnut oil was 70.5% to 85.3% of total fatty acid content and was significantly related to the linoleic acid in the oil. In consistence with the above-mentioned reports, the present study showed that 82% and 8.75% of the fatty acid content of hazelnut kernel were oleic acid and linoleic acid, respectively. Changes in the percentage of fatty acids were associated with the hazelnut culture area. The ratio of total fatty acid to linoleic acid in the present study was similar to previous studies, which means that the higher the fatty acid content, the lower the percentage of linoleic acid.[14]

Li *et al*. (2011)[15] showed that hazelnut extract could effectively inhibit proliferation in cancer cells probably through acting as a strong eliminator of free radicals. According to their results, Oregon hazelnut at a concentration of 6 mg/mL could considerably decrease the growth rate of HT29 human colon cancer cell line. Also, Turkish hazelnut extract at a concentration of 5 mg/mL could prevent 89% of cancer cells from proliferation and growth after 4 days of treatment at two- and four-day intervals. They identified that the presence of antitoxic and phenolic compounds are responsible for the anticancer effect of hazelnuts.

Olofinnade *et al.* (2021)[16] reported that the activity of the extract of hazelnuts and its contents on human melanoma cell lines and the Hela cell line inhibited cancer cells at an IC_{50} range of 459–584 mg/mL in a dose-dependent manner. They reported that hazelnut extract could induce apoptosis in all cell lines studied. In addition, hazelnut extract induced the cleavage PARP-1 after 24 h and 48 h in Hela cells and SK-Mel-28 cells. Apoptosis induced by hazelnut extract is dependent on Caspase 3 activation.

Caimari *et al*. (2015)[17] studied the effect of a hazelnut skin extract (FIBEROX) on the lipid and glucose metabolism in Golden Syrian hamsters as animal models. The hamsters were fed a high-fat diet (HFD) for 8 weeks. They administered FIBEROX either over the whole course of 8 weeks or only during the second half of the study course. In this way, they found that FIBEROX had reversed the HFD feeding-induced increase trend of plasma cholesterol (both total and LDL) in both groups. It also reduced the levels of free fatty acids and triglycerides circulating in the animal blood which had consumed FIBEROX for 4 weeks. Also, the increased bile acid excreted in animal feces from both groups suggested that from a mechanism-directed view, FIBEROX contributes to the cholesterol-lowering function of the extracted materials. Furthermore, FIBEROX intake sharply decreased the lithocholic/deoxycholic bile acid fecal ratio, a risk factor for colon cancer in both HFD-FBX groups. They concluded that FIBEROX consumption can improve various risk factors not only associated with colon cancer but also with cardiovascular disease.

The cell toxicity of hazelnut oil was evaluated using the MTT assay. In this method, the reducing activity of mitochondrial succinate dehydrogenase of live cells was detected. Our observation showed that hazelnut oil resulted in decreased cell viability in a dose-dependent manner.

Various types of cell death occur in the cell after cellular stress. Apoptosis and necrosis are two known cell death reported after treating with anticancer agents. In this study, two important genes involved in the intrinsic pathway of apoptosis, *bax* and *bcl-2,* were selected and the effects of hazelnut oil on their expression were assessed. *Bcl-2* is recognized as an antiapoptotic gene while the *bax* is recognized as a proapoptotic gene. Any alteration in the expression level of these genes is important for promoting apoptotic signals. Increased apoptotic index (*bax/bcl-2* ratio) more than 1 could be the indicator of apoptotic condition. Our findings showed that cell treating with hazelnut oil can increase the percentage of apoptotic cells and the apoptotic index (*bax/bcl-2* ratio).

A daily intake of 25 to 30 g of hazelnuts shows that it can completely supply daily vitamin E requirements. Recent studies have shown that the high levels of beta-sitosterol in hazelnut oil improve clinical conditions such as colon, prostate, and breast cancers. It also prevents the growth of tumors by stimulating apoptosis.[18,19]

Conclusion

The current study is the first of its kind that surveys the effect of hazelnut oil on the toxicity against and induction of apoptosis in a colorectal cancer cell line. Hazelnut is recognized as an adequate source rich in useful fatty acid compositions playing an essential role in human health and well-nutrition. Its lipid profile contains triacylglycerols, tocopherols, tocotrienols, phytosterols, and several other bioactive compounds. Triacylglycerols are a significant fraction of herb-originated oils including hazelnut oil that constitute 95%–98% of its whole oil content. Furthermore, in this study, the potential of hazelnut oil in inducing cytotoxicity in HT29 cells after a 24-h regimen was clearly observed. It also could increase the number of apoptotic cells, raise the apoptotic index, and express the genes involved in mitochondrial apoptosis.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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