



Antiviral effect of honey extract Camelyn against SARS-CoV-2

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ABSTRACT

This study aimed to evaluate the potential antiviral effects of honey extract "Camelyn" against Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2). The baby hamster kidney cell line 21 (BHK-21), bone marrow-derived hematopoietic stem cells (HSCs), and splenic cells were used for Camelyn cytotoxicity assay. After the isolation procedures, cell viability was assessed by trypan blue dye exclusion under a microscope using a hemocytometer. The in vitro cell growth rate was carried out using the cell counting Kit 8 (CCK-8) assay. The cells were seeded in growth media with various Camelyn concentrations (35 µg, 50 µg, 70 µg, 100 µg, 150 µg, and 200 µg). The absorbance at 450 nm was determined by the multiplate reader. The antiviral effect was assessed by plaque reduction assay for the determination of drug susceptibility against SARS-CoV-2. Serial dilution of the selected compounds was pre-incubated with 40 to 100 plaque-forming units (PFUs) of SARS-CoV-2. The pre-incubated mix of Camelyn and SARS-CoV-2 was then added to the confluent Vero E6 cells after incubation cells were fixed and stained and the number of PFUs was counted under an inverted microscope and plotted against the logarithm of antiviral concentrations. Our study showed that Camelyn is not cytotoxic, has a stimulatory effect on cell proliferation, and has an inhibitory effect against SARS-CoV-2 with EC50 (half-maximal effective concentration) from 85.7 µg/mL to 192.4 µg/mL depending on product concentration and viral plaque per cell.

INTRODUCTION

The current pandemic shows a great demand for every possible treatment and prevention approach against COVID-19 including existing natural products. Various organic compounds including bee honey, propolis, royal jelly, curcumin, resveratrol are extensively studied and utilized as potential treatment options for different infections [1]. Despite the critique of modern medicine in recent years, honey has got great attention due to its wide range of therapeutic

properties including antimicrobial, anti-inflammatory, and antiviral activity [2, 3]. Researchers have described various phytochemical factors such as hydrogen peroxide, volatile organic acids, lysozyme, glucose oxidase, catalase as effective antibacterial factors [4]. The beeswax, pollen, and propolis are important chemical compounds that provide antimicrobial properties to honey [5, 6]. Honey also contains oligosaccharides in small quantities related to the growth inhibition of various microbes, such as intestinal bacteria [7]. Phenolic compounds, including

flavonoids, of honey, propolis, and royal jelly are attributed to biologically active molecules that demonstrate antimicrobial effects [8, 9]. These physical and chemical factors give honey unique properties. It is determined that honey eliminates wound infections, provides minimization of scarring, suppresses inflammation, stimulates angiogenesis and epithelium growth [10]. The honey's anti-inflammatory activity showed an inhibition of the expression of cytokines [11]. It is known that honey can improve the proliferation of T and B lymphocytes, stimulates phagocytosis, and regulates the production of cytokines from monocytes, such as tumor necrosis factor (TNF), interleukin 1 beta (IL-1 β), and IL-6 [12]. It was determined that honey and its several components block the cell cycle of colon cancer cell lines in the G0/G1 phase [13, 14].

In vitro studies have shown the antiviral activity of honey against different types of viruses [15-17]. The antiviral effect of honey is attributed to its various ingredients, for instance, copper, which is a trace element part of honey inactivates viruses. The phenolic compounds, such as flavonoids, ascorbic acid, or hydrogen peroxide cause viral growth inhibition by interrupting viral transcription, translation, and replication [1], [18], [19]. To elucidate the possible action of honey, plaque inhibition assays were used in Watanabe *et al.* study [20]. It was reported that Manuka honey efficiently inhibited influenza virus replication (EC₅₀ = 3.6 \pm 1.2 mg/mL), which is related to its antiviral effects. In the presence of 3.13 mg/mL Manuka honey, the EC₅₀ of zanamivir or oseltamivir was reduced to nearly 1/1000th of their single-use. The results showed that honey has a strong inhibitory activity against the influenza virus, and demonstrated a possible medicinal value it may have.

The different kinds of honey from eight floral sources were analyzed to evaluate their anti-HIV-1 activities as well as their effects on lymphocyte proliferation. The anti-HIV-1 activity of eight different kinds of honey was performed by quantitative polymerase chain reaction (PCR) assay. The study revealed that monofloral (the same plant species) kinds of honey had anti-HIV-1 activity depended on plant sources and the amount of methylglyoxal in these plants biomass [21].

The study of Abedi *et al.* [22] provided some evidence of the potential effects of honey and its compounds against the coronavirus due to their ability to regulate the attachment and entry of the virus into the host cell

and RNA replication. Honey and its components may also regulate cellular signaling pathways including oxidative stress, inflammation, and apoptosis. One mechanism of the anti-viral action is inhibition of the viral proteins necessary for attachment and entry into host cells [23]. It has been pointed out that honey can affect the disulfide bonds in hemagglutinin protein HA receptors, which prevents the attachment of the influenza virus to the host cell surface. The coronavirus spike protein belongs to the same class of protein family [24]. It has been reported that [25,26] honey compounds such as quercetin, chrysin, kaempferol, galangin, and caffeic acid have anti-viral activity against COVID-19 through strong binding affinity to main protease and viral replication. The main compounds of honey, such as kaempferol, galangin, and caffeic acid can inhibit virus adsorption, invasion, and replication. Chrysin can prevent virus entry into the host cells and virus replication. Guercerin can inhibit virus coating, invasion, and replication [27-29]. The recent studies and the review article of the potential pharmacological effects of honey [30] indicate that honey and its main components have potential implications for the prevention and treatment of coronavirus infection, including COVID-19.

Although the antimicrobial activities of honey have been well studied against many bacteria and fungi [31] [32], its antiviral activities still need extensive investigations that it can be used as prevention and treatment of various viral infections. This study aimed to evaluate the antiviral effects of honey extract Camelyn against Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2).

MATERIALS AND METHODS

Commercial product Camelyn ampoules, obtained from JSC "Silicon Biotechnology", is made from a selected honey extract. The contents of the ampoule consist of 35% of Camelyn, and 65% water for injection. Camelyn contains ketones, ethers, bioorganic acids, phenols, aldehydes, and furfural. For cytotoxicity and antiviral assays, Camelyn was diluted to a final concentration from 10 μ g/mL to 2500 μ g/mL.

Experimental animals

Six-week-old BALB/c mice (n=3) were bred and housed in a breeding facility at the State Research

Institute Centre for Innovative Medicine (Lithuania). All procedures were carried out under the institutional guidelines of the European Union and were approved by the Lithuanian Ethics Committee on the Use of the Laboratory Animals under the State Veterinary Service No. G2-124 (2019.07.11). Animals were maintained in an environment of controlled temperature (23 ± 1 °C). Food and water were provided *ad libitum*.

Cells preparation

The baby hamster kidney cell line 21 (BHK-21) was obtained from Vilnius University Life Sciences Center (Lithuania). Parental BHK-21 cells were seeded in high glucose Dulbecco's Modified Eagle Medium (DMEM) (4.5 g/L) (Life Technologies, USA), supplemented with 10% FBS (Lonza, Switzerland) and 1% antibiotics (penicillin and streptomycin 10.000 U (Lonza, Switzerland)). Cultures were maintained at 37 °C and 5% CO₂ atmosphere. The cell monolayer was dispersed using a 0.25% trypsin – EDTA (Lonza, Switzerland) mixture.

Bone marrow-derived hematopoietic stem cells (HSCs) were isolated by flushing femur and tibiae of BALB/c mice as previously described by Juppeperi *et al.* [33] with some modifications. Splenic cells were isolated by gentle pressure-dissociation of tissue using PBS and then passed through a 70 µm sterile cell strainer. Collected HSCs and splenic cell suspensions were washed with PBS and then fractionated in a density gradient using Lympholyte M (Cedarlane, USA) media according to the manufacturer's recommendations. The isolated HSCs and splenic cells were washed three times in Roswell Park Memorial Institute RPMI-1640 media containing 10% FBS (Lonza, Switzerland), and 1% antibiotics (penicillin and streptomycin 10.000 U (Lonza, Switzerland)), centrifuged for 10 minutes at 300 × g, resuspended, and counted. After the isolation procedures, cell viability was assessed by trypan blue dye (0.4%, w/v) exclusion under Nikon ECLIPSE 50i (Nikon, Japan) microscope using a hemocytometer.

Camelyn cytotoxicity assay

The in vitro cell growth rate was carried out using the cell counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Japan) according to the recommendation by the manufacturer. Two × 10⁵ BHK-21, HSCs, and 5

× 10⁵ splenic cells were seeded in growth media into 96-well plates and for 72 hours incubated with control and various Camelyn concentrations (35 µg, 50 µg, 70 µg, 100 µg, 150 µg, and 200 µg) at 37 °C in a 5% CO₂ atmosphere. The absorbance at 450 nm was determined by the multiplate reader Sunrise (Tecan, Austria). The viability of the Camelyn treated cells was compared to control cells (untreated) and treated with DMSO (positive control). All assays were performed in three independent experiments.

SARS-CoV-2 plaque reduction assay

The compound was assessed by plaque reduction assay for the determination of drug susceptibility against SARS-CoV-2/Quebec City/21697/2020. The selected compounds were assessed by plaque reduction assay, the gold standard phenotypic method for the determination of drug susceptibility against SARS-CoV-2. Briefly, confluent Vero E6 cells were seeded at 1 × 10⁵ cells/well into 6 well plates. Serial dilution of the selected compounds was pre-incubated with 40 to 100 plaque-forming units (PFUs) of SARS-CoV-2 for 60 min at 37 °C in a 5% CO₂ atmosphere. The pre-incubated mix of compound and SARS-CoV-2 was then added to the confluent Vero E6 cells and incubated for 60 minutes at 37 °C in a 5% CO₂ atmosphere. Then inoculum was removed and the infected cells were incubated for three days (without the compound) in Minimum Essential Medium (MEM) (Merck, Germany) with 2% fetal bovine serum (Thermo Fisher Scientific, USA) containing 0.6% SeaPlaque agarose (Lonza, Switzerland). Cells were fixed and stained and the number of PFUs was counted under an inverted microscope and plotted against the logarithm of antiviral concentrations. The EC₅₀ values then were calculated. In parallel, the antiviral drugs favipiravir and remdesivir were assessed by plaque reduction assay according to a standard protocol (no pre-incubation of a virus with drugs) for the determination of drug susceptibility against SARS-CoV-2.

Statistical analysis

Statistical analyses were done using Microsoft Excel and IBM SPSS Statistics software package 25. Spearman rank correlations were used to calculate relationships between variables. A probability level of p-value < 0.05 was taken as statistically significant.

RESULTS

Cytotoxicity assay

Cytotoxicity assay is a quantitative determination of the difference between cell death and cell growth and has been used in our experiments to evaluate the possible effect of the honey product on cell growth and proliferation. Each ampoule of honey extract Camelyn contained 2 ml of an amber-colored solution for injection. The content of active compounds was 0.035g/mL. None of the Camelyn concentrations (35 µg, 50 µg, 70 µg, 100 µg, 150 µg, and 200 µg) tested had an adverse cytotoxic effect (Figure 1a, 1b, 1c). The higher Camelyn concentration significantly increased the hematopoietic stem cell amount. Compared with control, the number of HSCs ranged from 114%, when Camelyn concentration was 70 µg/mL, to 206% when concentration reached 200 µg/mL. Similar trends were observed in cell line BKH-21 assay (Figure 1a, 1b). The number of cells increased from 116% to 203% when the concentration increased from 70 µg/mL to 200 µg/mL. The stimulatory effect of Camelyn was even more notable for spleen cell growth. Starting from Camelyn concentration of 50 µg/mL the growth of spleen cells was increased, and at the end of the experiment, the average number of treated cells was more than 3 times higher compared with control (Figure 1c).

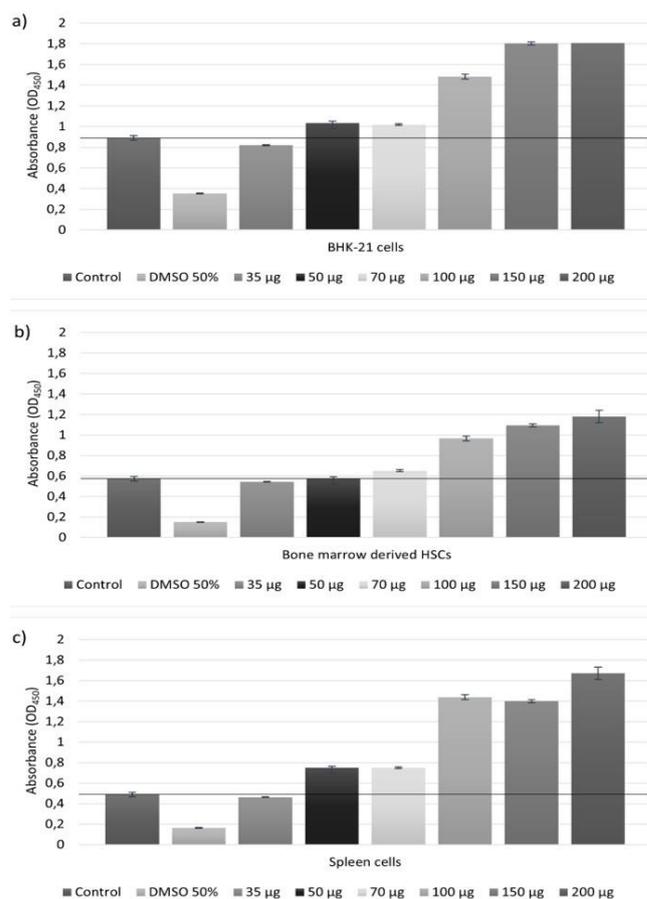


Figure 1. Influence of different concentrations of honey extract Camelyn on baby hamster kidney cell line 21 (BHK-21) (a), hematopoietic stem cells (HSCs) (b), and spleen cells (c) growth; black horizontal line – a value of control OD.

SARS-CoV-2 plaque reduction assay

To assess the antiviral activity of the honey product Camelyn against SARS-CoV-2, its half-maximal effective concentration (EC₅₀) was determined. Confluent Vero E6 cells were seeded in 6-well plates. Two-fold serial dilutions of Camelyn were pre-incubated with about 50–100 plaque-forming units (PFUs) of SARS-CoV-2/Quebec City/21697/2020 for 60 minutes and used to infect cells. After 3 days of incubation (without Camelyn) cells were fixed and stained with crystal violet. The number of PFUs was counted under an inverted microscope and plotted against the logarithm of antiviral concentrations to obtain the EC₅₀.

Concentrations of honey extract Camelyn from 9.08 µg/mL to 72.6 µg/mL, had an insignificant effect on virus plaque reduction when cells were infected with 100 PFU. The number of virus plaques decreased by 4.5–13.5 %. The higher concentration of 145.3 µg/mL

has reduced the number of virus plaques to 53.85% (Figure 2). The EC₅₀ was 192.4 µg/mL.

When virus inoculum was reduced to 25–30 PFU, Camelyn concentrations from 9.08 µg/mL to 36.3 µg/mL decreased virus plaque number 30–33% (Figure 2). Starting from 72.6 µg/mL concentration, the number of virus plaques was reduced significantly compared to control. This assay revealed, the concentration of honey extract Camelyn has shown inhibitory activity with EC₅₀ of 85.7 µg/mL. An additional test was done using the concentrate product, Camelyn tablets, to verify the similarity of the profile of inhibition. The number of virus plaque had a similar effect on Camelyn tablet inhibition. However, the dilutions of the concentrated Camelyn

tablet's solution have shown a stronger inhibitory effect with EC₅₀ of 116.27 ± 73.39 µg/mL when infected with 100 PFU. Our results showed that Camelyn extract seems to have an inhibitory activity at the beginning of the replicative cycle of SARS-CoV-2.

In comparison, the antiviral drugs favipiravir and remdesivir activities were found to have EC₅₀ of 15.71 µg/mL (100 µM) and 0.616 µg/mL (1.16µM), respectively. Wang et al. [34] identified favipiravir to have activity in vitro against SARS-CoV-2, albeit requiring a high concentration compared with remdesivir (EC₅₀ = 61.80 µM). Notably, remdesivir potently blocked virus infection at low-micromolar concentration (EC₅₀ = 0.77 µM) [35].

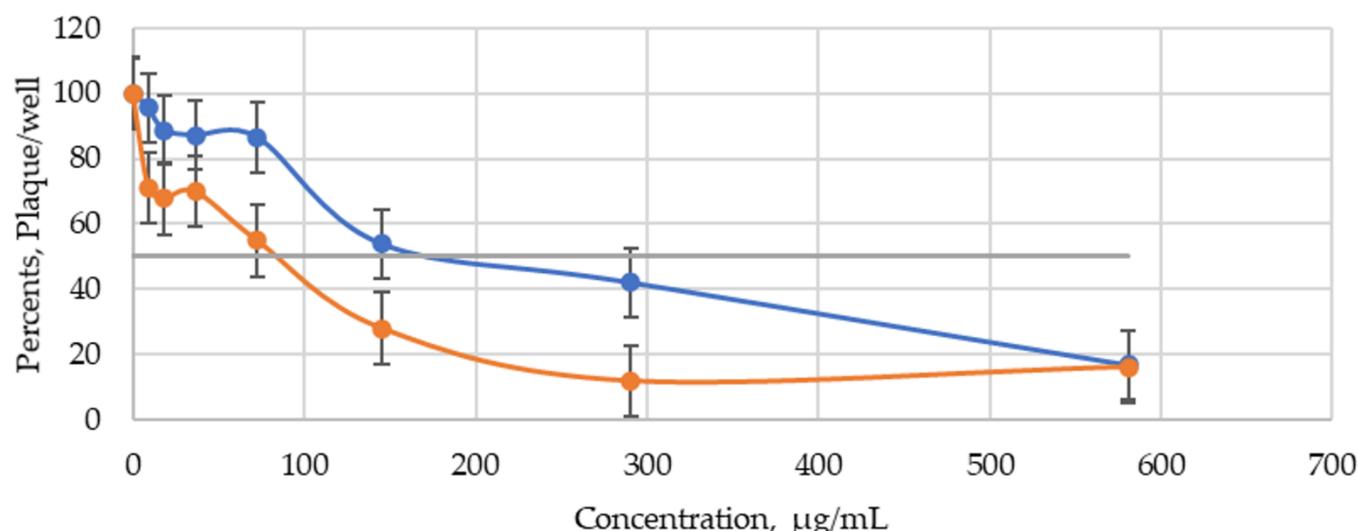


Figure 2. Effect of different Camelyn concentrations on SARS-CoV-2 plaque reduction in VERO E6 cells: blue line – average virus plaque per well was 100 (Spearman rank correlations between virus plaque per well and the concentration is $r_{s1} = -1.000$, and significant at the 0.01 level (2-tailed)); red line – average virus plaque per well was 30 (Spearman rank correlations between virus plaque per well and the concentration $r_{s2} = -0.952$, are significant at the 0.01 level (2-tailed)); grey line – half-maximal effective concentration (EC₅₀).

DISCUSSION

Honey is known for its medicinal benefits and receiving attention as natural medicine. The growing number of scientific and clinical reports suggest that honey could be used not only for home treatment but also for wound healing and tissue repair [36], [37], [38]. The beneficial effects of honey on wound healing mostly were attributed to antibacterial activity. High sugar content, which leads to high osmotic pressure, and low pH cause bacterial cell dehydration and cell wall disruption. Studies show that the antimicrobial activities of honey are related to increases in reactive oxygen species (ROS) hydrogen peroxide activity [39]. The antioxidant effect of honey is correlating with its anti-inflammatory and wound healing activity [40].

The honey samples may release hydrogen peroxide that is produced by the enzyme glucose oxidase and is responsible for the antimicrobial effect [39]. The harmful oxidizing effect of hydrogen peroxide is not observed on skin cells due to the honey polyphenolic component, which can antagonize the action of this ROS. Some researchers have noted the potential of honey to induce stem cell proliferation, stimulate hematopoietic stem cell migration, and also mediate the healing process by increasing tissue blood flow. On the other hand, the honey exhibited inhibitory effects on cellular growth by reducing the proliferation ability, inducing cell apoptosis, and inhibiting the cell cycle in a dose-dependent manner [41].

In many instances, honey should be used as it is. However, as needed, the active ingredients of honey, such as small peptides, amino acids, polyphenols, sugars, vitamins, can be extracted from honey. Camelyn is an original honey extract that was received from the special sort of honey by a patented extraction method and is a mixture of sugars, proteins, polyphenols, vitamins, minerals, and free amino acids. The data showed that Camelyn is not cytotoxic, and has a strong stimulatory effect on cell proliferation. Due to this property, Camelyn would be useful in wound healing as other honey products.

The data of Bakradze *et al.* clinical study of inflammatory diseases of parodontium revealed that Camelyn possesses immunostimulation, anti-inflammatory action, activates regeneration process has an analgesic effect. 56 patients with various forms of the disease (gingivitis, parodontitis) were under clinical observation. The study results have confirmed clinical appropriateness to use Camelyn for gingivitis, parodontitis, and periodontitis in combined treatment [42]. The research of Chumburidze *et al.* [43] showed an excellent regenerating and healing effect of Camelyn on damaged tissues. The features of Camelyn for treatment of different types of infections and tumors and pharmacokinetics of Camelyn in rat's plasma were described in this study. The minimum inhibitory concentration (MIC) of Camelyn was determined against some of the bacterial and fungal strains in the study of Maglakelidze *et al.* [44]. Camelyn was seen to possess powerful inhibitory action (0,012-0,150 µg/mL) against most test bacteria in vitro studies. Camelyn exhibited potent in vitro activities against fluconazole-resistant strains of *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, and *Candida krusei*, with MICs at which 90% of isolates were inhibited of 0.012 µg/ml, respectively.

As far as is known, no published scientific or clinical studies have observed the effects of honey on SARS-CoV-2. To date, four registered clinical trials estimate the efficacy of honey and its active compounds in patients with COVID-19 (NCT04323345, NCT04345549, NCT04347382, NCT04468139) [45]. Several studies that predicted the binding affinity of Manuka honey polyphenolic compounds to SARS-CoV-2 viral proteins have been conducted. Most of these studies investigated a possible antiviral effect of polyphenols based on the predicted binding to SARS-CoV-2 main protease (Mpro) [28] [46]. The study of Heba *et al.* [28] screened the biological activity of six

compounds present in honeybee and propolis against the COVID-19. The study revealed that four compounds have strong binding affinity and may inhibit the COVID-19 virus replication. Polyphenols are among these bioactive compounds and are currently under phase 3 of clinical investigation as a treatment of COVID-19 patients [46]. Results from Watanabe *et al.* study [20] showed that honey, and particularly Manuka honey, has potent inhibitory activity against the influenza virus, demonstrating a potential medicinal value. Manuka honey efficiently inhibited influenza virus replication (EC₅₀ = 3.6 ± 1.2 mg/mL). To compare with Camelyn inhibitory activity, this concentration is 10–20 times higher.

Based on scientific studies review, Hossain *et al.* [30] summarized that honey might be useful for COVID-19 patients by several major mechanisms; direct antiviral properties, regulating/boosting host immune signaling pathways, and curing and/or improving comorbid conditions. The use of drugs faces several problems such as bacterial multidrug resistance and possible side effects. This makes to think about new therapeutic alternatives such as honey and honey products.

CONCLUSIONS

The research has shown that honey extract Camelyn has no cytotoxic effect, is safe, and has demonstrated antiviral properties against SARS-CoV-2 as well. However, the molecular studies of the Camelyn effect on virus replication or immune system need to be done in detail in the future. There is no doubt that Camelyn doesn't work in the same way as other investigational drugs currently used to treat COVID 19, however, given the current emergency caused by the COVID-19 pandemic and the limited therapeutic options, Camelyn is presented as a promising and relevant therapeutic option that is safe, easy to administrate orally, and is readily available as a natural supplement.

AUTHOR CONTRIBUTIONS

Conceptualization, L.K.; methodology, M.B., L.K., and G.B.; formal analysis, I.G.; investigation, M.B and A.L.; writing - original draft preparation, L.K.; writing - review and editing, M.B, A.L., N.J.; visualization, I.G.; supervision, R.B.; funding acquisition, P.J. All authors

have read and agreed to the published version of the manuscript.

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CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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