Study of some biological activities of aqueous extract of ginger (*Zingiber officinale*)

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Purpose
This study was designed to evaluate the potential of two aqueous extracts of ginger rhizome under different extraction conditions (cold and hot water).

Materials and methods
Anticoagulant, fibrinolytic, antimicrobial, prebiotic, and antitumor activities were examined for the two aqueous extracts. The two aqueous extracts were then used for evaluation of yield, total carbohydrates, protein, and monosaccharide contents. The sulfation of crude extracts was carried out by chlorosulfonic acid as a sulfating agent in formamide.

Results
The results showed that sulfation modification of the two aqueous extracts increased significantly both the anticoagulation and the fibrinolytic activities, but did not affect the antimicrobial, prebiotic, or antitumor activities. However, the two native water extracts showed antibacterial activity against *Escherichia coli* only, but not for *Staphylococcus aureus*, and antifungal activity against *Candida albicans*.

Conclusion
The chemical modification of the two aqueous extracts of ginger by sulfation will increase significantly its anticoagulation and fibrinolytic activities while not affecting or improving the prebiotic and antimicrobial activities. However, the antitumor activity is high for both the native and the sulfated aqueous extracts of ginger. Thus, this result does not mean that the sulfation modification of the water extracts studied affected the antitumor activity.

Keywords:
anticoagulation activity, antimicrobial activity, antitumor activity, fibrinolytic activity, ginger (*Zingiber officinale*), prebiotic activity

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Introduction

Ginger, the rhizome of *Zingiber officinale*, is one of the most widely used species of the ginger family (Zingiberaceae) and is a common condiment for various foods and beverages. It has a long history of medicinal use dating back 2500 years in China and India for conditions such as headaches, nausea, rheumatism, anticancer and antioxidant effects, and colds [1]. Ginger is native to Southern Asia, but it is now cultivated extensively in Jamaica, Nigeria, China, India, Fiji, Sierra Leone, and Australia.

The extracts of ginger show antibacterial activity against the pathogens *Staphylococcus aureus*, *Staphylococcus pyogenes*, *Staphylococcus pneumoniae*, and *Haemophilus influenza*, and extracts of ginger may contain compounds with therapeutic activity [2].

Ginger has been studied widely for its pharmacological activities and has been reported to show anti-inflammatory, antipyretic, hypoglycemic, antimigraine, antischistosomal, antioxidant, hepatoprotective, diuretic, hypocholesterolemic [3], hypotensive [4], and gastrointestinal prokinetic activities [5].

The consumption of a high-fat diet may lead to an increase in serum cholesterol and plasma fibrinogen levels, which in turn may result in decreased fibrinolytic activity and blood coagulation time. These changes would also increase the risk of atherosclerosis and heart diseases. The importance of serum cholesterol levels and of lipoproteins in relation to atherosclerosis and coronary heart disease is well known. Thus, there has been an ongoing search for blood cholesterol-lowering agents and dietary adjuncts have especially been screened for this as it would be most advantageous. Some of the commonly consumed spices were naturally evaluated for a possible hypocholesterolemic action in a variety of experimental situations in both animals and humans [6].

Ginger inhibits platelet aggregation in healthy individuals [7] and patients with coronary artery disease [8]. The concurrent use of ginger and

anticoagulants may result in an increased risk of bleeding [9].

In Ayurvedic science, ginger has been described as an excellent tonic for the heart. It helps prevent various heart diseases by reducing blood clotting that can lead to plaque formation or thrombosis. It can also open the blockage in the blood vessels, thus decreasing peripheral vascular resistance and hence blood pressure. Ginger may also help to lower high cholesterol, making the heart healthy [2]. Srivastava *et al.* [10] found that aqueous extracts of ginger inhibited platelet aggregation induced by ADP, epinephrine, collagen, and arachidonic acid *in vitro*. The antiplatelet action of 6-gingerol was also mainly because of the inhibition of thromboxane formation [11].

Materials and methods

Materials

Collection of plant materials

Ginger (the rhizome of *Z. officinale*) was purchased from the local market and stored in a deep freezer at −20°C until analysis.

Microorganisms and media

Bacterial strains

Lactobacillus rhamnosus, *Lactobacillus reuteri*, and *Lactobacillus acidophilus* were obtained from Chr. Hansen (A/S Lab.Inc., Fanøgade, Copenhagen, Denmark).

The pathogenic isolated *Escherichia coli* and *S. aureus* were obtained from the clinical laboratory of Faculty of Medicine, Ain Shams University (El-Demerdash Hospital).

Yeast strain

A pathogenic yeast fungus (*Candida albicans*) was obtained from the clinical laboratory of the Faculty of Medicine, Ain Shams University (El-Demerdash Hospital).

Culture media

Unless otherwise stated, all of the following used media were adjusted at pH 7.2 before sterilization. This media were as follows:

Potato dextrose agar

This medium was used for inoculum growth and maintenance of the yeast strain *C. albicans*.

Nutrient agar medium

Nutrient agar medium was used for the growth and maintenances of the pathogenic bacterial strains *E. coli* and *S. aureus*.

De Man–Rogosa–Sharpe medium

This medium was used for the growth and maintenance of the three probiotics *L. rhamnosus*, *L. reuteri*, and *L. acidophilus* [12].

De Man–Rogosa–Sharpe (MRS) broth medium was used for determination of the growth density of the investigated probiotics by the replacement of the carbon source and use of the studied samples instead. The prepared MRS broth tubes were inoculated and incubated anaerobically at 37°C for 24 h.

Nutrient agar, nutrient broth, potato dextrose broth, and potato dextrose agar media were purchased from Diffco Company (PO Box 178, Cypress, TX 7741 DIFFCO, LLC, USA).

MRS broth and MRS agar media were purchased from Fluka Company (Egyptian International Center for Import, Cairo, Egypt by license from Fluka, Switzerland).

Penicillin (1 000 000 IU) and fluconazole (150 μg) antibiotics were used as antimicrobial controls and purchased from Pfizer Company (235 East 42nd Street, New York, NY, 10017, USA).

Chemicals, solvents, and reagents were obtained from Sigma Chemical Co (Egyptian International Center for Import, Cairo, Egypt by license from Sigma co., New York, United States).

Cell culture

A human colon carcinoma cell line, Caco-2, was obtained from the American Type Culture Collection (ATCC) (HTB-37) [from *Homo sapiens* (human; organ – colon], and cultivated in Eagle's minimum essential medium (ATCC) supplemented with 20% fetal bovine serum (ATCC) and a 1% antibiotic antimycotic solution (containing 10 000 U/ml penicillin G, 10 mg/ml streptomycin sulfate, and 25 μg/ml amphotericin B; Sigma-Aldrich, St. Louis, Missouri, USA). Confluent monolayers were subcultured by incubating with 0.05% trypsin and 0.2% EDTA in Ca^{2+}-free and Mg^{2+}-free PBS (Sigma-Aldrich). Cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. For all experiments, cells were seeded at high density (10^6 cells/ml, 0.2 ml/well) onto test surfaces contained within 96-well plates and proliferated for 2 days.

Methods

Analytical methods

For each studied sample, the chemical properties of moisture, ash, crude protein, and crude lipids were determined according to Association of Official Analytical Chemistry (AOAC) [13]. Total

carbohydrates were determined after complete acid hydrolysis according to Dubois *et al*. [14]. The resulting acid hydrolysates were examined by Paper Chromatography using Whatman no. 1 sheets and using n-BuOH–MeCO–H_2O (4:5:1) [15] as a solvent and aniline phthalate [16] as spraying reagents. Quantitative determination of the separated sugars was carried out according to Wilson [17]. Total nitrogen of the investigated samples was determined using the conventional micro-Kjeldahl's method [13].

Preparation of aqueous extracts

Ginger samples were dried for 48 h in a hot-air drier at 50°C. One hundred gram of dried samples were extracted with 2000 ml of deionized hot and cold water (pH 7) for 3 h. After cooling to room temperature and then filtering (Whatman no. 1), the extracts were dialyzed against distilled water for 48 h., dried under vacuum, and weighed.

Preparation of sulfated extracts

This was performed by the modification of the method reported by Yang *et al*. [18]. The sulfation of the extract was performed as follows: 0.1 g of each extract was suspended in 0.5 ml dry formamide and the mixture was stirred at room temperature for 24 h to disperse it into the solvent. The sulfating agent was prepared by dropping 1 ml of $HClSO_3$ in 4.0 ml of formamide under cooling in an ice-water bath and then added to the extract. The reaction was cooled in ice, neutralized by a 30% NaOH solution, and dialyzed against running water for 48 h and then lyophilized.

Biological activities

Anticoagulation activity

Anticoagulation activities of the various extracts (crude and sulfated) preparations were determined according to the time for clot formation as recorded [19] and compared with that of the standard heparin sodium preparation.

Fibrinolytic activity

This was done according to the method of USA Pharmacopoeia [20] as lysis percentages of the plasma clots when incubated at 37°C; a standard preparation of Hemoclar (El-Nile pharmeuticals co., El Amerya, Cairo, Egypt) (pentosan sulfonic polyester) was used.

Antimicrobial assay

The aqueous extract as well as the four isolated compounds were evaluated *in vitro* for their antimicrobial activity against the bacteria and the fungus species (*C. albicans*) using the well diffusion method [21,22]. The experiment was conducted using a culture at 37°C for 24 h on 10 ml of nutrient broth for bacteria and 48 h on potato dextrose broth for yeast. Agar (20 ml) was poured into sterile Petri dishes and allowed to solidify. Wells were prepared in agar plates using a sterile cork pore of 7 mm diameter. The cultures were adjusted to ~10^6 CFU/ml with a sterile saline solution. One hundred and fifty microliters of the suspensions were spread over the agar plates using a sterile cotton. Preliminary antimicrobial screening of the isolated studied samples was performed with 150 mg dry weight. Each tested sample was dissolved in 1 ml of distilled water and sterilized by filtration through a 0.22 μm membrane filter (using Milliopore membrane filter apparatus; Milliopore (Ground Floor, Building 1,885, Mountain Highway, Bayswater, VIC 3153, Australia)). One hundred and fifty microliters of each sample was added separately to the appropriate wells in the Petri dishes.

The antimicrobial activity was recorded by measuring the diameter of the clear inhibition zone. In these tests, streptomycin (15 μg/disc) and griseofulvin (20 μg/disc) were used as experimental positive controls for strains (bacteria and yeast, respectively) and sterile distilled water was used as a negative control for which no inhibitory effect could be observed. Experiments were conducted in triplicate and evaluated. The reported results are the average value with SD.

Prebiotic activity

The prebiotic activity was assayed by comparison of the densities of growths of the probiotic bacteria *L. acidophilus*, *L. rhamnosus*, and *L. reuteri* with that of the pathogenic *E. coli* as they were grown on MRS medium at 37°C for 24 h (containing polysaccharides (PS) of ginger extracts as the carbohydrate source). However, *E. coli* was grown on nutrient broth medium, both at 37°C for 24 h. Aliquots of 0.1 ml of each of the resulting bacterial culture were used as an inoculum for 10 ml studied medium containing ginger extract as the carbohydrate source. This medium was prepared at a concentration of 150 mg carbohydrate source per 10 ml MRS-base medium (according to the carbohydrate content of ginger extracts). After incubation at 37°C for 24 h, the resulting bacterial growth was measured at 625 nm against a blank composed of uninoculated medium [23]. The prebiotic activity was calculated as the 'prebiotic index' (I):

Prebiotic index (I)=optical density of bifidogenic bacterial culture/optical density of *E. coli* culture.

Antitumor activity

Antitumor activity of different Ginger water extracts

Quantitative assay of cytotoxicity

Quantification of fixated monolayer cells was performed spectrophotometrically with crystal violet as a DNA stain. Staining of cell nuclei of fixed cells

with crystal violet allows for rapid, accurate, and reproducible quantification of cell numbers in cultures grown in 96-well plates [24]. Crystal violet assays were used to measure the total count of cancer mammalian cells that well reflect the cytotoxic effects of the hot and aqueous extracts of ginger (native and sulfated) using the human colon carcinoma cell line, Caco-2, in 96-well plates. Experiments were conducted in basal tissue culture (BTC) medium buffered with 1 mol/l HEPES (pH 7.4), 2.20 g of $NaHCO_3$, and 10 ml of fetal bovine serum per liter maintained by incubation in 5% CO_2. Briefly, the cell culture type (Caco-2) was subcultured as follows: the growth medium was discarded and the cell sheet was washed with sterile 0.15 mol/l PBS (pH 7.5). A cell dispersing solution (trypsin) was added for 1 min and then discarded. Cells were incubated at 37°C. After cell separation and roundation were observed microscopically, 50 ml of fresh medium (BTC medium) was added to the flask. Suspended Caco-2 cells were counted and diluted to about 10^5 cells/ml. Cell suspension (100 μl) was distributed in a 96-tissue culture plate, and then incubated in a humid 5% CO_2 incubator at 37°C for 24 h to allow for attachment. After incubation, the medium was discarded and cells were exposed to a dilution series ranging from 0.02 to 0.005 mg/ml aliquots. The suspension of each sample of hot or cold water extracts (native and sulfated) (suspended in fresh BTC medium) was inoculated in eight wells and incubated for 48 h in a 5% CO_2 incubator at 37°C including controls (contains only BTC medium). After 48 h of exposure, the medium was discarded and cells were fixed with 100 μl of glutaraldehyde of 1% for about 30 min. The excess glutaraldehyde solution was removed and then washed with tap water. A crystal violet solution (0.1%), 100 μl, was added for about 30 min. Wells were immersed at a very slow rate of running tap water for 15 min. The plates were dried and then the stained wells were eluted with 100 μl of a 10% acetic acid solution. The color intensity in each well was measured using a microplate reader at 590 nm. Eight replicates were used for each isolate, and all tests were performed in triplicate. The absorption reading of different aliquots of ginger extracts (average reading of eight wells) was divided by the control reading (average reading of eight wells containing 100 μl of sterile BTC) multiplied by 10^4 (the total number of Caco-2, cells per well). This yields the number of viable Caco-2 cells. Subsequently, the previous number was subtracted from 10^4 to obtain the number of dead Caco-2 cells.

Statistical analysis

Data were statistically analyzed using SPSS (version 10.00; SPSS Inc., Chicago, Illinois, USA) for Windows. Data were presented as mean±SD.

Results and discussion

Chemical composition of extracts

Data of the chemical composition of investigated ginger extracts (*Z. officinale*) are shown in Table 1. The results showed a wide range of variation in the yield of the ginger extracts investigated. The highest yield was recorded in the hot water extract of 31.8% and the lowest yield in the cold water extract (27.3%).

The data in Table 1 show a variation in the proportions of this constituent. The highest value was observed in the hot extract of 34.6% and the lowest value in the cold extract (32.1%).

Another wide range of variation was also observed in the soluble-protein content of the ginger extract studied (Table 1). Thus, the percentages of protein varied from 28.6% in the cold extract to 39.0% in the hot extract. However, the highest sulfate content was recorded in the hot extract (8.2%) and the lowest in the cold water extract (7.8%).

The results in Table 1 show that the highest values of glucuronic acid (3.0%) proportions were found in the hot extract and the lowest value in the cold extract (1.6%), and the highest values of galactose (14.1%) were found in the hot water extract and the lowest value in cold water extract (11.1%). In all cases, glucose represents the major monosaccharide component of the two extracts, the highest values of glucose in the cold extract (51.0%) and the lowest value in the hot extract (48.9%). Furthermore, the highest value of mannose was recorded in the cold extract (12.3%) and lowest value in the hot extract (10.0%). It has also been reported that the highest values of arabinose were found in the cold extract (20.5%) and the lowest value in the hot extract (18.8%). However, the highest values of xylose were found in the hot extract (5.2%) and the lowest value in the cold extract (3.5%).

Table 1 Yield, total carbohydrates, monosaccharide constituents, protein, and sulfate content of aqueous extracts of ginger (*Zingiber officinale*) (mg/100 mg)

Extracts	Yield	Total carbohydrates	Protein	Sulfate content	Monosaccharide composition (%w/w)					
					Uronic acid	Galactose	Glucose	Mannose	Arabinose	Xylose
Cold water extract	27.3	32.1	28.6	7.8	1.6	11.1	51.0	12.3	20.5	3.5
Hot water extract	31.8	34.6	39.0	8.2	3.0	14.1	48.9	10.0	18.8	5.2

Biological activities

Investigations focused on the promotion of the biological activities (anticoagulant, fibrinolytic, antimicrobial, prebiotic, and antitumor) throughout the chemical modification of the native aqueous extracts were performed by introducing sulfate ester groups on carbohydrate moieties of aqueous extracts.

Testing aqueous extracts for its anticoagulation activity *in vitro*

Data on the anticoagulation activity of aqueous extracts and their corresponding sulfated derivatives are shown in Table 2. Heparin was used as the standard anticoagulant, which takes 90 min for complete clot formation.

The results showed that most of the aqueous extracts studied had no anticoagulation activities at 2000 μg/ml concentration.

However, the results showed promising anticoagulation activities of the sulfated extracts at different concentrations (2000, 1000, 500, 200, 100, and 50 μg/ml) compared with corresponding native extracts.

The results in Table 2 indicated that the two native extracts have no anticoagulation activity, whereas the two sulfated aqueous extracts studied have significant anticoagulation activity. The lowest sulfated hot water extract concentration yielding anticoagulation activity was found at a concentration of 50 μg/ml, which had a clotting time of 40 min. However, the lowest anticoagulation activity was reported using the same concentration (50 μg/ml) of sulfated cold water extract (20 min). From these data, it was concluded that the addition of a sulfate group to the extracts enhanced anticoagulation activities.

Testing of aqueous extracts for its fibrinolytic activity *in vitro*

The data presented in Table 3 show that original aqueous extracts have weak fibrinolytic activity (60% for the cold water extract and 50% for the hot water extract), whereas the chemically modified extracts have higher fibrinolytic activities than the standard Hemoclar (80% for each extract). Thus, sulfation modification of the original extract increases its fibrinolytic activity, and this is in agreement with the result of Hussein *et al*. [19].

Antimicrobial activity

Antimicrobial activities of the four aqueous extracts (two native and two sulfated) obtained from ginger at a concentration of 1.6 mg/ml saline are shown in Table 4. Streptomycin (15 μg/disc) was used as a reference material or as a positive control for antibacterial activity and griseofulvin (20 μg/disc) was used as a positive control for antifungal activity. The results indicated that all extracts showed antibacterial activity against studied Gram-negative bacterium *E. coli*, but not the Gram-positive bacterium *S. aureus*.

The highest recorded antibacterial activity of water extracts against Gram-negative bacteria was less effective than streptomycin by 80% for all water extracts in the *E. coli* bioassay. These data are in agreement with those of Okoli and Iroegbu [25], who reported that water and methanolic extracts of some plants showed significant antimicrobial activity. Similarly, Basri and Fan [26] reported that the aqueous and acetone extracts of galls of *Quercus infectoria* (Oak) showed similarities in antimicrobial activity in bacterial species and as such, it could be a potential source of antimicrobials.

Interestingly, water extracts of ginger (Table 4) showed effective antifungal activity compared with the reference commercial fungicidal griseofulvin. The results also showed moderate activity against yeast (*C. albicans*) for all the extracts studied (about 66.6% activity compared with griseofulvin). These results were in agreement with those of Zahid *et al*. [27], who reported that the aqueous extract of fennel (*Foeniculum vulgare* Mill) had potential antifungal activity against three soil-borne fungi, namely, *Macrophomina phaseoli*, *Rhizoctoina solani*, and *Fusarium moniliforme*.

From the above data, it is clear that sulfation modification of the two water extracts studied has no effect as antimicrobial agents.

Table 2 Anticoagulation activity of aqueous extracts of tested plants and their sulfated aqueous extracts against human plasma (min)

Extracts	Clotting time (min)							
	Standard (heparin)	Control (distilled water)	Concentrations of extracts (μg/ml)					
			2000	1000	500	200	100	50
Cold extract	90	6	10	–	–	–	–	–
Sulfated cold extract	90	6	>600	>600	>600	>600	110	20
Hot extract	90	6	10	–	–	–	–	–
Sulfated hot extract	90	6	>600	>600	>600	>600	>600	40

Table 3 Fibrinolytic activities of aqueous extracts of ginger and their corresponding chemically modified extracts (2000 μg/ml)

Extracts	Fibrinolytic activity (2000 μg/ml) (%)	
	Before sulfation	After sulfation
Cold water extract	60	80
Hot water extract	50	80
Standard (Hemoclar)	75	

Table 4 Antimicrobial activity of aqueous extracts of ginger and their corresponding chemically modified extracts

Compounds	*Staphylococcus aureus* (mm)	*Escherichia coli* (mm)	*Candida albicans* (mm)
Ginger hot	Negative	20	20
Ginger cold	Negative	20	20
Sulfated hot extracts of ginger	Negative	20	19
Sulfated cold extracts of ginger	Negative	20	19
Standard streptomycin	28	25	–
Standard griseofulvin	–	–	30

Table 5 Prebiotic activity of aqueous extracts of ginger and their corresponding chemically modified extracts

Compounds	*Lactobacillus acidophilus*	*Lactobacillus rhamnosus*	*Lactobacillus reuteri*
Ginger hot	0.394	0.714	0.820
Ginger cold	0.410	0.767	0.846
Ginger hot sulfated	0.837	0.849	0.815
Ginger cold sulfated	0.373	0.440	0.129

Table 6 Antitumor effect of water ginger extracts

Ginger extract concentration	Sample	OD	Number of dead cells	Number of viable cells	% of cytotoxicity
At concentration 0.02 mg/ml	Control	1.277	0	10 000	0
	Cpd 1	0.359	7185	2815	71.85
	Cpd 2	0.351	7254	2746	72.54
	Cpd 3	0.359	7188	2812	71.88
	Cpd 4	0.348	7274	2726	72.74
At concentration 0.01 mg/ml	Cpd 1	0.806	3688	6312	36.88
	Cpd 2	0.766	3999	6001	39.99
	Cpd 3	0.812	3638	6362	36.38
	Cpd 4	0.774	3939	6061	39.39
At concentration 0.005 mg/ml	Cpd 1	0.908	2890	7110	28.90
	Cpd 2	0.911	2866	7134	28.66
	Cpd 3	0.909	2885	7115	28.85
	Cpd 4	0.914	2840	7160	28.40

Cpd 1, crude cold water extract of ginger; Cpd 2, sulfated cold water extract of ginger; Cpd 3, crude hot water extract of ginger; Cpd 4, sulfated hot water extract of ginger; OD, optical density.

Prebiotic activity of PS

The basis for the determination of the prebiotic activity depends on comparison of the densities of growths of the probiotic-beneficial bacteria *L. acidophilus*, *L. rhamnosus*, and *L. reuteri* with that of the pathogenic *E. coli* as they were grown on MRS medium (containing ginger extracts as a carbohydrate source) (Table 5).

The results clearly show no prebiotic activity for the four extracts studied as all prebiotic indexes (I) for all extracts were less than one and the sulfation modification of the ginger carbohydrates did not improve the prebiotic activity of the two native extracts.

The prebiotic activity was negative for all the studied extracts, it may be because that the pathogenic *E. coli* can utilize all the studied ginger extracts as well as the examined probiotics.

Antitumor activity

Water ginger extracts and its sulfated derivatives were tested in an in-vitro study for their antitumor activity. The data in Table 6 indicate that all the tested concentrations of the native and sulfated water ginger extracts showed higher cytotoxicity that ranged between 2815 and 7185 of dead cells, compared with the control, with the concentration of water ginger extracts and their sulfated ranging from 0.005 to 0.02 mg/ml. Hassan *et al.* [28] reported that there is a positive correlation between antitumor activity and carbohydrate concentration. Carbohydrate–protein conjugates are involved in interactions with an animal's immune system and as a result enhance immune system activity [29]. They also play a role in animal antioxidant and antimutagenic defense [30]. The results of Table 6 are in agreement with those of Liu *et al.* [31] and Abdel-Fattah *et al.* [32], who reported that a chemically modified group, acetyl, phosphoryl, or benzyl group in a polysaccharide molecule can significantly enhance the antioxidant and antitumor activities of levan.

Conclusion

From the above data, chemical modification of the two aqueous extracts of ginger by sulfation increased its anticoagulation and fibrinolytic activities significantly while not affecting or improving the prebiotic and antimicrobial activities. However, the antitumor activity was high for both the native and the sulfated aqueous extracts of ginger. This, this result does not imply that sulfation modification of the water extracts studied affects the antitumor activity.

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Conflicts of interest

None declared.

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