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Calendula arvensis L. extracts: GC-MS and HPLC-DAD quantification of the main phenolic components and their pharmacological potential

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Abstract: The aim of the current research was to examine and quantify the chemical composition of the chloroform extract of *Calendula arvensis*, as well as investigate the pharmacologicala ctivities of two different extracts using enzymatic, chemical and chromatographic methods. The plant was preserved after being dried, while HPLC and GCMS chromatographic techniques were used for quantitative analyses of the chloroform extract. Both extracts (chloroform and *n*-butanol) were examined for their total phenol and flavonoid contents. GC-MS analysis of the chloroform extract revealed the presence of 77 compounds; germanicol (10.25%), arachidonicacid (8.17%), hexadecanoicacid (6.94%), 17-octadecynoic acid (5.99%), syringicacid (5.22%) and *p*-coumaricacid (3.04%) were found to be the major constituents. Syringic and *p*-coumaricacids were confirmed and quantified by HPLC-DAD analysis of chloroform extract. Both extracts had a high concentration of polyphenols and demonstrated moderate antioxidant activity using five methods (DPPH, ABTS, CUPRAC reducing power and β-carotene). The chloroform extract presented an interesting inhibition against butyrylcholinesterase (BChE) with an IC_{50}= 69.06 ± 1.33 µg/mL, near that exerted by galantamine. These results indicated that *C. arvensis* extracts might be used as a promising natural alternative source of antioxidant and anti-Alzheimer molecules.

Keywords: *Calendula arvensis*; plant extract; antioxidant activity; anticholinesterase; GC-MS; HPLC−DAD

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INTRODUCTION

Many research studies have demonstrated that medicinal plants have many different types of compounds, including polyphenols, flavonoids, alkaloids, tannins, coumarins, terpenoids and many others. (Navarrete *et al.,* 2024; Flouchi *et al.,* 2024). Antioxidant, antiviral, anticarcinogenic, anti-inflammatory and potential anti-Alzheimer and antiproliferative effects on tumor cells are only some of the many important pharmacological characteristics revealed *in vitro* by natural products, flavonoids, polyphenols and phenolic acids. (Khalfallah *et al.,* 2017; Kowalska *et al*., 2021;Tafrihi *et al.,* 2021; Elhouda *et al*., 2024). One possible explanation for some of these effects is that these molecules serve as antioxidants, free-radical scavengers and peroxidation inhibitors (Sivasothy *et al*., 2013). Owing to the variety of health hazards to which humans are exposed, such as neurotoxicity, nephrotoxicity and cancer, natural antioxidants have become a viable alternative to synthetic compounds with their powerful effects and lower adverse effects on human health (Srief *et al.,* 2023). A wide variety of illnesses can be treated using medicines found in the rich Algerian flora. *Calendula*, a genus of over 25

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species in the Asteraceae family, is one such plant used for therapeutic purposes (Gonçalves *et al.,* 2023). *C. arvensis* Linn, *C. officinalis* Linn, *C. suffruticosa* Vahl. And *C. stellata* Cav. are the most abundant in the Mediterranean region (Arora *et al*., 2013)(Gu *et al.,* 2022). The *Calendula* genus has traditionally been used to treat skin problems, bruises and open wounds, eye infections and impaired eyesight, abnormal menstrual cycles, varicose veins, hemorrhoids, duodenal ulcers. and other diseases (Tugba *et al.,* 2012;Paşa, 2022). As a sedative, diaphoretic and emmenagogue, it has been used medicinally for centuries (Neal*et al.,* 2019; Paşa, 2022). In fact, *C. arvensis* has been the subject of several ethno pharmacological studies (Tugba *et al.,* 2012; Arora *et al.,* 2013; Neal *et al.,* 2019; Paşa, 2022; Gonçalves *et al.,* 2023; Khouchlaa *et al.,* 2023). As well as the previously mentioned properties, *C. arvensis*'s extracts and essential oils have further been shown to have antioxidant, antifungal and hemolytic properties (Çelik *et al.,* 2012; Belabbes *et al.,* 2017; Servi *et al.,* 2020). Sesquiterpenes, saponins, triterpenoids, phenols and flavonoids have all been identified in *C. arvensis* due to its chemical composition.(Paolini *etal.,* 2010; Fiorentino *et al.,* 2022). Despite many investigations on the chemical composition of *C. arvensis*, there is no data on the bioactive molecules present in their solvent extracts or on the anti-Alzheimer and antioxidant activities of *C. arvensis.* Therefore, the objective of this investigation was to search for its chemical characteristics using GC-MS analysis and the anti-Alzheimer and antioxidant effects of *C. arvensis* chloroform ($CHCl_3$) and *n*-butanol (*n*-BuOH) extracts. In addition, the phenolic compounds of chloroform extract were studied and determined using HPLC-DAD.

MATERIALS AND METHODS

Plant identity

Calendula arvensis L., was collected during the flowering season at Taghit, Bechar (North Western Algerian Sahara: 30° 51' 20.75"N 2° 0' 21.95"W). A voucher specimen (CA.05.2019) of the vegetal material has placed in the herbarium of the research unit VARENBIOMOL, University of Constantine 1 (Constantine, Algeria).

Sample preparation

Approximately 1.2 kg of *C. arvensis* (leaves and flowers) were macerated in MeOH-H_2O (8/2:v/v) for 24 hours. Following filtering, this process was done three times. At reduced pressure, the filtrates were mixed and condensed to temperatures of up to 38 °C. The resulting residue was melted in 560 mL of distilled water for one night. Following filtration, chloroform was used to extract the resultant solution to obtain (yield 12.34 g), ethyl acetate to obtain (yield 5.3 g) and *n*-butanol to yield (20.1 g), successively.

Analysis by gas chromatography-mass spectrometry (GC-MS)

Analyzes were performed using Bruker scion SQ equipment consisting of a variable VF5-MS capillary column (0.25 µm (film thickness); 30 m; 0.25 mmi.d) coupled to a signal quadrupole mass detector with the following experimental conditions: mobile phase: helium gas; flow rate: 1 mL/min; injector temperature: 280 °C; ionization voltage: 70 eV). Comparing the mass (MS) and fraction retention times (Rt) with those of the current commercial spectrum library NIST 2011 allowed for the identification of the constituents. (Singleton and Rossi, 1965) (Mikaia *et al.,* 2014).

Analysis by HPLC−DAD

The analysis of *C. arvensis* chloroform extract was achieved using HPLC-DAD working between 220 and 400 nm. The chromatographic runs were done using a Kinetex C-18 column (100 × 21.2 mm, pore size 100 Å, particle diameter 5 µm) at room temperature and eluted with a gradient system using methanol-water. Both compounds 1 and 2 were found at 280 nm and 320 nm, respectively.

Total phenolic content (TPC)

The Folin-Ciocalteu (FC) reagent was utilized to determine the TPC. (Singleton and Rossi, 1965) with a microplate assay previously reported (Müller *et al.,* 2010). Water extract (20 µL), diluted FC reagent (100 µL) and a solution of sodium carbonate (75 g/L) were mixed together (75 L). After exposing the samples to darkness for two hours at 765 nm. The TPC was calculated as gallic acid equivalents (GAE) in µg/mg.

Total flavonoid content (TFC)

The flavonoid assay in extracts was evaluated according to the original method with some modifications for a 96-well microplate assay (Topçu *et al.,* 2007). The extract (50 µL, 1 mg/mL methanol) was combined with methanol (130 µL), potassium acetate (10 µL, 98 g/L) and aluminum nitrate (10 µL, 100 g/L). Following 40 minutes of incubation at room temperature, the absorbance was detected at 415 nm. The TFC was calculated as quercetin equivalents (QE) in µg/mg.

Antioxidant activity

The *in vitro* antioxidant effect of *C. arvensis* chloroform and *n*-butanol extracts was examined in five ways: 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) reduction, antioxidant capacity reducing cupric ions (CUPRAC), reducing power activity andβ-carotene bleaching inhibition. Standardized protocols were used for all tests (Blois, 1958;Marco, 1968; Oyaizu, 1986; Re *et al.,* 1999; Apak *et al.,* 2004). In the case of DPPH, $ABTS^+$ and β-Carotenelinoleicacid,

the findings were determined the concentration of 50% inhibition (IC_{50} value (µg/mL).

Anticholinesterase activity

The inhibitory activity of $CHCl_3$ and *n*-BuOH extracts against AChE and BChE was determined according to a process prescribed elsewhere (Ellman *et al.,* 1961). The AChE of the electric eel and the BChE of the horse were utilized and acetylthiocholine iodide and butyrylthiocholine chloride served as the reaction's substrates. (5, 5'-dithio-bis (2-nitrobenzoic) acid) DTNB was utilized for measuring the activity. The results were compared with galantamine.

STATISTICAL ANALYSIS

The findings of all laboratory experiments are given as the mean± standard error (SD) of three tests. One-way ANOVA was used to record the findings. (Graph Pad Prism 9 software) accompanied by post hoc test of tukey for several comparisons and statistical significance was assumed at the $P<0.05$ level.

RESULTS

Chemical composition

GC-MS recognized numerous substances (77) present in the chloroform extract (table S1).

Analysis by HPLC−DAD

Chromatograms showing the findings are presented in fig.1.

Total content of phenolic and flavonoid

The *n*-butanol extract presented the highest TPC (423.78±2.49µg gallic acid equivalent (GAE)/mg), followed by the chloroform extract (366.058±2.35µg GAE/mg), Fig. 2. In good agreement with the above reported results, the flavonoids richest extract was the *n*-butanol extract (10.97±0.58 µg quercetin equivalent QE)/mg), followed by chloroform extract (7.34±0.14µgQE/mg).

Antioxidant activity

The DPPH scavenging results (table 1) reveal that the extract of *n*-butanol displayed the strongest antioxidant activity (IC_{50}= 75.98±18.17 µg/mL), higher than the $CHCl_3$ extract. In addition, all samples had weak activity in comparison to the reference compounds: BHA, BHT and ascorbic acid. Furthermore, an estimation of the positive correlation between polyphenols and antioxidant activity for both *n*-butanol and $CHCl_3$ extracts has been determined (table 2).

Anticholinesterase activity

Table 3 compares the anti-AChE and anti-BChE effects of *C. arvensis* extracts with those of galantamine, which was employed as a positive control. Among the tested extracts, chloroform extract was the only one giving better activity against BChE (IC_{50} value: 69.06 ± 1.33 µg/mL). The *n*-butanol extract was inactive; all extracts were inactive against AChE activities.

DISCUSSION

The major compounds were: Germanicol (10.25%), arachidonic acid (8.17%), hexadecanoic acid (6.94%), 17-Octadecynoic acid (5.99%), syringic acid (5.22%), *p*-coumaric acid (3.04%). The majority of the constituents present in the chromatograms are similar to phenolic compounds. The identification of compound one (C1: syringic acid) and compound two (C2: *p*-coumaric acid) were identified from the chloroform extract of *C. arvensis* by comparing their UV spectra to a perfect match obtained by photodiode array detection of the unknown peaks and the Rt (retention times) with those of the corresponding standard. GC-MS examination of the chloroform extract of *C. arvensis* confirmed those results, where (C1) and (C2) were found among the major constituents. (table S1) Studies on the phytochemistry of *C. arvensis* revealed that it includes compounds from a number of different classes, including phenolic acids and flavonoids (Paolini *et al.,* 2010; Reznicek and ZitterI, 2003; Fiorentino *et al.,* 2022).To our knowledge, syringic acid and *p*-coumaric acid are reported in *C. arvensis* for the first time. In comparison with other *Calendula* species, syringic acid has been reported in a flower sample from *Calendula officinalis* L. (Frum, 2017). and *p*-coumaric acid has been isolated in *n*-butanol extract from *Calendula tripterocarpa* Rupr (Al-Rifai, 2018). In comparison with other *C. arvensis* from Turkey, according to the result, the quantity of phenolic compounds received from *C. arvensis* from Algeria is higher than that obtained from *C. arvensis* from Turkey (118.18±10.29 µg GAE/mg methanol extract). The content of flavonoid substances obtained from Algerian *C. arvensis* is lower than that of Turkish *C. arvensis* (74.14±3.09 µg QE/mg methanol extract) (Tugba *et al.,* 2012). It should be noted that factors contributing to the quantitative variations in phenolic concentration include variations in extraction method, standard solution, location and climate (Sujana *et al.,* 2013; Paié-Ribeiro *et al.,* 2024). The antioxidant effect of the organic *C. arvensis* extracts has never been investigated before in Algeria. Numerous reports of *Calendula* species, especially *C. arvensis*, have shown antioxidant activity in a variety of assays to date. For instance, *C. arvensis* dichloromethane extract exhibited very low antioxidant activity in the DPPH and reducing power tests. However, the methanol *C. arvensis extract* showed the greatest scavenging activity against DPPH in this investigation (Neal *et al.,* 2019; Tugba *et al.,* 2012).In another study, the antioxidant potential of eleven species (among them *C. arvensis*) employed in Sardinian traditional medicine was examined by Dall'Acqua*et al.* (2008).

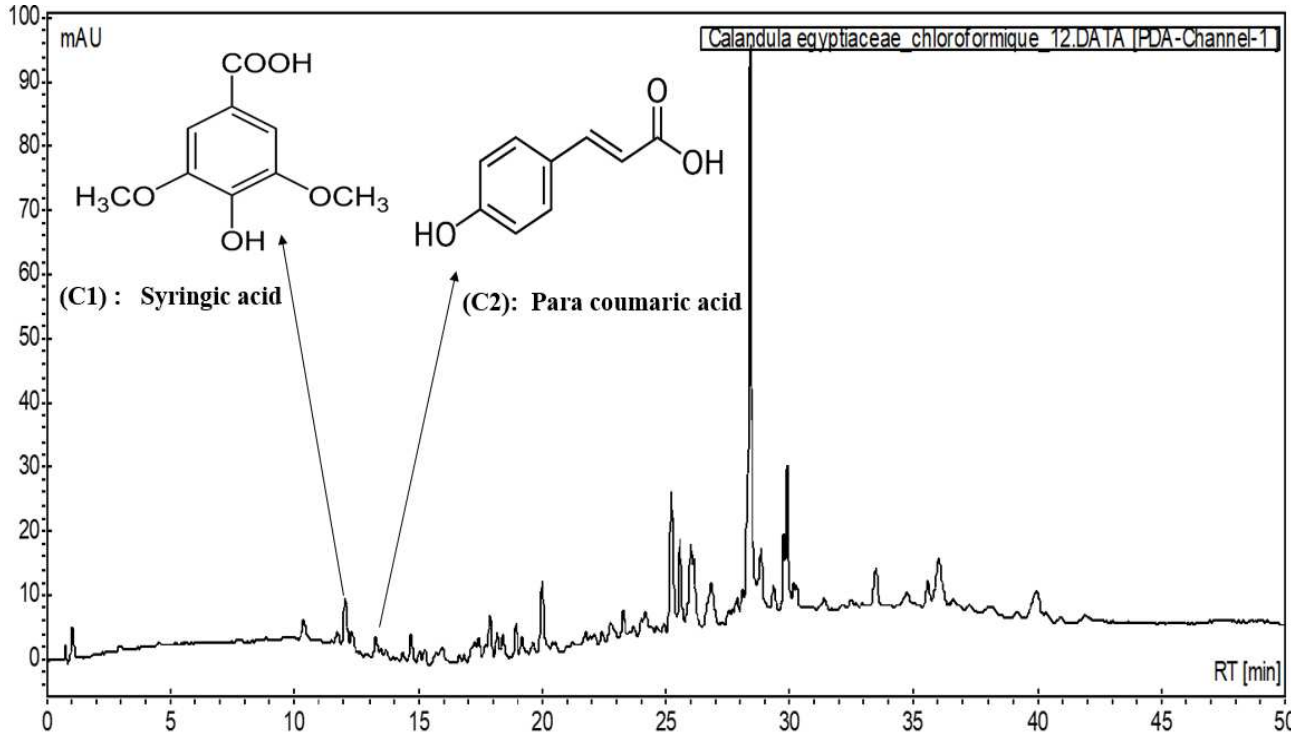

Fig. 1: HPLC-DAD chromatogram of chloroform extract of *C. arvensis,* (C1): Syringic acid; (C2): *p*-coumaric acid

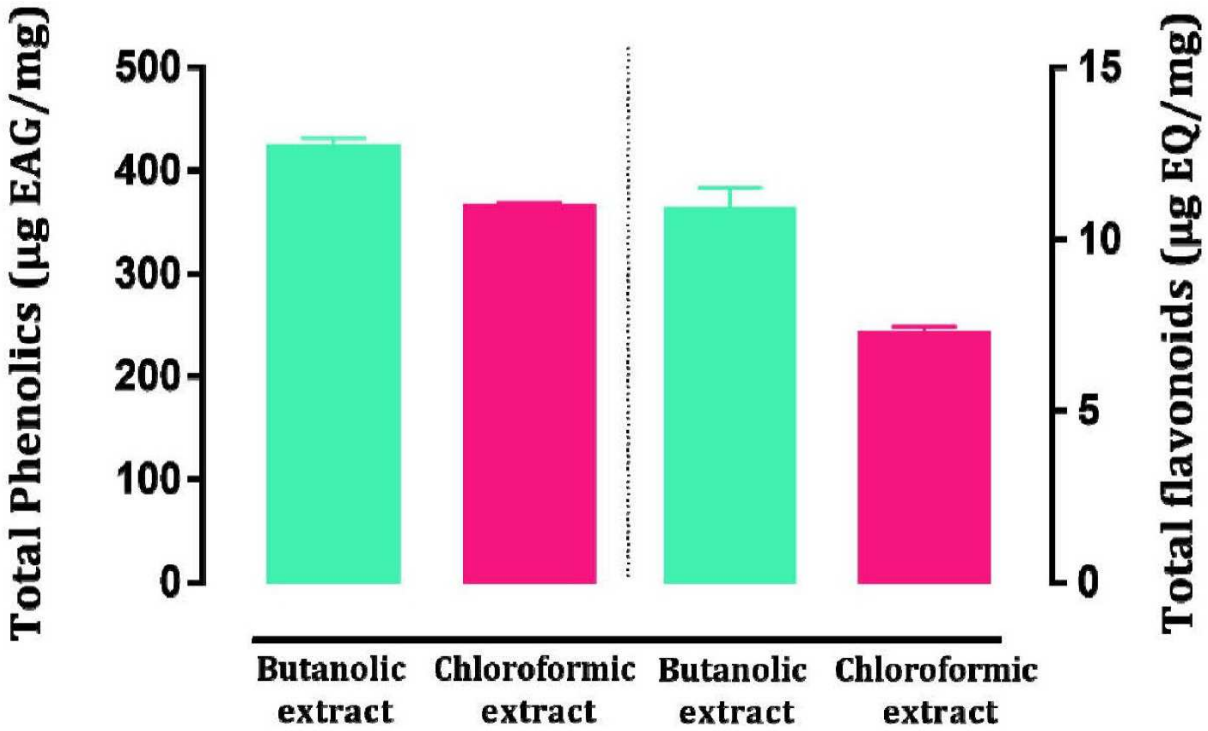

Fig. 2: Total contents ofphenolic compounds and flavonoids in*C. arvensis* methanol and chloroform extracts.

Table 1: Antioxidant activity of chloroform and *n*-butanol extract of *C. arvensis* according to different chemical assays.

Extracts	DPPH testing IC_{50} (µg/mL)[a]	ABTS*test IC_{50} (µg/mL)[a]	β-Carotene linoleic acid testing IC_{50} (µg/mL) [a]	CUPRAC testing $A_{0.5}$ (µg/mL)[a]	Reducing power testing $A_{0.5}$ (µg/mL)[a]
Chloroform extract	> 800	96.67 ± 3.30	98.49 ± 5.64	103.44±1.36	> 800
n-butanolextract	75.98 ± 18.17	31.63 ± 0.93	186.57 ± 5.70	51.64 ± 1.74	175.78 ± 2.12
Ascorbic acid[b]	13.94 ± 2.81	1.74 ± 0.10	52.59 ± 1.98	12.43 ± 0.09	6.37 ± 0.42
BHT[b]	12.99 ± 0.41	1.29 ± 0.30	22.32 ± 1.19	8.97 ± 3.94	152.24 ± 2.43
BHA[b]	6.14 ± 0.41	1.81 ± 0.10	0.90 ± 0.02	6.62 ± 0.05	7.99 ± 0.87

[a]IC50 and A0.50 Values shown are the means standard deviations of three separate measurements (p<0.05). [b]Reference compounds. BHA: *butylatedhydroxyanisole,* BHT: *butylatedhydroxytoluene*

Table 2: Correlation coefficients between antioxidant assays of chloroform and *n*-butanol extract of *C. arvensis*

Chloroform extract	*DPPH* testing	*ABTS*+ testing	*CUPRAC* testing	*Reducing power* testing	*β-Carotene linoleic acid* testing
DPPH testing	1				
ABTS+ testing	0.615157292	1			
CUPRAC testing	0.425895948	0.994879682	1		
Reducing power assay	0.561324998	0.998499141	0.987851366	1	
β-Carotene linoleic acid testing	0.900494617	0.83661912	0.776972702	0.865364225	1
n-butanol extract	*DPPH* testing	*ABTS*+ testing	*CUPRAC* testing	*Reducing power* testing	*β-Carotene linoleic acid* testing
DPPH testing	1				
ABTS+ testing	0.990418474	1			
CUPRAC testing	0.882791415	0.939206878	1		
Reducing power assay	0.682884131	0.575456283	0.259668246	1	
β-Carotene linoleic acid testing	0.687374852	0.580487285	0.265616839	0.999980996	1

Table 3: AChE and BChE inhibiting effects of different *C. arvensis* extracts

Extracts	AChE testing IC_{50} (µg/mL)	BChE testing IC_{50} (µg/mL)
Chloroformextract	NA[C]	69.06 ± 1.33
n-butanol extract	NA[C]	NA[C]
Galantamine[b]	6.27±1.1	34.75±1.9

[b]Reference compound Not active

Using the DPPH radical scavenging test, the methanol extract of *C. arvensis* ranked seventh among the studied plant species, as it showed an EC_{50}= 80.9 ± 1.5 µg/mL. These findings are consistent with those obtained duringour investigation of the polar extract. Indeed, the ABTS analysis data, CUPRAC and reducing power assay revealed that the *n*-butanol extract is more active than the chloroform extract but lower than that of the tested standards. On the other hand, *n*-butanol extract indicated the significantly highest activity with the β-carotene bleaching test; however, when compared to the standards, it remained weak. In many studies, the extract with high total phenol and total flavonoid contents was possibly associated with its good antioxidant effect (Jabir *et al.,* 2018; Chaves *et al.,* 2020; Rudrapal *et al.*, 2022; Lang *et al.,* 2024).Our GC-MS analysis showed that this extract appeared rich in polyphenols at varied concentrations, especially *p*-coumaric acid, which might explain its significant activity. In fact, many polyphenols, including caffeicacid, cinnamic acid, caffeine, quercetin, curcumin, resveratrol and gallocatechins are known to have an impact on Alzheimer's disease treatment by inhibiting cholinesterase (Jabir *et al*., 2018; Takao *et al.,* 2017; Diniso *et al.,* 2022).

CONCLUSION

This is the first analysis of the phytochemical constituents, antioxidant and anticholinesterase properties of *C. arvensis.* Seventy-seven different chemical compounds were identified in the chloroform extract by GC-MS analysis. In addition, the identification of polyphenolic compounds was confirmed by HPLC-DAD. Our results showed that *C. arvensis* extracts obtained in chloroform and *n*-butanol exhibited moderate antioxidant

activity using various methods. Indeed, the chloroform extract was shown to have significant butyrylcholinesterase (BChE) inhibitory activity, suggesting they merit further investigation into the identity of the active compounds of these extracts, as well as associations and interactions, in order to become more familiar with the use of the plant and to better benefit from its potential beneficial effects. Alzheimer's disease and oxidative substances are considered serious problems facing humans due to several reasons, the most important of which are the industrial materials that we use in our daily lives, especially food. This requires the necessity of introducing natural materials to reduce or eliminate these problems due to the fact that they are rich in natural products with high pharmacological effects, the most important of which are medicinal plants. Therefore, it is necessary to delve into research and determine the type of these compounds and their quantity in this plant, as well as further study on the extent of their pharmacological activities.

Conflicts of interest

There are no conflicts to declare.

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