Chemical Composition, in vitro Cytotoxic, and Antibacterial Activities of Moroccan Medicinal Plants

Euphorbia resinifera and Marrubium vulgare

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Abstract: The purpose of our study was to bring elements of knowledge on the anticancer and antibacterial effects of two plants, widely used in Moroccan traditional pharmacopeia. These plants are: Marrubium vulgare and Euphorbia resinifera. The aerial parts of each plant were extracted successively with Hexane, Dichloromethane, and finally with methanol. The MTT-based method was applied to evaluate the cytotoxicity of the cancer cells: animal cells BSR and Vero and human cell RD. We evidenced an anticancer activity of the extract of the Marrubium vulgare and the dichloromethane extract of Euphorbia resinifera against the studied cells. The antibacterial activity was evaluated for three species of Rhodococcus: Rhodococcus equi, and strains GK1, GK3, grown in a liquid medium, or this medium solidified with agar. In the last test, the method is based on substance diffusion from well throughout the solid medium. The obtained profiles showed that the growth of bacteria is strongly inhibited by the extracts of Marrubium vulgare. However, the extracts of Euphorbia resinifera had no significant effect on bacterial growth. The chemical analysis of the raw extracts of Marrubium vulgare and Euphorbia resinifera by GC-MS analysis showed the presence of several major chemical compounds, mainly: octadecane, 2,6,10,15-tetramethylheptadecane, 2,6,10-trimethyltetradecane, linoleic acid, and deisopropylatrazine. Our observations an encouraging for deepening the studies of the extracts, in order to target better the active molecules, isolate them and to determine their mechanisms of action. The suggested studies would result in the much better valorization of these two medicinal plants.

Keywords: Euphorbia resinifera; Marrubium vulgare; bioactive compounds; antibacterial activity; anticancer activity.

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1. Introduction

Cancer is a complex disease that constitutes the second cause of death worldwide after cardiovascular diseases. It continues to present the largest cause of mortality and morbidity in the world. The cancer is caused by deregulation in the genetic and epigenetic programs that affect cell division [1, 2]. The treatment of cancer is based essentially on chemotherapy using synthetic drugs. This treatment presents many seed effects such minor ones as vomiting, diarrhea, or major ones such as neurological, cardiac, pulmonary, and renal toxicity. On the
other hand, infectious diseases continue to cause major health problems for developing countries. Indeed, bacteria have developed sophisticated mechanisms that allowed them to resist antibiotics action [3, 4]. The resistance of bacteria to antibiotics has today constituted a veritable question in the health system toward infection diseases [5, 6].

*Rhodococcus equi* infection is commonly encountered in HIV-infected patients; recipients of organ transplants; and in those with lymphoma, chronic renal failure, alcoholism, lung cancer, leukemia, diabetes mellitus, and other states of immunodeficiency. The most common manifestations of *Rhodococcus equi* infections are multiple abscesses in the lungs, and extra-pulmonary infections may include wound infections, subcutaneous abscesses, brain abscesses, meningitis, pericarditis, osteomyelitis, cervical adenopathy, endophthalmitis, lymphangitis, or mastoiditis [7]. Recently, the search on natural and synthetic molecules with antibacterial and anticancer activities has received much interest over the past few years [8, 9, 10]. Among the potential sources of new agents, medicinal plants have long been investigated. The secondary metabolites of medicinal plants contain several chemical bioactive compounds that have shown numerous biological activities such as antibacterial, anticancer, antileishmanial, antithiatic, and antifungal effects [11, 12]. Morocco is one of the developing countries which have an enormous diversity of plants, and yet the majority stays scientifically neglected and undiscovered [13]. In Morocco, the use of traditional medicine is a widespread practice. The ethnobotanical and ethnopharmacological surveys conducted in different areas allowed the compilation of numerous medicinal plants used to treat several complications [14, 15]. Various of these medicinal plants have been investigated for their *in vitro* and *in vivo* pharmacological effects such as antimicrobial, antitumor, antiparasitic, antifungal, and antileishmanial properties [16, 17, 11, 12]. These plants contain several chemical compounds, such as terpenoids, flavonoids, tannins, and alkaloids.

In the context of the valorization of Moroccan medicinal plants, two medicinal plants have been studied: *Euphorbia resinifera* and *Marrubium vulgare*. The choice of these plants was based on their use in Moroccan traditional medicine. The biological properties were based on the fact that to the best of our knowledge, there is no reported study regarding the anticancer and antibacterial activity of *Euphorbia resinifera* and *Marrubium vulgare* extracts. Antibacterial assays have been performed on *Rhodococcus* species, which present a similar morphology and growth characteristics with *Mycobacterium tuberculosis* (TB). An attempt has been made to discover new anti-TB agents. The extracts were also tested against RD: Embryonal Rhabdomyosarcoma cancerous cell lines, BSR: Kidney adenocarcinoma of hamster and Vero: Monkey kidney cancerous cell lines.

2. Materials and Methods

2.1. Plant material and preparation of extracts.

Extracts were prepared from *Euphorbia resinifera* and *Marrubium*. Table 1 summarizes the taxonomic classification of the plants, vernacular name, different parts of plants collected, traditional use, and pharmacological activities. Each plant was dried in the shade under the normal environmental condition and homogenized to coarse powder before use, the powdered materials were then weighed (300 g) and charged into soxhlet apparatus, and extraction was carried out with following solvents successively: hexane, dichloromethane, and methanol. The filtrate obtained was concentrated in a rotary evaporator to obtain the crude extract. The crude extracts were kept at 4 °C until further uses.
Table 1. Ethnobotanical data and some reported pharmacological activities of *Euphorbia resinifera* and *Marrubium vulgare* plants.

| Plant species       | Trivial name | Part plant collected | Traditional use      | Pharmacological activities                                      |
|---------------------|--------------|----------------------|----------------------|-----------------------------------------------------------------|
| *Euphorbia resinifera* | Tikîut       | Aerial parts         | Analgesic            | Antioxidant and antibacterial activity [18]                     |
| *Marrubium vulgare*  | Merryût       | Aerial parts         | Liver disorders      | Antioxidant properties [19]                                      |
|                     |              |                      |                      | Hepatoprotective activity [20]                                 |

2.2. Analytical techniques.

Gas chromatography-mass spectrometry (GC/MS) analysis of the different extracts was performed on a TRACE GC ULTRA Polaris Q (Thermo Electron Corporation) equipped with non-polar VB-5 (5% phenyl, 95% methylpolysiloxane) capillary column (30 mm x 0.25 mm x 0.25 μM film thickness), directly coupled to a mass spectrometer (Polaris Q). The electron ionization energy was set at 70 eV [21]. The oven temperature was programmed from 60 to 280 °C at 4 °C/min, then for 280 to 300 °C at 20 °C/min. The components of the extracts were identified by comparison of their mass spectra with those in the Willey NIST 7th Edition Library of mass spectra data. The composition of the extracted sample was calculated from GC-MS peak areas and given by percentages.

2.3. Cell Viability Assays.

The *in vitro* cytotoxic effect of the various extracts was evaluated on RD: Embryonal Rhabdomyosarcoma cancerous cell lines (ATCC N° CCL-136), BSR: Kidney adenocarcinoma of hamster (ATCC N° CCL-10), Vero: Monkey kidney cancerous cell lines (ATCC N° CCL-81). Cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) (GIBCO) supplemented with 10% heat-inactivated fetal calf serum and 1% Penicillin-Streptomycin mixture. Cultures were maintained at 37 °C in 5% CO₂ and 100% relative humidity atmosphere. The effect of the isolated extracts on cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which measures the metabolic activity of mitochondria [22]. MTT assays are presently the preferred methods of cytotoxicity assessment in our laboratory [23, 24, 25]. The tests were conducted on 96-well microplate. Before treatment with extracts, 100 μL medium DMEM (GIBCO) containing 3 - 4x10⁶ cells/mL were placed in each well containing DMEM (GIBCO) and cultured at 37 °C in 5% CO₂/humidified air for 24 h. After 24 h incubation and attachment, cells were treated with crude extracts. Exactly from the stock solution (80 mg/mL), each extracted sample was applied in a series of 6 dilutions (final concentrations ranging from 12.5 to 400 μg/mL) in dimethylsulfoxide (DMSO 1%). Test solution (100 μL) was added in decreasing concentrations in duplicate. The microplate was then incubated for 48 h at 37°C in the air condition of 5% CO₂. After, a 20 μL MTT solution (5 mg/mL) (SIGMA) was added to the wells containing cells. The cells were incubated for 4 - 5 h at 37 °C in 5% CO₂. Tetrazolium salts are cleaved to formazan dye by the cellular enzyme (only in the viable cells). A solubilization solution (Isopropanol/hydrochloric acid) is added to dissolve the insoluble purple formazan product into a coloration solution. The absorbance was measured at 545 nm, using a microplate reader (Statfax 2100). Tests were carried out in duplicate. Cells were incubated with different concentrations. Data are expressed as means ± SD of three independent experiments.
2.4. Antibacterial Activity.

2.4.1. Microorganisms and inoculum preparation.

The bacteria studied were three species of Gram\(^+\): *Rhodococcus equi* isolated from Poulain (France), *Rhodococcus spGK1* isolated from soil polluted with petrol (France), and *Rhodococcus sp GK3* obtained from the soil. Each isolate was inoculated into sterile medium mixture: \((\text{NH}_4)_2\text{SO}_4, \text{Na}_2\text{HP}_4, \text{KH}_2\text{PO}_4, \text{Thiamine}, (\text{MgSO}_4, 7\text{H}_2\text{O}), (\text{CaCl}_2, 2\text{H}_2\text{O}), (\text{FeSO}_4, 7\text{H}_2\text{O}), (\text{MnSO}_4, 3\text{H}_2\text{O}), (\text{ZnSO}_4, 3 \text{H}_2\text{O})\) and agar for solid medium bacteria growth [26].

2.4.2. Agar well diffusion method.

Agar well diffusion method is widely used in many microbiology laboratories to evaluate the antimicrobial activity of plants or microbial extracts [27, 28]. In this well-known procedure, the test samples were first dissolved in DMSO (1%) who thus did not affect the microbial growth. Briefly, the test was performed in sterile Petri plates containing medium agar. 30 mL of sterilized medium was poured into sterile Petri plates. After solidification, 100 \(\mu\)L of fresh cultures of *Rhodococcus sp* (one microorganism per Petri dish) were swabbed on the respective plates. Then, 100 \(\mu\)L of extracts were placed in wells previously punched over the agar plates using sterile Pasteur pipette, at various concentrations (6.25, 12.5, 25 and 50 \(\mu\)g/mL). All Petri plates were then incubated at 30 °C for 48 h. The diameters of inhibition zones were measured in millimeters. In addition, the antimicrobial activities of two selected plant extracts on *Rhodococcus sp* were compared with the commercially available antibiotics. The antibiotic discs such as Chloramphenicol and Ampicillin were placed on the surface of the plates. DMSO 1% was used as a negative control. The plates were incubated at 30 °C for 48 h, and after incubation, the diameter of the inhibition zones was measured in mm and recorded [29]. Tests were carried out in duplicate. Bacterial Cells were incubated with plant extracts. Data are expressed as means of inhibition zone in mm ± SD of three independent experiments.

2.4.3. Time-kill dynamic curves.

*Rhodococcus sp* were grown overnight at 30 °C in 25 mL medium broth. Each extracts 50 \(\mu\)g/mL were prepared in DMSO (1%) and placed in viable bacteria and were shaken and incubated at 30 °C. The density of each culture (designed as bacterial growth) was measured by spectrophotometer at a wavelength of 600 nm after each time point, indicating the bacterial biomass present in the suspension. The suspension of bacteria strains with no extracts was used as control. Tests were carried out in duplicate. Data are expressed as means of bacterial biomass (g/L) ± SD of three independent experiments.

3. Results and Discussion

3.1. Phytochemical Analysis

Freshly prepared extracts were subjected to a preliminary phytochemical screening for various constituents by GC-MS analysis. This study has exhibited the presence of phytochemicals considered as active medicinal chemical constituents (Table 2). The result of the phytochemical analysis shows that important medicinal phytochemicals such as flavonoids, terpenoids, alkaloids, and phlorotannins were present in the samples of two selected plant extracts.
effect on the cell growth BSR. For the Vero cancerous cell lines, we noted that the cancerous inhibition effect with the inhibition values of IC\textsubscript{50} (Figure 2 and Table 3). In the case of inhibition (IC\textsubscript{50}) of the MM and DM extracts on the RD cell growth was at 79.2 µg/mL. In addition, ME extract presents less important inhibiting effects on the cell growth RD, in particular hexane extract of E. resinifera with IC\textsubscript{50} of 50 µg/mL.

### Table 2: Percentage (%) of the main components of the Euphorbia resinifera and Marrubium vulgare plant extracts

| EREH | Main components | % |
|------|----------------|---|
|      | Heptacosane     | 26.24 |
|      | 1,3,4-Trimethyl-3-cyclohexanyl-1-carboxaldehyde | 14.04 |
|      | Ethyl linoleate | 13.63 |
| ERDE | 1,3,4-Trimethyl-3-cyclohexanyl-1-carboxaldehyde | 70.27 |
|      | Ledane          | 13.11 |

|      | cis-Z-α-Bisabolene epoxide | 7.08 |
|      | 1,4-bis-(2′-cyclopropyl-2′-methylcyclopropyl)-but-2-en-1-one | 5.89 |
| ERME | Methyl arachidonate | 9.92 |
|      | cis-Z-α-Bisabolene epoxide | 5.70 |
|      | Methyl ester 9,11-(1,1′-bicyclopropyl)-octanoic acid | 4.71 |
| MVHE | 8-Heptyl pentadecane | 26.22 |
|      | 2,6,10-Trimethyl-tetradecane | 7.14 |
|      | Linoleic acid | 4.82 |
|      | Octadecane | 4.59 |

| MVDE | 2-Methylene-5α,6α,7α,8α,9α,10α,11α,12α,13α,14α,15α,16α,17α,18α-octadecanediol | 6.20 |
|      | Methyl ester 9,11-(1,1′-bicyclopropyl)-octanoic acid | 6.00 |
|      | 9-octadecanol | 5.22 |
| MVME | 1,3,5-Triazine-2,4-diamine-6-chloro-4-n-ethyl | 4.81 |
|      | Methyl arachidonate | 7.92 |
|      | Methyl ester 9,11-(1,1′-bicyclopropyl)-octanoicacid | 7.36 |
|      | 3-Cyclopropyl carbonyl oxy tridecane | 5.02 |

### 3.2. Cytotoxicity Effects.

The investigation of the cytotoxic potential of six extracts from Euphorbia resinifera and Marrubium vulgare Moroccan plants that are used in traditional medicine for the treatment of various diseases were conducted on three tumor cell lines RD, BSR, and Vero. Cancerous cell lines were exposed to increasing concentrations ranging from 12.5 to 400 µg/mL. Assay by the MTT assay, as described above, indicates that the extracts revealed different cytotoxic activities towards the three cancer cell lines investigated. In general, a dose-dependent decrease in the survival of the three cancerous cell lines.

As shown in Figure 1 and Table 3, Hexane extract (HM) and Dichloromethane extract of M. vulgare (DM) are present a good inhibiting effect on the RD cell growth with a total inhibiting effect at 400 µg/mL. However, the cytotoxic effects of M. vulgare Mehtanolic (MM) extract are not significantly varied with increasing concentrations. Whereas, the extracts of E. resinifera presents less important inhibiting effects on the cell growth RD, in the particular hexane extract of E. resinifera with IC\textsubscript{50} of 50 µg/mL.

### Table 3: Inhibition concentration (IC\textsubscript{50} in µg/mL) values from M. vulgar and E. resinifera on RD, BSR, and Vero as determined by the MTT assay.

| Cells | M. vulgar | E. resinifera |
|-------|-----------|---------------|
|       | HM        | DM | MM | HE | DE | ME |
| RD    | 23.67±2.20 | -  | -  | 50.7±4.89 | -  | 67.57±4.15 |
| BSR   | 128.8±7.73 | 26.42±2.7 | 97.8±5 | -  | 77.2±4.21 | 200±8.32 |
| Vero  | 30.12±3 | 50±3.68 | 145±8.2 | 266.43±10.20 | 79.2±4.63 | - |

On the other hand, MM and DM extract induced a complete inhibitory effect on BSR cancerous cell lines was at a concentration of 400 µg/mL. The concentrations providing 50% inhibition (IC\textsubscript{50}) values of the MM and DM extracts 97.8 µg/mL and 26.42 µg/mL, respectively (Figure 2 and Table 3). In the case of E. resinifera extracts, DE extract present a significant inhibiting effect with the inhibition values of IC\textsubscript{50} 77.2 µg/mL. In addition, ME extract present a moderate cytotoxicity effect (IC\textsubscript{50} 200 µg/mL). In contrast, HE extracts present less important effect on the cell growth BSR. For the Vero cancerous cell lines, we noted that the cancerous
cell line was more resistant to MM (IC₅₀ 145 µg/mL) and more sensitive to HM and DM with IC₅₀ 30.12 µg/mL and 50 µg/mL respectively (Figure 3 and Table 3). In the case of E. resinifera extracts, HE and DE present low inhibit effects on the Vero cell lines with IC₅₀ of 266.43 µg/mL and 79.2 µg/mL, respectively.

Figure 1. Cytotoxic activity of extracts from 2 medicinal plants against RD cell lines. HM: Hexanic extract from Marrubium vulgare; DM: Dichloromethanic extract from Marrubium vulgare; MM: Methanolic extract from Marrubium vulgare; HE: Hexanic extract from Euphorbia resinifera; DE: Dichloromethanic extract from Euphorbia resinifera; ME: Methanolic extract from Euphorbia resinifera.

3.3. Antibacterial Activity.

The results of agar well diffusion and broth dilution methods showed that each extract showed different degrees of growth inhibition. The screening for antibacterial activity indicates that at a concentration of 50 µg/mL, MM and DM extract was found to possess a relatively high antibacterial activity against R. equi with a diameter of inhibition about 32 mm and 25 mm respectively (Figure 4) and high of growth inhibition (Figure 5). In addition, both DE and HE present a moderate antibacterial activity (Diameter of inhibition about 18 mm). Furthermore, each extract from M. vulgare was found to be more active at 50 µg/mL concentration against Rhodococcus sp GK1 (inhibition zone ranged from 17 to 25 mm) (Figure 6 and Figure 7). In the case of E. resinifera, DE presented a moderate antibacterial activity (Diameter of inhibition about 15 mm).

Figure 2. Cytotoxic activity of extracts from 2 medicinal plants against BSR cell lines. HM: Hexanic extract from Marrubium vulgare; DM: Dichloromethanic extract from Marrubium vulgare; MM: Methanolic extract from Marrubium vulgare; HE: Hexanic extract from Euphorbia resinifera; DE: Dichloromethanic extract from Euphorbia resinifera; ME: Methanolic extract from Euphorbia resinifera.
Figure 3. Cytotoxic activity of extracts from 2 medicinal plants against Vero cell lines. HM: Hexanic extract from *M. vulgare*; DM: Dichloromethanic extract from *M. vulgare*; MM: Methanolic extract from *M. vulgare*; HE: Hexanic extract from *E. resinifera*; DE: Dichloromethanic extract from *E. resinifera*; ME: Methanolic extract from *E. resinifera*.

Figure 4. Antibacterial activity of extracts from two Moroccan medicinal plants against *Rhodococcus equi* as determined by diffusion technique on solid media.

Figure 5. Bacterial growth registered after 40 h of exposition of each extract on *Rhodococcus equi*.
Figure 6. Antibacterial activity of extracts from two Moroccan medicinal plants against *Rhodococcus* sp *GK1* as determined by diffusion technique on solid media.

Figure 7. Bacterial growth registered after 30 h of exposition of each extract on *Rhodococcus* sp *GK1*.

As shown in Figure 8 and Figure 9, the Methanolic and Dichloromethanolic extracts of *M. vulgare* present important inhibiting effects against *Rhodococcus* sp *GK3* (inhibition zone about 35 mm). In addition, *E. resinifera* extracts exhibited low antibacterial activity against *Rhodococcus* sp *GK1* (Diameter of inhibition about 15 mm at concentration 50 µg/mL). Chloramphenicol used as reference antibiotics showed important and similar antibacterial activity against all the *Rhodococcus* population tested with an inhibition zone of 30 mm. So, there was no preferential activity against bacteria strains studied. While the three strains were resistant to ampicillin. In addition, no zone inhibition was observed with DMSO 1% [21].
Figure 8. Antibacterial activity of extracts from two Moroccan medicinal plants against *Rhodococcus sp* GK3 as determined by diffusion technique on solid media.

Figure 9. Bacterial growth (measured as bacterial biomass (g/l)) registered after 30 h of exposition of each extract on *Rhodococcus sp* GK3.

3.4. Discussion.

Medicinal plants have been used to fight against several diseases, including infectious diseases and cancer. These medicinal plants showed several *in vitro* pharmacological properties such as antibacterial [30], antioxidant [31], anti-lithologic [12], antileishmanial [11] and antitumor activities [32, 33]. Phytochemical compounds of medicinal plants contain a wide variety of bioactive molecules such as essential oils, polyphenols, and flavonoids [9, 8].

In this study, the antibacterial and anticancer activities of *Euphorbia resinifera* and *Marrubium vulgare* extracts have been evaluated. Extracts are rich in bioactive compounds that could be involved in biological activities. However, the chemical composition varies between plant species and solvents extraction. Indeed, the synthesis and secretion of secondary...
metabolites depend on genetic factors of each species, geographical location, and methods of extraction [34]. Moreover, the used solvents for extraction have a different polarity, which explains the variation in bioactive chemical compounds between extracts [35, 36]. Bacteria have established resistance against antibiotics. This situation has caused the emergence of infectious diseases [37, 38].

In our study, the antibacterial effect was evaluated against gram$^+$ species: *Rhodococcus equi*, *Rhodococcus sp* GK1, and *Rhodococcus sp* GK3. Extracts have shown the remarkable antibacterial effect measured by the diameter of inhibition. Moreover, extracts have tested on the kinetic growth of *Rhodococcus* and showed inhibition of growth at different bacterial time growth. The action of medicinal plant extracts against bacteria has not been well understood. However, some works suggested that secondary metabolites and their derivatives compounds have several mechanisms of action against bacterial [39, 40, 41]. They can target cell membranes (especially against gram$^+$ bacteria) disturb the respiratory chain of electrons and caused morphological changes in bacteria cells [42, 43]. Moreover, recent studies have shown that bioactive compounds extracted from medicinal plants could deregulate quorum sensing signaling pathways, thus inducing decreasing in cell viability [4].

On the other hand, our extracts are tested against cancer cell lines. We have shown an interesting antiproliferative effect measured by MTT assay. Dichloromethane and Methanol extracts of *E. resinifera* have demonstrated remarkable anticancer activity against Vero cell lines. Moreover, Methanol extract of *M. vulgare* was active against BSR cell lines. The anticancer effects of *E. resinifera* and *M. vulgare* have certainly attributed to the main bioactive compounds present in extracts. However, the minor compounds also could participate in these activities by their synergetic effects with the main compounds and/or by their additive effects. The action of natural products on cancer cell lines has been studied by several researches [44, 21]. They may affect the cell division, the apoptosis, the telomerase activity, and angiogenesis [45]. The anticancer activity of our extracts could be attributed to the bioactive chemical compounds such as heptacosane, ethyl linoleate, octadecane and methyl arachidonate.

Other studies have reported the antibacterial effects of *E. resinifera* [46, 47]. These effects seem to be related essentially to the presence of bioactive compounds such as the phenols [46]. Moreover, *M. vulgare* extracts have also shown important antibacterial and antiproliferative properties [48, 49]. However, to the best of our knowledge, any study has reported the anticancer properties of *E. resinifera* extracts.

4. Conclusions

This study was investigated by the chemical compounds of *E. resinifera* and *M. vulgare* extracts and evaluated their antibacterial and anticancer properties. Dichloromethane and Methanol extracts of *E. resinifera* showed interested anticancer effects against Vero cell lines, and Hexane extract of *M. vulgare* revealed remarkable activity against BSR cell lines. Moreover, our extracts have shown remarkable antibacterial effects against *Rhodococcus* species. The findings of our study revealed that *E. resinifera* and *M. vulgare* extracts are a potential source of antibacterial and anticancer agents. However, further studies regarding the mechanisms of action involved in these effects are needed.

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Conflicts of Interest

The authors declare no conflict of interest.

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