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Impact of Extraction Solvent Polarity: Antioxydant Activity of Methanolic, Hydromethanolic and Aqueous Decocted Extracts of Algerien *Thymelaea hirsuta* (L.) Endl. Areal Parts

Roumaissa Ounis[1,a,*], Fatima Benchikh[1,b], Smain Amira[1,c], Hassiba Benabdallah[1,d], Hind Amira[1,e], Walid Mamache[1,f], Bensouissi Chawki[2,g], Khaoula Hellal[3,h]

[1]*Laboratory of Phytotherapy Applied to Chronic Diseases, Department of Biology and Animal Physiology, Faculty of Nature and Life Sciences, University of Setif 1, 19000, Algeria.*
[2]*Biotechnology Research Center (CRBt), UV 03 BP E73, Nouvelle Ville Ali Mendjli, Constantine, Algeria*
[3]*Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[*]*Corresponding author*

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ABSTRACT

Thymelaea hirsuta (L.) Endl. , known as 'Methnane' in Algeria, is a widely medicinal plant used in folk medicine. In the present study, *In vitro* antioxidant activity of *T. hirsta* extracts and the impact of extraction solvent polarity on the antioxidant potential were investigated. Three types of polar solvents with decreasing polarity were chosen; water for decocted extract, methanol-water at 50% and absolute methanol for macerated extracts. Total phenolic and flavonoid contents were evaluated and showed a high amount wihch decreases with increasing polarity. Antioxydant activity was assessed with different methods: ABTS assay for evaluation of scavenge activity, CUPRAC and reducing power for assessement of the reduction potential of *T. hirsuta* areal parts. The results showed that *T. hirsuta* areal parts exhibited a strong scavenging activity with significant difference between extracts in terms of their polarity. In the same line, the most polar aqueous decocted extract exhibited a considerable reducing activity followed by hydromethanolic and methanolic extract with increasing potential. These findings suggest the suitability of polar solvents for the extraction of phytochemical compounds from *T. hirsuta* areal parts and so, their antioxidant activity against several radicals and ions.

[a] *bio_ounis@yahoo.com* *https://orcid.org/0000-0003-4437-9748*
[b] *f.ben@univ-setif.dz* *https://orcid.org/0000-0001-6863-8818*
[c] *smainamira@gmail.com* *https://orcid.org/0000-0003-4457-359*
[d] *benabdallahhas2015@gmail.com* *https://orcid.org/0000-0003-4457-3591*
[e] *hindaamira12@gmail.com* *https://orcid.org/0000-0002-6686-2207*
[f] *mamache_w@univ-setif.dz* *https://orcid.org/0000-0002-8567-5634*
[g] *chawkiislam@yahoo.fr* *https://orcid.org/0000-0003-4612-4642*
[h] *khaoulahellal@gmail.com* *https://orcid.org/0000-0002-1223-271X*

Introduction

Since ancient times, plants have been employed by primitive communities for therapeutic purposes (Sevindik et al., 2023). In recent years, there has been a growing interest in the biological characteristics of medicinal plants in order to identify and assess their therapeutic potential, as well as to identify the primary bioactive chemicals and potential synergies (Martins et al., 2014; Uysal et al., 2023). *T. hirsuta*, a medicinal plant, known as 'Methnane' in Algeria, Morroco and Tunisia. (Jamila et al., 2014), belonguing to Thymelaeaceae familly, a flowering plant family with 50 genera (Galicia et al., 2006). The Thymelaea genus includes roughly 31 species of xerophyllous shrubs and herbs with extremely tiny leaves and yellowish or greenish blooms. *T. hirsuta* is from the most prominent species among the thymelaea genus, division of Magnoliophyta, Subclass of Rosidae, and Myrtales Order (T.A.P. Group, 1998).

Traditionally, *T. hirsuta* was used to treat hypertension, respiratory problems and inflammation, (Jamila et al., 2014; Le Floc'h, 1983; Miara et al., 2019). A variety of degenerative disorders such as diabetes, cancer, brain dysfunction, cardiovascular disease, and immune system decline are caused by oxidative stress (Halliwell, 2007; Valko et al., 2007; Mohammed et al., 2022). Chemically, oxidative stress is linked to an increase in the formation of reactive oxygen species (ROS) or a considerable reduction in the antioxidant defenses' efficiency (Lushchak, 2014; Unal et al., 2022). Antioxidants occur in two ways to neutralize free radicals. Although the final result will be the same; radicals can be deactivated by either hydrogen donation (HAT) or electron transfer (SET). HAT and SET pathways can coexist, with the dominant mechanism dictated by antioxidant structure and other characteristics (solubility, partition coefficient, system solvent ...) **(**Prior

et al., 2005; Mohammed et al., 2021). Polyphenols are the most common antioxidants extracted from plants. Flavonoids, which have antioxidant and anti-inflammatory activities (Greenspan et al., 2005; Castila et al., 2006), belong to this family of secondary metabolites. Therefore, characterization, and extraction of antioxidants from natural plant sources have all been attempted. The goal of an extraction procedure is to obtain the highest concentration of target compounds and extracts with the highest antioxidant activity (Spigno et al., 2007; Sevindik et al., 2017). The popular method for extracting bioactive compounds from plantsis Solvent extraction. It is a method that uses a liquid matrix (solvent with different polarity) to remove soluble antioxidant chemicals from a solid matrix (plant tissue) (Makitra et al., 1988). Solvent polarity was defined by ritchard as "Overall solvation capability for reactants complexes and molecules in the excited states, which is dependent on the action of specific, nonspecific and intermolecular interactions between solvent and solute molecules," Depending on chemical nature, various bioactive compounds in plants are extracted in solvents of different polarity.while, There is no way to extract the total of the phytochemicals and antioxidant components with a single solvent (Lapornik et al., 2005; Iloki-Assang et al., 2015).

In this ponder, two diverse polar solvents of different index polarities were used, specifically methanol and water with different concentrations, to extract the bioactive components from *T. hirsuta* areal parts and to evaluate the total antioxidant activity using different *in vitro* assays: ABTS, CUPRAC and reducing power assay, in order to determine the effect of solvent polarity on the evaluation of T. hirsuta antioxidant activity and to highlight the proper selection of method for these process.

Materials and Methods

Plant Collection

The areal parts of *T. hirsuta* were collected in 2021 during february from Batna province, commune of Barika (North East of Algeria). The identification and taxonomic classification of the plant were developed by Professor Smain Amira, Department of Animal Biology and Physiology, University Setif 1, Algeria. The voucher number 91 Th 12/05/20 Bat/OR was deposed at the laboratory of Phytotherapy Applied to Chronic Diseases. The aerial parts of the plant were dried in shadow and grounded to a fine powder using an electric grinder.

Samples Preparation

Methanolic and hydromethanolic crude extracts

The dried powder of *T. hirsuta* areal parts was extracted by maceration as described by Benchikh et al. (2018) (20 g dried powder sample in 400 mL of 95% methanol) for methanolic extract and (20 g dried power sample in 200 distilled water and 200 mL of 95% methanol) for the hydromethalic extract. The preparations were left for 72 h at room temperature with continious stirring. The filtrates were pooled and the solvents were removed under vacuum at 45°C using a rotary evaporator. The obtained crude extracts were stored at 4°C.

Aqueous Decoction Extract

The aqueous extraction was conducted according to the method of Ferreira et al. (2005) with slight modification, 20 g of dried powder of *T. hirsuta* areal parts were extracted with 400 mL of distilled water at 100°C for 10 min after boiling. It was filtered with four layers of muslin cloth then a filtration was applied twice on the mixture through a Whatman filter paper. The water was evaporated to dryness and the residue was storred at 4°C.

Parameters Evaluated

The extraction yield is defined as the quantity of extract recovered in mass compared to the initial amount of dry bark and is a measure of the solvent efficiency to extract certain components from the original material.

Extraction yield of the different extracts were calculated using the equation bellow:

$$\text{Extraction yield (\%)} = \frac{\text{Weight of extract}}{\text{Weight of sample}} \times 100$$

Quantitative Phytochemical Analysis

Determination of total phenolic content

Folin–Ciocalteu reagent was used to determine total phenolic content (TPC), as described by Li et al (2007). A volume of 100 µL of each extract or various concentrations of the standard (gallic acid) were combined with 500 µL of Folin–Ciocalteu reagent (diluted 10 times). Four minutes later, 400 µL of 7.5% Na_2CO_3 solution was added. The mixture was incubated in the dark for 1 hour at room temperature, and the absorbance was measured at 760 nm. Using a gallic acid calibration plot, TPC was expressed as mg of gallic acid equivalent per gram of dried plant extract weight (mg GAE/g DW).

Determination of total flavonoid content

Aluminum chloride assay was used to determine total flavonoid content (TFC) (Bahorun et al., 1996). To 1 mL of test extract or varied standard (quercetin) concentrations, 1 mL of $AlCl_3$ (2%) was added. After 10 minutes of incubation, the absorbance was measured at 430 nm. Using the quercetin calibration curve, the quantity was reported as quercetin equivalent per gram of dry plant extract weight (mg QE/g DW).

Evaluation of Antioxidant Activity

Three different *in vitro* assays were performed using serial dilutions: scavenging effects on ABTS radical, reducing power (measured by ferricyanide Prussian blue assay) and CUPRAC (Cupric reducing antioxidant capacity) assay.

ABTS radical scavenging assay

The free radical scavenging test toward ABTS was evaluated using the slightly modified method of Re et al (1999). The solution stock for ABTS radical was prepared by mixing 2.45 mM potassium persulfate and ABTS (7 mM in water) and kept in the dark at room temperature for 16 hr. The solution was then diluted to give an absorbance of 0.7 to 0.75 at 734 nm with methanol. A total of 40 µL of the sample was mixed with 160 µL of ABTS mixture in a 96-well microplate and kept in the dark for 10 min. The reaction mixture absorbance was measured to 734 nm. Butylhydroxytoluene (BHT) has been used as a positive

control. Scavenging capacity of the test compounds was estimated from the equation below:

$$\% \text{ inhibition} = (AC - AS)/AS \times 100$$

AS: Test simple absorbance
AC: Control absorbance

Reducing power assay

The extract and BHT reduction power were carried out according to Oyaizu's method (1986). A total of 10 µL of each extract or standard was combined with 40 µL of 0.2M phosphate buffer (pH 6.6) and 50 µL of 1% potassium ferricyanide in a 96-well microplate. After 20 minute of incubation period at 50°C, the mixture was added to 50 µL of 10% trichloroacetic acid. Finally, 10 µL of 0.1 $FeCl_3$ and 40 µL of distilled water were mixed together, and the absorbance at 700 nm was measured. The concentrations at which the absorbance was 0.50 were reported.

Cupric reducing antioxidant capacity (CUPRAC)

The cupric lowering ability of the extracts was tested using the technique described by Apak et al (2004). To 40 µL of sample or standard, 50 µL of 10 mM $CuCl_2$, 50 µL of 7.5 mM neocupronin, and 60 µL of 1 M ammonium acetate solution were added, respectively. The reagent combination was kept at room temperature for 60 minutes in the dark. At 450 nm, the reaction's absorbance was measured. As a positive control, BHT was used. The findings were reported as $A_{0.5}$ (µg/mL), indicating a concentration of 0.5 absorbance.

Statistical Analysis

All samples were assayed in triplicate. Results are presented as means ± standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test using GraphPad Prism Version 8.0.2 (GraphPad Software, Inc, USA). The p values $P<0.0001$ were considered significantly different.

Results

Extraction Yield

The choice of solvent is crucial for obtaining extracts with notable yields. The yields of the extracts using various solvents are listed in the following order: Water > Hydromethanol > absolute methanol (Table 1).

Determination of Total Phenolic and Flavonoid Content

In this study, the total content of phenols and flavonoids has been affected by two factors, the method of extraction and the solvent polarity. Overall, the highest content of total phenols was marked by methanol crude extract with maceration method (200.86±2.20 mg GAE/g DW), followed by hydromethanolic extract with (180.56±1.91 mg GAE/g DW), while the aqueous decoction extract showed the lowest phenolic content (114.00±1.35 mg GAE/g DW).

The flavonoid content decreased in the same order: where, methanolic, hydromethanolic and aqueuous decoction extracts showed the following values: 18.26±1.03 mg QE/g DW, 17.01±1.03 mg QE/g DW and 16.80±1.03 mg QE/g DW, respectively (Table 2).

Table 1. Yield extraction of *T. hirsuta* areal parts extracts

	Yield of extracts (%)
ADE	11.6
HME	12.05
ME	12.80

Abreviations: ADE: aqueous decoction extract; HME: hydromethanolic extract at 50%; ME: absolute methanolic extract

Table 2. Total polyphenols and flavonoids in *T. hirsuta* areal parts extracts

	TPC(mg GAE/g DW)	TFC(mg QE/g DW)
ADE	114.0±1.35	16.80±1.03
HME	180.56±1.91	17.01±1.03
ME	200.86±2.20	18.26±1.03

Abreviations : ADE: aqueous decoction extract; HME: hydromethanolic extract50%; ME: methanolic extract; TPC: Total polyphenol content; TFC: Total flavonoid content; DW: dry weight; GAE: gallic acid equivalent; QE: quercetin equivalent.

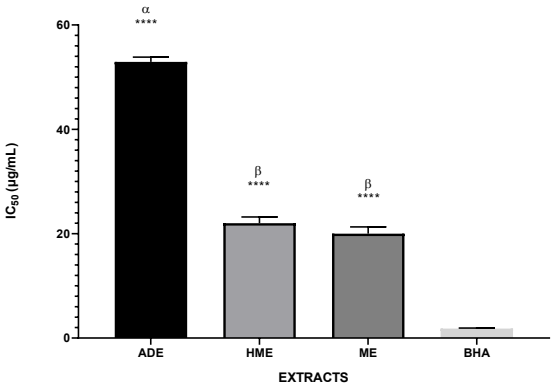

Figure 1. ABTS radical scavenging activity of *T. hirsuta* L. areal parts extracts.
ADE: aqueous decoction extract; HME: hydromethanolic extract 50%; ME: methanolic extract. Data were presented as IC_{50} means±SD (n=3) (****P≤0.0001; ns: not significant) vs BHA as standard. Columns carrying different letters are significantly different at P<0.0001

Evaluation of Antioxydant Activity

The decrease in ABTS radical scavenging effect of the extracts took the following order: ME, HME and ADE. The IC_{50} values were estimated to be respectively: (20.86±1.00), (22.01±1.20) and (52.93±0.90) µg/mL (Table 3). The methanolic and hydromethanolic extract had a better significant activity than aqueous decoction extract (Figure 1).

Reducing Power Assay

The most effective extract in iron reduction were the methanolic one with a concentration ($A_{0.5}$=81.20±0.64 µg/mL) followed by hydromethanolic and aqueous decoction extracts with ($A_{0.\ 50}$=85.37±0.38 µg/mL) ($A_{0.50}$ =124.5±1.79 µg/mL), respectively (Figure 2).

A high significant difference between ADE capacity and both of the methanolic and hydromethanolic one.

Cupric Reducing Antioxidant Capacity (CUPRAC)

The reductive capacity of different extracts against cupper ions was significantly different. The effective absorbances decreased in the following order:

ME (31.09±1.56 µg/mL) > HME (70.46±0.92 µg/mL) > ADE (134.5±0.44 µg/mL) (Figure 3).

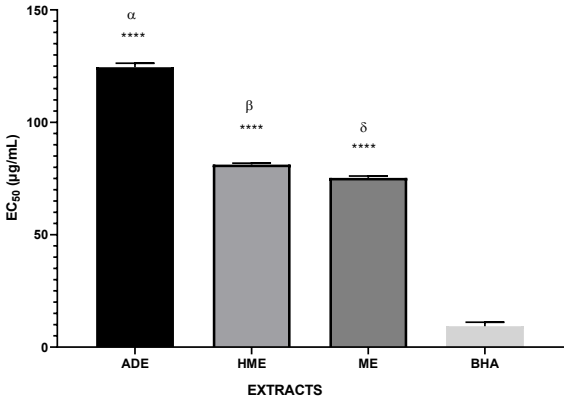

Figure 2. Reducing power activity of *T. hirsuta* L. areal parts extracts.

ADE: aqueous decoction extract; HME: hydromethanolic extract at 50%; ME: methanolic extract. Data were presented as IC_{50} means ± SD (n=3) (****P≤0.0001; ns: not significant) vs BHA as standard. Columns carrying different letters are significantly different at P <0.0001; β vs δ: are significantly different at P<0.001.

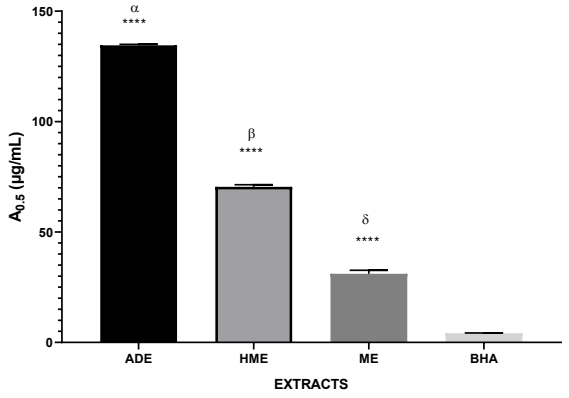

Figure 3. Cupric reducing antioxidant activity of *T. hirsuta* areal parts extracts.

ADE: aqueous decoction extract; HME: hydromethanolic extract 50%; ME: methanolic extract. Data were presented as IC_{50} means ± SD (n=3) (****P≤0.0001; ns: not significant) vs BHA as standard. Columns carrying different letters are significantly different at P<0.0001

Discussion

Several techniques and different solvents have been applied to extract the bioactive components from *T. hirsuta*, few of them foccused on the effect of solvent polarity and extraction techniques used. In the present study, the chosen solvents were 100% methanol, 50% methanol and water with polarity index values of 6.6, 7.8 and 9, respectively (Kumoro et al., 2006) in order to highlight the effect of solvent polarity for the extraction of bioactive components from *T. hirsuta*, and its antioxidant activity.

A variability of extraction yields was detected. Where, the aqueous decocted extract of *T. hirsuta* showed the lowest yield with 11.6% in comparison with the hydromethanolic and methanolic extract obtained with maceration method. This difference can be explained by the effect of temperature in the breakdown of cellular constituents during the decoction process (Hamrouni-Sellami et al., 2012), since some bioactive compounds are extremely sensitive to oxygen and heat (Ishida et al., 2005).

The highest extraction yield was recovered by absolute methanol (12.8%), followed by the hydromethanolic extract at 50% methanol (12.05%). A similar remarque was marked by Yahyaoui et al (2018) in different conditions of temperature and duration of extraction with Tunisian genus of *T. hirsuta*. Such variation can be explained by difference of polarity's degree, where water the most polar solvent showed the minutest yield then the subsolvant of water/methanol mixture, arriving to absolute methanol with low polarity. These results showed that the increasement of solvent polarity with water addition induce a decrease in extraction yield. This is consistent with what has been found in previous studies (Yahyaoui et al, 2018; Djermane et al., 2020).

Flavonoids and phenolic compounds are reported to be natural antioxidants (Shahidi et al., 2015). The antioxidant activity of polyphenols is mostly owing to their redox characteristics, which allow them to function as reducing agents, hydrogen donors, singlet oxygen quenchers, and metal chelators (Javanmardi et al., 2003). The mechanisms of action of flavonoids are exerted through scavenging or chelating process (Schmitt-Schillig et al., 2005). *T. hirsuta* was shown to contain flavonoids and polyphenols in several studies (Trigui et al., 2014; Kristanti et al., 2018; Djermane et al., 2020).

The present study revealed that all extracts were rich in polyphenols, values were higher than those cited by Yahiaoui et al. (2020) regarding methanolic and hydromethanolic extracts. This difference may be due to extraction techniques condition. Our results were slightly less than those reported by Djermane et al. (2020), with aqueous and hydromethanolic extracts, this variance can be explained by temperature effect on chemical compound and /or methanol proportion. A considerable total flavonoid content has been found in *T. hirsuta* areal part in all extracts. These findings are in accordance with those reported by Amari et al. (2014) showing an acceptable flavonoid content in the diffrent areal parts of *T. hirsuta.* Additionally, Trigui et al. (2014) demonstrated a higher flavonoid content by treatement of the areal parts of *T. hirsuta* with solvents with increasing polarity.

Overall, the absolute methanolic extract had the highest total content of both phenolic and flavonoid compounds, followed by hydromethanolic and aqueous decocted extract. Such variation can be explained by the simple fact that the chemical composition varied considerably according to the solvent polarities. Such differences have been reported in the littérature by Javaprakasha et al. (2001). Lesjak et al. (2011) indicated that TPC is affected by the solvent type and polarity. In the same line, Do et al. (2014) declared that the TFC level in extracts is influenced by solvent type and polarity index, plant species, and plant parts used.

From the following results, the methanolic extract with the highest amount of total phenolics and flavonoids was the stronger radical scavenger of ABTS radical, followed by hydromethanolic and aqueous decocted extract with decreasing in total phenols and flavonoids content, respectively. These findings, were comparable to those reported by Amari et al. (2014) with different areal parts of *T. hirsuta* and also to those described by Yahiaoui et al. (2018). Harvesting season and geographic region can also

affect the phytochemical content of extracts, declared Ben Farhat et al. (2015)

From our results of method based on free radical scavenging, a highly significant difference was marked between aqueous decocted extract and both hydromethanolic and methanolic extracts, whereas these lasts were nearly similar and not significantly different (P>0.0001) marking a more powerful scavenging effect against the radical ABTS. Overall, these findings are in accordance with findings reported by Djermane et al. (2020). A similar conclusion was reached by Ioannou et al. (2015). A number of studies have found a link between free radical scavenging activity and total phenolic components (Zheng and Wang, 2001; Wangensteen et al., 2004; Kanatt et al., 2007; Sarikurkcu et al., 2008).

The reductive capacity of the extracts assessed by two different assays:

CUPRAC, an electron-transfer (ET)-based method, where, a redox reaction of the CUPRAC reagent with chain-breaking antioxidants form the neocoprine (Nc) chelate. The redox potential of the extracts in study were significantly different, aqueous decoction extract had the lowest capacity to reduct the copper ions with a very low value in comparison with previous study by Djermane et al. (2020) on aqueous maceration extract of *T. hirsuta*. This difference may be justified by the fact of temperature in the dielectric constant of water **(**Owe et al, 1961; Dyer et al., 2006 Carr et al, 2011). Where, increasing temperatures cause an overall decrease in the water interactions known as "polarity." (Zahra et al., 2016). Wherease, methanolic and hydromethanolic extracts showed a considerable reduction ability of copper ions from CU(I) to CU(II). This result ties well with previous study, wherein a 70% hydromethanolic extract of *T. hirsuta* showed remarquable reduction ability (Djermane et al., 2016).

In the reducing power assay, the reduction of the Fe3+/ferricyanide complex to its ferrous form is due to the presence of antioxidant reductants in the extracts and is monitored by measuring the formation of Perl's Prussian blue of ferrous form at a wavelength of 700 nm (Gülçin, 2006). The results of the present study clearly indicated that the organic methanolic extract with the highest amount of phenolic compound was the strongest reductant one, which proved its richeness with compound with electron transfer ability, followed by hydromethanolic and aqueous decoction extracts with a considerable potential. These studies also indicated sub solvent water-methanol in maceration to be a better solvent than water in extraction of these compounds. Contrary, Djermane et al. (2020), declared a higher reducing potential of aqueous macerated extract than the hydromethanolic extract at 70%. These results suggest that *T. hirsuta* areal parts are rich in antioxydants with high reductant activity.

Conclusion

The polarity-dependent decrease in extraction yield, antioxidant activity, free radical scavenging potential and reducing properties of *T. hirsuta* may be attributed to the high affinity of antioxidant compounds in areal parts towards slight polar solvents as compared to more polar ones. However, high values of TPC and TFC in polar solvents with low polarity index reflects the nature of the phenolic compounds present in *T. hirsuta* areal parts.

Findings suggest the suitability of polar solvents for the extraction of antioxidant compounds from plant materials. The present study also suggests that the assessement of the phytochemical compounds content and their antioxydant activity against several radicals and ions by different methods in a broad range of conditions is crucial for determination of the plant antioxydant potential and its mode of action. These results are a good agreement of the popular use of *T. hirsuta* which may constitute a good source of healthy compounds.

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Conflict of interest statement

We declare that we have no conflict of interest.

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