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Original scientific paper

PHYSICOCHEMICAL CHARACTERISATION OF *THYMUS SERPYLLUM* EXTRACTS PREPARED USING NATURAL DEEP EUTECTIC SOLVENTS

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Abstract

Thymus serpyllum L. (wild thyme) extracts were prepared using 1 g of plant material and three natural deep eutectic solvents (30 mL, malic acid+maltose, lactic acid+menthol, or citric acid+glycerol with 50% of water) in maceration (90 min). The extracts were characterized in terms of total polyphenol content (TPC), total protein content, extraction yield, zeta potential, conductivity, pH, density, surface tension, and viscosity. TPC was the highest in citric acid+glycerol extract (35.06±1.13 mg gallic acid equivalents (GAE)/g of plant material), whereas malic acid+maltose and lactic acid+menthol extracts possessed similar TPC (29.62±1.11 and 28.94±1.15 mg GAE/g, respectively). Total protein content amounted to 5.55±0.20 mg/g in citric acid+glycerol extract, while significantly lower values were determined in malic acid+maltose and lactic acid+menthol extracts (1.22±0.30 and 1.73±0.18 mg/g, respectively). The extraction yield was the highest for citric acid+glycerol extract, 1.57±0.11%, followed by malic acid+maltose and lactic acid+menthol extracts, 1.28±0.10 and 1.27±0.13%, respectively. Zeta potential (absolute value, as a predictor of potential application in water treatment) was low in all extracts (0.86±0.06 mV for citric acid+glycerol, -0.35±0.09 mV for lactic acid+menthol, and -0.17±0.05 mV for malic acid+maltose extract). The conductivity (as a predictor of antioxidant capacity) was in the range of 2.35±0.21 and 2.71±0.29 mS/cm (citric acid+glycerol and malic acid+maltose extracts) to 5.70±0.23 mS/cm (lactic acid+menthol extract). pH ranged from 1.44 in citric acid+glycerol extract to 1.78 and 1.85 in malic acid+maltose and lactic acid+menthol extracts. Density varied from 1.067±0.003 g/mL for lactic acid+menthol extract to 1.169±0.002 and 1.195±0.005 g/mL for citric acid+glycerol and malic acid+maltose extracts. Surface tension was the highest in citric acid+glycerol extract (38.0±0.4 mN/m), followed by malic acid+maltose and lactic acid+menthol extracts (31.5±0.4 and 25.9±0.1 mN/m, respectively). The viscosity of lactic acid+menthol extract was significantly lower (1.83±0.30 mPa•s) in comparison to malic acid+maltose and citric acid+glycerol extracts (6.64±0.15 and 7.84±0.10 mPa•s, respectively). The highest TPC, total proteins, and extraction yield were measured in citric acid+glycerol extract, while lactic acid+menthol extract possessed the highest conductivity and the lowest density, surface tension, and viscosity. Therefore, the constitution of natural deep eutectic solvent should be optimized depending on the future application of T. serpyllum extract.

Key words: *Natural deep eutectic solvent, polyphenols, Thymus serpyllum, physical properties.*

Introduction

Thymus serpyllum L. (wild thyme, Lamiaceae) is a perennial subshrub plant, which has known applications in traditional medicine as antiseptic, anthelmintic, diuretic, tonic, carminative, antispasmodic, expectorant, emmenagogue, sedative, tonic, anticholesterolemic, immunostimulant, and analgesic agent. The dried plant has widespread use in ethnomedicine for the treatment of gastrointestinal and respiratory disorders, rheumatism, menstrual disorders, wounds, and eczema (Jarić et al., 2015; Jovanović et al., 2017a).

The extraction of biologically active components, such as polyphenols, carotenoids, alkaloids, or terpenoids from various plant sources represents an initial step to remove ballast substances and isolate target compounds (Hikmawanti et al., 2021). Maceration, as a traditional extraction method, is a simple and low-cost procedure that also possesses disadvantages, such as lower extraction and polyphenol yields, prolonged extraction time, consumption of the large content of plant material and extraction solvents (Hikmawanti et al., 2021; Jovanović et al., 2017b). Therefore, natural deep eutectic solvents (NADESs) can be applied as a tool for improving polyphenol release in maceration. The advantages of NADESs, which can extract both hydrophilic and lipophilic molecules, include environmentally friendly materials and procedures, easy preparation, fewer hazards, lower energy, and biodegradability (Hikmawanti et al., 2021; Promila and Singh, 2018).

In the present study, *T. serpyllum* extracts were obtained using three types of NADESs (malic acid+maltose, lactic acid+menthol, or citric acid+glycerol, all with 50% of water), and maceration. The obtained extracts were characterized *via* analysis of total polyphenol content (TPC), total protein content, extraction yield, zeta potential, conductivity, density, surface tension, and viscosity.

Materials and Methods

Plant material and reagents

The dried *T. serpyllum* herb was from the Institute for Medicinal Plants Research "Dr Josif Pančić", Belgrade, Serbia. The following reagents were used: malic, lactic, and citric acids (Fisher Bioreagents, Belgium), maltose and menthol (Acros Organics, China), Folin-Ciocalteu reagent, Coomassie® Brilliant blue G 250, phosphoric acid, ethanol, and gallic acid (Merck, Germany), sodium carbonate and glycerol (Fisher Scientific, UK), and ultrapure water (Simplicity UV® water purification system, Merck Millipore, Merck KGaA, Germany).

Preparation of NADESs

The mixture of compounds and water was prepared at 60°C with constant mixing for approximately 20 min until a stable transparent liquid was formed. In order to evaporate water from the mixture and to create NADESs, the samples were placed in a rotary evaporator, Heizbad Hei-VAP (Heidolph, Germany) at 60°C, pressure of 50 mbar and rotation speed of 200 rpm for 2 h. After that, all NADES mixtures were diluted using ultrapure water (1:1).

Extraction procedure

The extracts were prepared employing three different NADESs (malic acid+maltose, lactic acid+menthol, or citric acid+glycerol, all with 50% of water) in maceration (incubator shaker at 200 rpm, KS 4000i control, IKA, Germany). The solid-to-solvent ratio was 1:30 g/mL and the extraction time was 90 min. The samples were filtered through filter paper after the extraction and the extracts were stored at 4°C until further analyses.

Determination of total polyphenol content

The total polyphenol content (TPC) of *T. serpyllum* extracts with NADESs was determined spectrophotometrically at 765 nm using the modified Folin-Ciocalteu method (Galván d'Alessandro et al., 2012). The results are expressed as milligrams of gallic acid equivalents per gram of plant material (mg GAE/g).

Determination of total protein content

The total protein content was measured using the assay described by Bradford (Bradford, 1976). The absorbance was measured at 595 nm and the results were expressed as mg of proteins per g of plant material (mg/g).

All spectrophotometric measurements were performed in a UV-1800 spectrophotometer (Shimadzu, Japan).

Determination of extraction yield

Extraction yield was calculated as:

$$EY\ (\%) = 100 - \frac{(a-b)*100}{m} \qquad \text{(Eq. 1)},$$

where *a* represents the weight (g) of the vessel containing the sample before drying, *b* represents the weight (g) of the vessel containing the sample after drying at 105°C to constant mass, and *m* represents the weight (g) of the sample.

Determination of zeta potential, conductivity, and pH values

The measurements of zeta potential and conductivity of *T. serpyllum* extracts with NADESs were performed using photon correlation spectroscopy in Zetasizer Nano Series, Nano ZS (Malvern Instruments Ltd., UK). Each extract was measured three times at room temperature. pH value of *R. canina* extracts was determined using pH meter HI 2211 (Hanna Instruments, USA). Each sample was measured three times at room temperature.

Measurement of density, surface tension, and viscosity

The density and surface tension of *T. serpyllum* extracts with NADESs were determined using silicon crystal as the immersion body and Wilhelmy plate, respectively, in Force Tensiometer K20 (Kruss, Germany). Each extract (20 mL) was examined three times at room temperature.

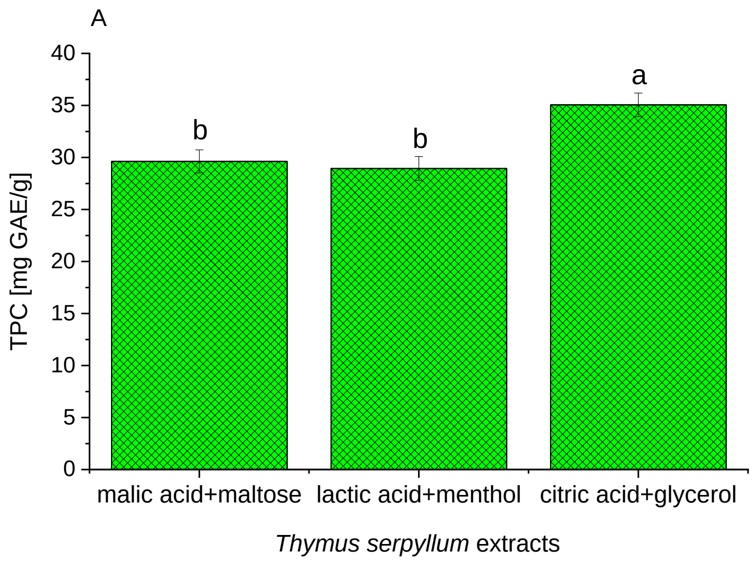
The viscosity was determined using Rotavisc lo-vi device equipment with VOL-C-RTD chamber, VOLS-1 adapter, and spindle (IKA, Germany). Each extract (6.7 mL) was examined three times at room temperature.

Statistical analysis

The statistical analysis was done by using analysis of variance (one-way ANOVA) and Duncan's *post hoc* test in STATISTICA 7.0. The differences were considered statistically significant at $p<0.05$.

Results and Discussion

The impact of three types of NADESs (malic acid+maltose, lactic acid+menthol, and citric acid+glycerol, all with 50% of water) on TPC, total proteins, extraction yield, zeta potential, conductivity, pH, density, surface tension, and viscosity of *T. serpyllum* extracts was examined. The results are presented in Figure 1 (TPC and total proteins), Figure 2 (extraction yield, zeta potential, and conductivity), and Figure 3 (density, surface tension, and viscosity).



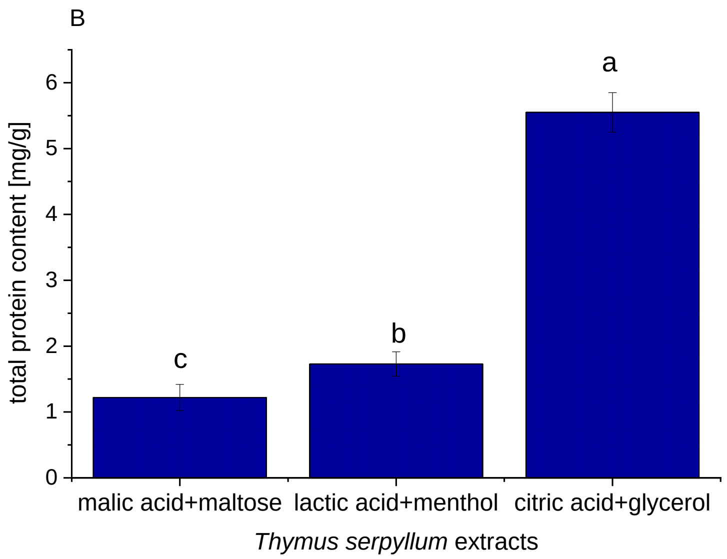
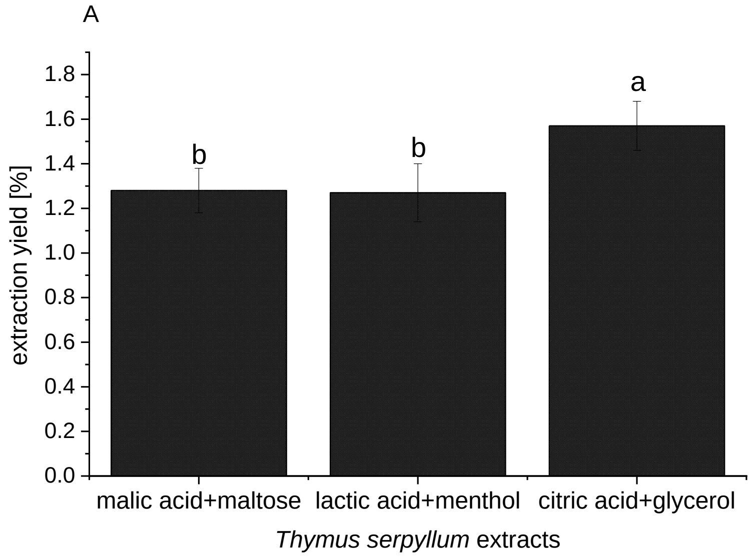
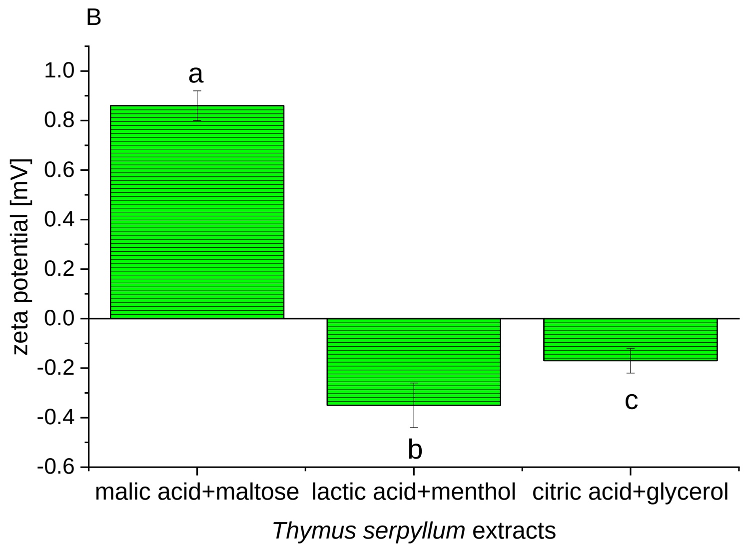


Figure 1. (A) Total polyphenol content (TPC) and (B) total protein content of Thymus serpyllum extracts prepared using natural deep eutectic solvents in maceration; gallic acid equivalent, GAE; the letters above bars showed statistically significant differences ($p<0.05$; $n=3$; analysis of variance, Duncan's post-hoc test).

As can be seen from Figure 1A, TPC was the highest in citric acid+glycerol extract (35.06±1.13 mg GAE/g), whereas malic acid+maltose and lactic acid+menthol extracts possessed similar TPC (29.62±1.11 and 28.94±1.15 mg GAE/g, respectively). NADESs can efficiently extract polyphenol compounds in a higher concentration compared to conventional extraction mediums (Hikmawanti et al., 2021; Jovanović et al., 2022). The difference between polyphenol concentration in the extracts obtained using various NADESs can be explained by the fact that the composition of the extraction medium should be optimized for every plant source (Jovanović et al., 2017). According to the literature data, NADESs have direct and indirect mechanisms of action. Namely, direct action implies the interaction with target compounds *via* hydrogen bonding, whereas indirect action implies the degradation of plant cells and higher recovery of polyphenols (Hikmawanti et al., 2021). Total protein content amounted to 5.55±0.20 mg/g in citric acid+glycerol extract, while significantly lower values were determined in malic acid+maltose and lactic acid+menthol extracts (1.22±0.30 and 1.73±0.18 mg/g, respectively, Figure 1B). Since the application of NADESs leads to a significant destruction of plant cells, it also causes the increase of large amounts of ballast substances, including proteins, sugars, and lipids into the extraction solvent, which results in a higher dry weight content at the end of the extraction process. The mentioned phenomenon is approved after the determination of the extraction yield where citric acid+glycerol extract possessed the highest value (1.57±0.11%), followed by malic acid+maltose and lactic acid+menthol extracts (1.28±0.10 and 1.27±0.13%, respectively, Figure 2A).





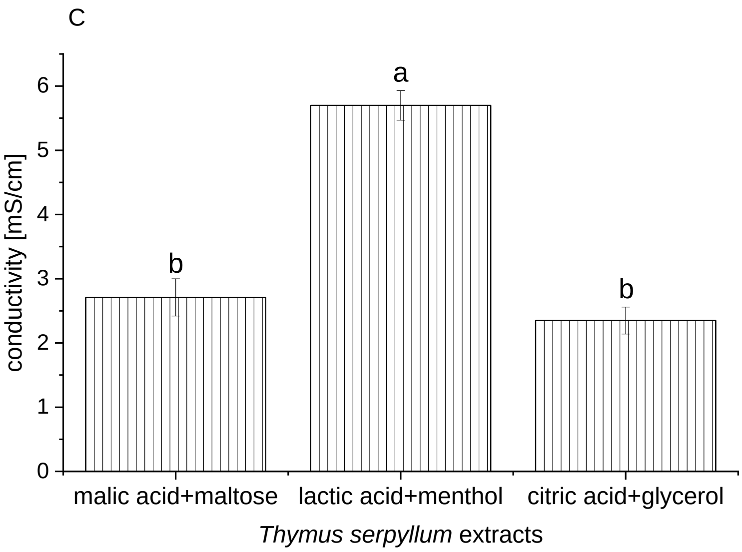
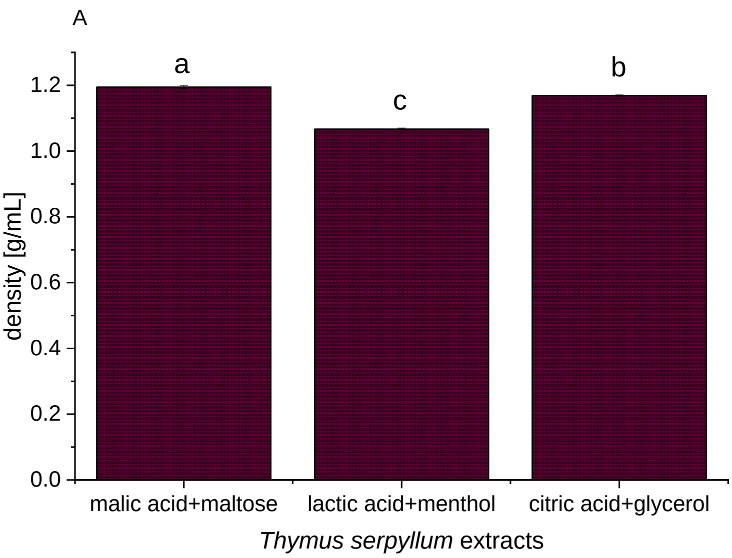
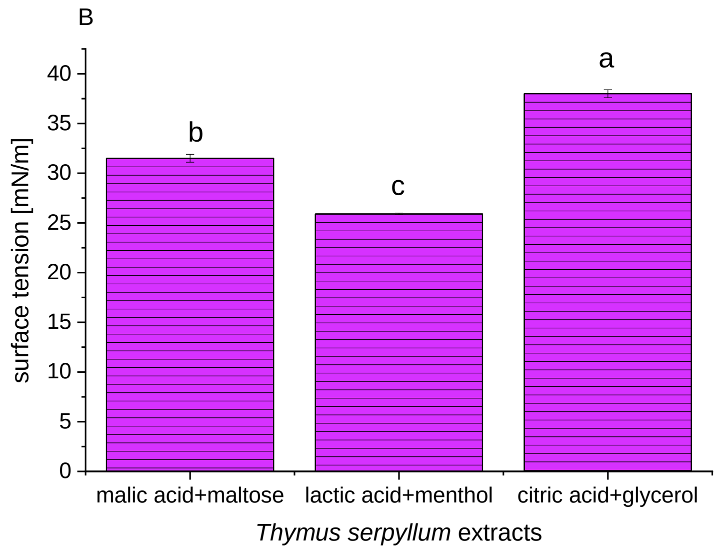


Figure 2. (A) Extraction yield, (B) zeta potential, and (C) conductivity of Thymus serpyllum extracts prepared using natural deep eutectic solvents in maceration; the letters above bars showed statistically significant differences (p<0.05; n=3; analysis of variance, Duncan's post-hoc test).

Zeta potential (absolute value, as a predictor of potential application in water and wastewater treatment and a measurement of the stability of the system) was low in all extracts (0.86±0.06 mV for citric acid+glycerol, -0.35±0.09 mV for lactic acid+menthol, and -0.17±0.05 mV for malic acid+maltose extract, Figure 2B). Skaf et al. (2021) have reported that the zeta potential of the extracts depends on extraction conditions and the composition of the extraction solvent. The conductivity (as a potential predictor of the antioxidant activity of the extracts) was in the range of 2.35±0.21 and 2.71±0.29 mS/cm (citric acid+glycerol and malic acid+maltose extracts) to 5.70±0.23 mS/cm (lactic acid+menthol extract, Figure 2C). According to the literature data, the extracts with a higher conductivity possess better antioxidant potential (Jurinjak Tušek et al. (2018). Nevertheless, ions from the eutectic solvents can significantly influence the conductivity, without changing the antioxidant capacity of the extracts, thus the application of *in vitro* antioxidant assays is necessary for the determination of extract antioxidant potential. pH values of the extracts ranged from 1.44 in citric acid+glycerol extract to 1.78 and 1.85 in malic acid+maltose and lactic acid+menthol extracts.





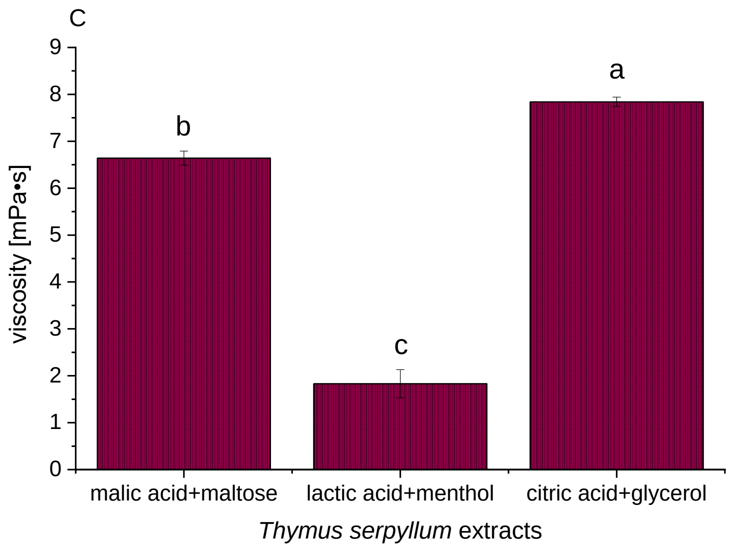


Figure 3. Density, surface tension, and viscosity of Thymus serpyllum extracts prepared using natural deep eutectic solvents in maceration; the letters above bars showed statistically significant differences ($p<0.05$; n=3; analysis of variance, Duncan's post-hoc test).

As can be seen from Figure 3A, the density varied from 1.067±0.003 g/mL for lactic acid+menthol extract to 1.169±0.002 and 1.195±0.005 g/mL for citric acid+glycerol and malic acid+maltose extracts, respectively. According to Mladenović et al. (2018), the density is correlated to the extraction yield. Nevertheless, it was not the case with the obtained *T. serpyllum* extracts prepared using NADESs, probably due to the more significant impact of the extraction medium (Florindo et al., 2014). Surface tension was the highest in citric acid+glycerol extract (38.0±0.4 mN/m), followed by malic acid+maltose and lactic acid+menthol extracts (31.5±0.4 and 25.9±0.1 mN/m, respectively). The viscosity of lactic acid+menthol extract was significantly lower (1.83±0.30 mPa•s) in comparison to malic acid+maltose and citric acid+glycerol extracts (6.64±0.15 and 7.84±0.10 mPa•s, respectively). Namely, the viscosity of eutectic solvents was in the range from 0.05 to 50 mPa•s and the composition of the eutectic solvents significantly affected plant extract viscosity (Florindo et al., 2014).

Conclusions

The aim of the present study was the examination of the influence of the three NADESs on TPC, total protein content, extraction yield, zeta potential, conductivity, density, surface tension, and viscosity of *T. serpyllum* extracts. The citric acid+glycerol extract possessed the highest TPC, total proteins, and extraction yield. The zeta potential of all extracts was significantly low, while conductivity achieved the highest value in lactic acid+menthol extract. Additionally, malic acid+maltose extract possessed the highest density, whereas citric acid+glycerol extract had the highest surface tension and viscosity. Thus, the composition of NADES, as an extraction medium, should be optimized depending on the further implementation of plant extracts. Nevertheless, due to higher TPC, total proteins, and extraction yield, *T. serpyllum* extract obtained using citric acid+glycerol was favored as an ingredient in food, functional food, pharmaceutical, and cosmetic products.

Acknowledgments

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Conflict of interest

The authors declare that they have no financial and commercial conflicts of interest.

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