

Effects of *Artemisia judaica* Essential Oil and Ethanolic Extract on Experimentally-Induced Benign Prostatic Hyperplasia

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Submitted: 28-May-2020

Revised: 15-Jul-2020

Accepted: 11-Aug-2020

Published: 30-Nov-2020

ABSTRACT

Background: Recent studies have shown that the essential oil (EO) and the ethanolic extract (EE) from *Artemisia judaica* L., a Jordanian medicinal plant, exhibit a potent anti-angiogenic and anti-inflammatory activities. Angiogenesis and inflammation processes are known to play a role in benign prostatic hyperplasia (BPH), a disease associated with aging in men. **Objectives:** The present study aimed to address the effects of EO and EE on experimentally-induced BPH in rats. **Materials and Methods:** Four experimental groups were assigned with six rats in each group, including the corn oil vehicle as a control. The three other groups were induced to develop BPH by testosterone injection. The BPH rats were randomized into the BPH-untreated group, BPH-EO treated group (200 mg/kg/subcutaneously) and BPH-EE treated group (500 mg/kg/orally). **Results:** The prostate weight/body ratio and epithelial thickness showed a significant reduction in the EO and EE treated groups compared to the BPH untreated. In addition, mRNA expression levels of the proliferating marker (proliferating cell nuclear antigen), the angiogenesis marker (vascular endothelial growth factor-A) and interleukin-6; an inflammatory cytokine, were significantly down-regulated in the BPH groups that were treated with EE or EO. **Conclusion:** Our results indicated that in experimentally-induced BPH, EO and EE from *A. judaica* ameliorate BPH development by inhibiting prostatic cell proliferation, angiogenesis, and inflammation.

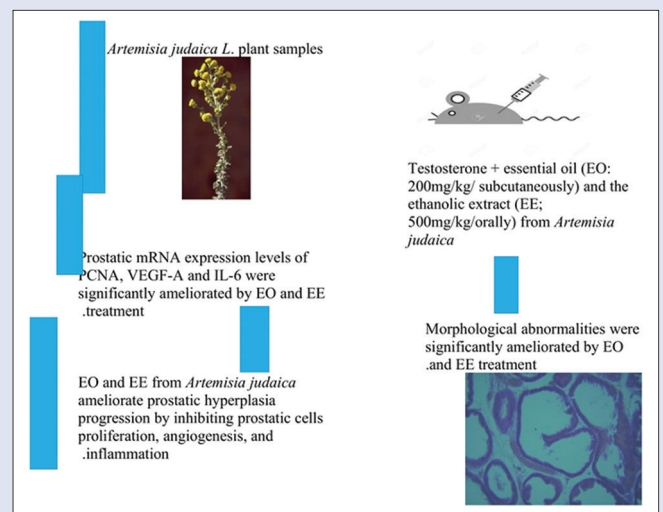
Key words: *Artemisia judaica* L, essential oil, ethanolic extract, prostatic hyperplasia, rats

SUMMARY

- The essential oil (EO) and ethanolic extract (EE) from *Artemisia judaica* significantly reduced prostate weight/body ratio and epithelial thickness in experimentally-induced benign prostatic hyperplasia (BPH)
- The EO and EE ameliorate BPH development by inhibiting prostatic cell proliferation, angiogenesis, and inflammation.

Abbreviations used: EO: Essential oil; EE: Ethanolic extract; BPH: Benign prostatic hyperplasia; PCNA: Proliferating cell nuclear

antigen; VEGF-A: Vascular endothelial growth factor-A; IL-6: Interleukin-6; TNF- α : Tumor necrosis factor; IL-1: Interleukin-1.



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DOI: 10.4103/pm.pm_216_20

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INTRODUCTION

Most urological disorders are considered to be caused by benign prostatic hyperplasia (BPH) and prostate cancer in men.^[1-3] BPH is characterized by progressive hyperplastic growth of glandular-epithelial tissue and stromal-muscle tissue in the prostate, which is related to acute urinary retention.^[2] Although the etiology of BPH has not been completely understood, however, prostatic inflammation and abnormal angiogenesis have been demonstrated as essential parameters that are involved in the development and progression of BPH.^[3] Specific inflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor, and IL-6 are known to be secreted by activated inflammatory cells. Besides, these cytokines have been reported to be released by prostatic stromal

and epithelial cells during the BPH. It has been observed that these cytokines mainly contributes to the development and growth of local angiogenesis factors, including the vascular endothelial factor of

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Cite this article as: Al-Trad B, Al Zoubi M, Migdady M, Lahham J, Aljabali AA, Shehab M, et al. Effects of *Artemisia judaica* essential oil and ethanolic extract on experimentally-induced benign prostatic hyperplasia. Phcog Mag 2020;16:S561-5.

growth-A (VEGF-A).^[3] Several studies showed an increase in VEGF-A and IL-6 expression during BPH progression.^[4,5]

At present, the two most important drugs of choice for the treatment of BPH are α_1 -adrenergic-receptor antagonists, which leads to smooth muscle relaxation and 5 α -reductase inhibitors, which lead to a reduction in the levels of circulating androgen.^[6,7] These treatments, however, are often associated with multiple complications such as reduced libido and erectile dysfunction.^[6,8] Considering this, there is a high demand for providing useful natural substances for the inhibition and treatment of BPH combined with proven safety with minimal side effects. Therefore, the phytotherapy approach affords a new route providing new treatment alternatives for many disorders. Interestingly, the phytotherapy treatment of BPH occupies more than 50% of the treatment options in Austria, Germany, and Italy.^[9] There are many clinical trials on the use of phytotherapy for BPH treatment.^[9] For instance, *Serenoa repens* (saw palmetto) has been reported to reduce BPH symptoms such as dysuria, prostate size, and inflammation sign with low side effects.^[10-13] Nevertheless, further clinical trials on previously discovered herbal medications and the potential use of new plant extracts are required to provide the most effective and safe phototherapeutics treatments for BPH.

Artemisia judaica L. (family *Asteraceae*) is a common desert flora in Jordan under the local name *Beithran* with a traditional-medicinal use for the treatment of some diseases such as digestive system disorders, diabetes, inflammation, and cancer.^[14,15] Recent studies have shown that the essential oil (EO) and the ethanolic extract (EE) from *A. judaica* exhibit a potent antiangiogenic and anti-inflammatory properties.^[15,16] In addition, extracts of *A. judaica* have been reported to have antioxidant^[17] and antidiabetic properties.^[18] On the other hand, different extracts of seven species of *Artemisia* showed antiproliferative activity on different human cancer cell lines.^[19] Therefore, *A. judaica* extracts may have a potential impact on treating proliferative inflammatory diseases such as BPH. In the current study, therefore, we tried to deal with a traditional-medicinal plant with the potential impact of EO and EE (*A. judaica*) on experimentally-induced BPH in rats.

MATERIALS AND METHODS

Plant samples Collection and identification

All plant samples of *A. judaica* L. were collected from the Southern desert of Jordan near to the Saudi-Jordan borders. The collection period was in the spring of 2018. *A. judaica* L. was identified by Prof. Ahmad El-Oqlah from the Biological Sciences Department at Yarmouk University, according to the local floras.

Preparation of essential oil and the ethanolic extract of *Artemisia judaica*

A single batch of the *A. judaica* was used for both EO and EE preparations. For EO extraction, 70 g of the powdered-dried plant was added to 700 mL of distilled water and then steam distilled for three h using a Clevenger-type apparatus.^[20] The obtained oil was subjected to drying procedure using anhydrous sodium sulfate and then stored at 4°C until analyses. For EE, after shade-drying 100 g of the dried herb were soaked in 95% ethyl alcohol for 72 h followed by filtration through filter paper, then concentrated using the rotator evaporator under vacuum, followed by percolation for several times till exhaustion. All EE were stored in the refrigerator (4°C) until use.

Experimental animals and design

Acute toxicity study

For all laboratory procedures and studies, the National Health Institute recommendations for the treatment and use of lab animals were observed

and procedures were conducted after official approval by the committee of research at the Department of Biological Sciences/Faculty of Science at Yarmouk University) permission number: YU-21/5/2018(. Different doses from the EO (subcutaneous) and EE (orally) of *A. judaica* were given to male Wistar rats (weighing 220–250 g; n = 5/group) as follows: EO: 100, 200, and 1000 mg/kg and EE: 100, 500, and 1000 mg/kg. The mortality and any sign of toxicity were observed regularly for the first 24 h and daily for 14 days.

Benign prostatic hyperplasia animal model and treatments

The animal house unit provided 3-month-old Wistar rats with an average weight of 220–250 g at Yarmouk University. Seven days of laboratory environment acclimation was performed before assigning the obtained animals into four experimental groups (n = 6 for each group). The first group is designated as a negative control group that received 2 ml/kg of corn oil vehicle orally. On the other hand, group 2, group 3, and group 4 were injected subcutaneously with 3 mg/kg/day of testosterone (testosterone enanthate 250 mg, Primoteston-Depot®, Schering, Germany) for BPH induction,^[4] testosterone and 200 mg/kg of EO subcutaneously and testosterone and 500 mg/kg EE orally, respectively. All injections and oral administration were given for 21 days once per day. A preliminary pilot study using the same experimental design was conducted for dose determination. Briefly, the oral dose was determined after applying 200 and 500 mg/kg of the EE. Though, the subcutaneous dose was determined by applying 100 and 200 mg/kg of the EO. The prostate weight to body weight ratio and the histological morphology changes were used to confirm the most effective dose in the BPH rats. Besides, EO and EE without testosterone groups were included in the pilot study. However, as no significant changes were observed on the prostate weight or histology with EO and EE treatment, we decide to exclude these groups from further analysis.

At the end of 21 days, the ventral and dorsolateral prostate was extracted after ether anesthesia and sacrificing protocol. All prostates were divided symmetrically. One of the prostate divisions was stored in liquid nitrogen then proceed for mRNA extraction. The second half of the prostate tissue was washed with normal saline, fixed with 4% formalin for 4 h and followed by histology protocol and finally embedded in paraffin blocks.

The prostate size and histological examinations

The prostate weight to body weight ratio was calculated as described before.^[4] The paraffin-embedded tissue from each group was sectioned at 5 μ m thickness sections and subjected to deparaffinization, rehydration in xylene alcohol serial concentrations, respectively. Finally, h and e staining was performed for histological examination. Blinded investigators investigated the morphological changes in the histological structure of prostate tissues. ImageJ software (NIH, Bethesda, MD, USA) was used for the calculation of the prostate epithelial thickness.

mRNA extraction, cDNA synthesis and quantitative reverse transcription polymerase chain reaction

The total RNA from prostate tissues extracted using total RNA isolation kit (iNtRON, South Korea) following the manufacturer's protocol. RNA concentrations were measured by QuantiFluor RNA stain and QuantusFluorometer (Promega, Madison, USA). Revert Aid First Strand cDNA Synthesis Kit was used for RNA reverse transcription (Thermo Scientific, USA). The prepared cDNA stored at –20°C for future use for quantitative reverse transcription polymerase chain reaction (qRT-PCR). Quantitative real-time PCR for the expression of proliferating cell nuclear antigen (PCNA), VEGF-A, and IL-6 was performed on Line-Gene 9600 thermal cycler system (Bioer Technology, Bingjiang, China) as the following amplification conditions: Activation step was started at 95°C for 3 min followed by 95°C for 5 s and 60°C for 30 s,

using beta-actin (β -actin) as non-regulated reference gene. Primers for the target sequences were designed using Primer3 (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) software and ordered from IDT [Integrated DNA Technologies, INC., IA; Table 1]. To ensure that the primers were accurate, a gel was tested that produced a single strip of the intended scale. The SYBR Premix Ex was used to carry out qRT-PCR reactions TaqII PCR master mix (Takara, USA) in 20 μ l final volume (10 μ l SYBR green, 1 μ l forward prime, 1 μ l reverse primer, 6 μ l nuclease-free water, and 2 μ l cDNA). The target genes expression levels were calculated based on $2^{-\Delta\Delta CT}$ approaches.^[21] The β -actin gene expression level was used as a standard control for relative expression calculation.

Statistical analysis

All statistical parameters, including one-way analysis of variance-*post hoc* test, were analyzed using the SPSS software (version 19.0) (SPSS Inc., Chicago, IL, USA). The *P* value was calculated to be significant when it is <0.05.

RESULTS

Acute toxicity study

As per observations, the LD₅₀ value for EO and EE treatments was found to be higher than 1000 mg/kg body weight when administrated subcutaneously or orally, respectively, indicating that the EO and EE from *A. judaica* might be a non-toxic and possibly safe treatment.

The prostate size and histological examinations

At the end of the experiment, there were no significant weight changes between the groups (control 267 \pm 4.5, BPH 256 \pm 10.5, BPH + EE 275 \pm 8.6, EO + BPH 255 \pm 9.5 g; *P* > 0.05). The histological examination of the normal prostate gland showed typical morphological features with normal glandular and stromal architectural histology as shown in Figure 1. As expected, the BPH untreated prostate glands demonstrated abnormal morphological features and showed a higher degree of proliferation in the glandular epithelial and the intervening fibromuscular stroma compared to the control group. Moreover, several intraluminal papillary folds have been noticed in the BPH untreated prostates compared to the healthy control group. In addition, as shown in Figure 2 and Figure 3a, the BPH untreated group significantly showed a higher prostate weight-to-body weight ratio and significant increase in the thickness of the epithelial layer compared to the control group that received corn oil vehicle (*P* < 0.05). On the other hand, the EO and EE of *A. judaica* treatment showed ameliorated histological morphology in the BPH treated rats compared to BPH-untreated group.

Prostate expression of vascular endothelial growth factor-A, proliferating cell nuclear antigen, and interleukin-6

The BPH untreated group showed a significant increase in the prostatic mRNA expression levels of PCNA [Figure 3b], a marker of cell proliferation, VEGF-A [Figure 4], a biomarker of angiogenesis and IL-6 [Figure 5], an inflammatory cytokine. In addition, a significant

decrease in the expression of VEGF-A, PCNA, and IL-6 was observed in the BPH + EO and BPH + EE groups compared to the BPH untreated group (*P* < 0.05).

DISCUSSION

The results of the current study underscoring the favor of the benefit of phototherapeutics as an alternative option for the treatment of BPH with the expected capability to tolerate natural products besides their low association with serious adverse events.^[9] The LD₅₀ of the current study demonstrated the potential tolerated toxicity of *A. judaica* extracts. *A. judaica* is widely used in Jordan herbal medicine for its medicinal properties in traditional practices. The present study demonstrated that EO and EE from *A. judaica* ameliorate prostatic hyperplasia progression in experimentally-induced BPH. This protection could be related to the inhibition of prostatic cell proliferation, angiogenesis, and inflammation.

In here, we showed that EO and EE from *A. judaica* potently inhibit the proliferation of prostatic epithelial cells as indicated by the decreased mRNA expression of PCNA, a marker of cell proliferation and prostatic epithelial thickness. The current findings are in agreement of the previous studies that showed the action of certain phototherapeutics against cell proliferation by reducing the expression of PCNA.^[22] PCNA is usually used as a marker for the level of proliferation of benign hyperplasia and the reduction in the PCNA expression is associated with the effective treatments against prostatic hyperplasia.^[23,24]

Interestingly, the prostate epithelial cells and stromal cells have been reported to secrete different pro-inflammatory cytokines, including IL-1, IL-6, and IL-8 cytokines, which may create a local inflammatory microenvironment and induce fibromuscular growth in BPH.^[5,25]

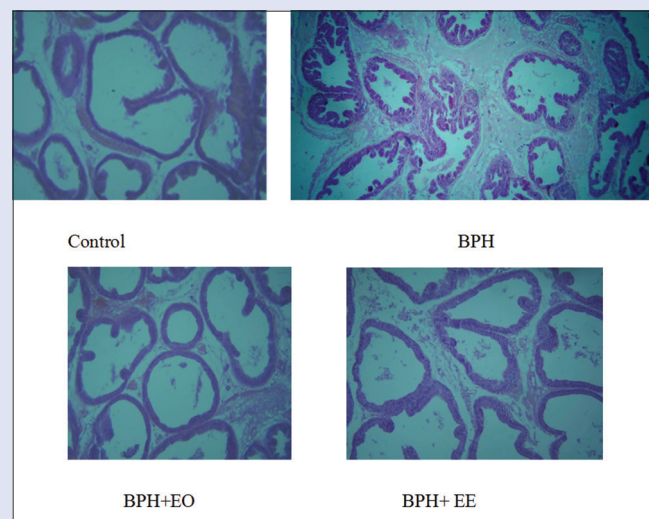


Figure 1: Effect of essential oil and ethanolic extract treatment on the histomorphological changes of prostate sections (hematoxylin staining; magnification, $\times 100$). Abbreviations: BPH: Benign prostatic hyperplasia; EO: Essential oil; EE: Ethanolic extract

Table 1: Sequence of primers used for quantitative real time reverse transcription polymerase chain reaction

Gene	Forward (5-3)	Reverse (3-5)
β -actin	CTTCCAGCCTTCCTCCTG	CAATGCCTGGGTACATGGTG
PCNA	AGGCCCTCAAAGACCTCATC	CGGTATGTGTCGAAGCCTTC
IL-6	TCAACTCCATCTGCCCTTCA	CTGTGAAGTCTCCTCTCCGG
VEGF-A	CGAACAGAGAGAGGGACAGG	GTCTGTCTGTCTGTCCGTCA

PCNA: Proliferating cell nuclear antigen; IL-6: Interleukin-6; VEGF- A: Vascular endothelial growth factor-A

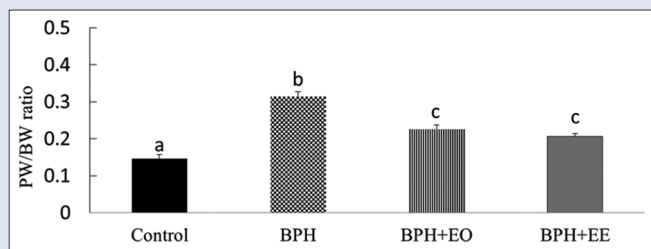


Figure 2: Effect of essential oil and ethanolic extract treatment on the prostate weight/body weight ratio. Data represent the mean \pm standard error of the mean. Means with different superscript letters are significantly different from one another ($P < 0.05$). Abbreviations: BPH: Benign prostatic hyperplasia; EO: Essential oil; EE: Ethanolic extract; PW: Prostate weight; BW: Body weight

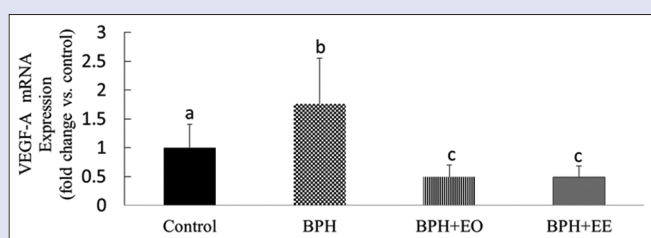


Figure 4: Effect of essential oil and ethanolic extract treatment on the vascular endothelial growth factor-A mRNA expression. Data represent the mean \pm standard error of the mean. Means with different superscript letters are significantly different from one another ($P < 0.05$). Abbreviations: BPH: Benign prostatic hyperplasia; EO: Essential oil; EE: Ethanolic extract; VEGF-A: Vascular endothelial growth factor-A

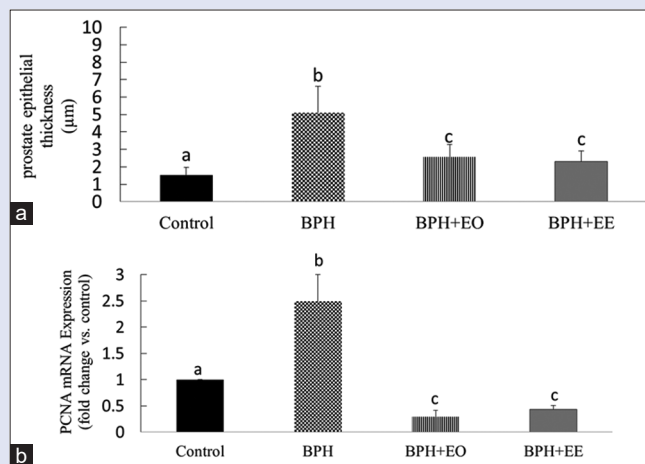


Figure 3: Effect of essential oil and ethanolic extract treatment on the prostate epithelial thickness (a) and proliferating cell nuclear antigen mRNA expression (b). Data represent the mean \pm standard error of the mean. Means with different superscript letters are significantly different from one another ($P < 0.05$). Abbreviations: BPH: Benign prostatic hyperplasia; EO: Essential oil; EE: Ethanolic extract; PCNA: Proliferating cell nuclear antigen

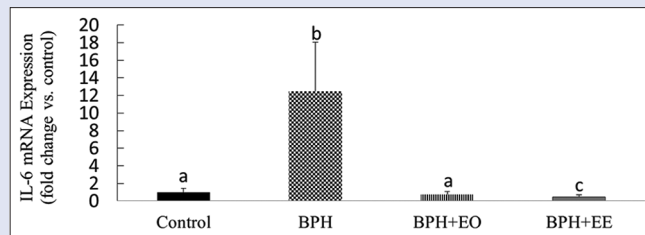


Figure 5: Effect of essential oil and ethanolic extract treatment on the interleukin-6 mRNA expression. Data represent the mean \pm standard error of the mean. Means with different superscript letters are significantly different from one another ($P < 0.05$). Abbreviations: BPH: Benign prostatic hyperplasia; EO: Essential oil; EE: Ethanolic extract; IL-6: Interleukin-6

Therefore, anti-inflammatory phototherapy may result in improved and more effective treatments for BPH. In the current study, we further confirmed an anti-inflammatory effect of EO in addition to the EE through suppressing the IL-6 mRNA expression in BPH treated groups. Our findings are consistent with the previous *in vitro* results, which reported an anti-inflammatory effect of EO from *A. judaica* from Jordan.^[15] According to our findings, *A. judaica* EO and EE extracts acted as anti-inflammatory agents by inhibition of the critical inflammatory cytokine (IL-6) that has been reported to play a role in the onset of BPH.^[4] In addition, our findings are supporting the previous reports that demonstrated the anti-inflammatory effect of phytotherapeutic treatments by reducing IL-6 in prostatic hyperplasia.^[26,27]

The reduction of these inflammatory and proliferating markers by *A. judaica* extracts is attributed to the interfering with the pathophysiology of BPH, which involves inflammatory signs and hyperplasia of both the fibro muscular stromal and glandular epithelial elements.^[2] The mechanism of the anti-inflammatory effect of the *A. judaica* extracts is not entirely understood. However, it has been proposed that the anti-inflammatory potency is due to the presence of a high amount of oxygen-containing monoterpenes in *A. judaica* EO.^[28] Similarly, the antiproliferative activity has been attributed to the presence of monoterpenes, as shown on a group of cancer cell lines.^[28,29] Moreover, the antitumorogenic effects of monoterpenes were accounted for their ability to inhibit the isoprenylation of cell growth-regulatory proteins such as Ras or by the induction of apoptosis.^[29] However, a previous study showed that the EE from *A. judaica* had low antiproliferative activity against human breast cancer cell line MCF7.^[16] These conductorly findings might be attributed to different cell applications or experimental designs.

Angiogenesis is a crucial tumor growth pathway, so the strategies for treating antiangiogenic are typical in the tumor. The results of the current study directly demonstrated that the EO, in addition to the EE possesses significant antiangiogenic activity in BPH, *in vivo*, by reducing the VEGF-A expression levels. These results are consistent with the previous findings that have shown the antiangiogenic activity of EE from *A. judaica*, *in vitro*.^[16] Besides, our findings provide supportive evidence about VEGF targeting by phototherapeutic treatments, as described previously.^[30]

CONCLUSION

In experimentally-induced BPH, EO and EE from *A. judaica* ameliorate prostatic hyperplasia progression by inhibiting prostatic cell proliferation, angiogenesis, and inflammation through the suppression of PCNA, VEGF-A, and IL-6 expressions levels.

Acknowledgements

We thank all persons from the Department of Biological Sciences/Faculty of Science at Yarmouk University for their contribution to complete all experiments.

Financial support and sponsorship

This work could not be completed or conducted without the financial fund from the Deanship of Research and Graduate Studies/Yarmouk University (Grant number 34/2017).

Conflicts of interest

There are no conflicts of interest.

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