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Comparative analysis of the chemical constituents and *in vitro* antioxidant activities of different aqueous extracts of the *Cistanche phelypaea* (L.) Cout. from Algeria

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ABSTRACT

Cistanches herba are well-known for their nutritional and therapeutic effects, but only few reports are available about the species *Cistanche phelypaea* (L.) Cout. and studies of aqueous extracts are scarce. This study aimed to elucidate the impact of aqueous extraction methods including decoction, infusion and cold maceration from *C. phelypaea* roots on phytochemical composition and *in vitro* antioxidant activities of the extracts. In order to accomplish this, determinations of total phenolic and flavonoid contents, liquid chromatography electrospray ionization mass spectrometry (LC/ESI-MS and LC/ESI-MS/MS) analyses and *in vitro* antioxidant activity studies were carried out. All extracts had significant levels of total phenolic content, but the decoction possessed the highest concentration and no significant difference was observed between infusion and cold maceration. Concerning flavonoids, decoction and infusion possessed the highest concentrations. Data obtained from LC/ESI-MS and LC/ESI-MS/MS analyses showed similar qualitative profiles for all aqueous extracts with 14 characteristic chromatographic peaks in negative mode and 6 in positive mode, which allowed the tentative identification of syringin, 14 phenylethanoid glycosides and 5 iridoids, including 5 pairs of isomers. However, significant variations were recorded for the relative abundance of some compounds depending on the method that was employed. Hot extraction procedures, decoction and/or infusion were more efficient in extracting acteoside, isoacteoside and two molecules corresponding to isomers of 2'-acetylacteoside/tubuloside B, and it seems that a longer time of heating-extraction was required for an improved extraction of isoacteoside, and one of the two isomers of 2'-acetylacteoside/tubuloside B, as decoction gave a significant higher amount of these compounds. However, 8-epiloganic acid and cistanoside F, with their respective isomers, and syringin were more extractable by the cold maceration process. All extracts had similar antioxidant properties in scavenging DPPH radical and total antioxidant activity assays, with the exception of the ferric-reducing power activity assay, in which cold maceration exhibited a significantly less potent activity. Aqueous extracts of *C. phelypaea* roots present an interesting antioxidant potential which is related to the synergistic effects of several antioxidant compounds.

Abbreviations: A, absorbance; AAEs, ascorbic acid equivalents; ANOVA, analysis of variance; CA, caffeic acid; CE, collision energy; CMA, cold maceration; DEC, decoction; DPPH, 2,2-diphenyl-1-picrylhydrazil; EC_{50}, effective concentration at which the absorbance was 0.5; EIC, extracted ion current; FA, formic acid; FRAP, ferric-reducing antioxidant power; GAEs, gallic acid equivalents; IC_{50}, inhibition concentration 50%; INF, infusion; LC- ESI-MS/MS, liquid chromatography electrospray ionization tandem mass spectrometry; PhG, phenylethanoid glycoside; QEs, quercetin equivalents; RSA, radical-scavenging activity; RT, retention time; SEM, standard error of mean; TAA, total antioxidant activity; TFC, total flavonoid content; TPC, total phenolic content

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1. Introduction

The *Cistanche* genus, belonging to the *Orobanchaceae* family, contains 22 species that are perennial parasite plants mainly distributed in arid and semi-arid areas as well as deserts of the northern hemisphere (Jiang and Tu, 2009). Because of its excellent applications in traditional medicine and its nutritional benefits, Cistanches herba have been honored as "desert ginseng" (Tian et al., 2017).

Furthermore, pharmacological studies of these species revealed a wide range of biological activities including antiapoptotic (Wat et al., 2016), antihyperglycemic and hypolipidemic (Xiong et al., 2013), hepatoprotective (Guo et al., 2016), neuroprotective (Lin et al., 2008), modulation of the immune response (Zhang et al., 2018) and lifespan extension (Lin et al., 2017). These activities are mainly related to the antioxidant potential of the *Cistanche* species. The main phytochemical constituents of this genus are phenylethanoid glycosides (PhGs), iridoids, oligo- and polysaccharides, lignans, alditols and volatile oils. In particular, PhGs and polysaccharides have been reported to be strongly associated with the observed pharmacological activities of the *Cistanche* species (Jiang and Tu, 2009).

According to Quezel & Santa (1963), the genus *Cistanche* is represented in Algeria by three species: *C. phelypaea* (L.) Cout. (Syn. *C. lutea* (Hoffm.) Link.), *C. violacea* (Desf.) Beck. and *C. mauritanica* (Coss. and Dur.) Beck. *Cistanche phelypaea* is a Saharo-Mediterranean species appreciated for its nutritional and medicinal properties. Given its constant abundance regardless of the rainfall regime, this species was one of the most valuable nutritional sources widely used by nomadic and sedentary populations of the Algerian Sahara during periods of famine and drought (Gast, 2000). Nowadays, it is used as a food condiment and as a remedy for diabetes, abdominal pain, diarrhoea, muscle aches and agalactia (Hammiche and Maiza, 2006). Young roots are the most frequently used; they are consumed as a vegetable after boiling in water or roasting under hot coals. Powders are obtained by drying the macerate obtained after crushing the roots by stones or by drying directly after collection. Furthermore, decoctions are prepared for medical use (Gast, 2000; Benchelah et al., 2011).

Limited studies have been published about the bioactivities of *C. phelypaea,* reporting *in vitro* antioxidant and anticancer activities (Aboul-Enein et al., 2012; Elkamali and Hamed, 2015). Recently, it was reported that solvent extracts and some purified compounds exhibited inhibitory effects on butyrylcholinesterase, α-glucosidase, α-amylase, tyrosinase and monoacylglycerol lipase enzymes which are involved in some pathological alterations (Beladjila et al., 2018; Trampetti et al., 2019).

The extraction method may have a decisive impact on the phytochemical composition, and consequently the biological activities of the herbal extracts will be influenced. Decoction and infusion are two classical extraction methods that use heating at different contact times. The products are largely consumed, accepted, and added to human food. However, thermal decomposition could reduce the bioactivity of the extracts through the loss of heat-labile substances during the application of these methods. Maceration is another classical procedure without heating and requires a much longer contact time. In this case, as well, obtaining certain active compounds which require heating would be restricted (Pisoschi et al., 2016). Besides that, antioxidant properties of extracts are largely related to the differences in their quantitative and qualitative composition resulting from different extraction settings.

Not much scientific validation has been achieved for this species for its medicinal uses. Taking into account that this species is traditionally exploited in water extracts, the present work was conducted to compare the phytochemical composition and *in vitro* antioxidant capacity of *C. phelypaea* root extracts resulting from various aqueous extraction methods including decoction, infusion and cold maceration.

2. Material and methods

2.1. Plant collection

Young roots of *C. phelypaea* were collected from the Beni Abbes community (30° 4′ 48″ N, 2° 6′ 0″ W) located in the South-West of Algeria in January, 2015. This arid area is located in the Grand Oriental Erg. The specimens belong to *C. phelypaea*. The taxonomic identification of the plant was confirmed by Dr. Rachid Amirouche, a specialist in systematic botany at the University of Sciences and Technology Houari Boumediene, Algiers. The voucher specimen was placed in the Official Herbarium of the National Superior School of Agronomy (ENSA), Algiers, Algeria. The collected plant materials were washed and cut into small pieces, and then air-dried in the shade at room temperature. The dry material was ground to a fine powder and stored carefully until used.

2.2. Preparation of the extracts

Three different aqueous extracts were prepared using the conventional methods of decoction, infusion and cold maceration. To prepare the decoction (DEC), 5 g of the powdered dry roots were added to 200 mL of boiling distilled water, and boiled for 30 min under agitation. To prepare the infusion, (INF) 5 g of the powdered dry roots were added to 200 mL of boiling distilled water, and the mixture was left under agitation for 30 min. For the cold maceration (CMA) process, 5 g of the powdered dry roots were added to 200 mL of cold distilled water and left to macerate under agitation at room temperature for 24 h. After extraction, each extract was filtrated using gauze and centrifuged at 3000g for 30 min. Supernatants were lyophilized and then stored at -20°C for further analysis. Each extraction process was repeated on 3 different samples.

2.3. Phytochemical studies

2.3.1. Determination of total phenolic content

Total phenolic content (TPC) was determined according to the Folin-Ciocalteu method with minor modifications (Singleton et al., 1999). Briefly, a 0.2 mL aliquot, prepared by dissolving each dry extract in distilled water at a concentration of 1 mg/mL was mixed with 1 mL Folin-Ciocalteu phenol reagent and 0.8 mL sodium carbonate solution (7.5%,w/v). After 30 min of incubation in the dark, the absorbance of each mixture was read against a blank at 765 nm. A calibration curve was plotted using gallic acid as standard and total phenolic content was expressed as the mg of gallic acid equivalents/g of dry extract (mg GAEs/g extract).

2.3.2. Determination of total flavonoid content

The total flavonoid content (TFC) was determined using the aluminium trichloride colorimetric method of Subedi et al. (2014) with slight modifications. Therefore, 0.5 mL aliquot, prepared by dissolving each dry extract in distilled water at concentration of 0.5 mg/mL was mixed with 1.5 mL of distilled water and subsequently with 150 μL of sodium nitrite solution (5%, w/v). After a 5 min interval at room temperature, 150 μL of aluminium trichloride solution (5%, w/v) was added and allowed to stand for 6 more minutes before 500 μL of sodium hydroxide solution (4%, w/v) was added. The absorption of the mixture against the blank was immediately recorded at 510 nm. A calibration curve of quercetin was prepared under the same conditions and the total flavonoid content was expressed as mg of quercetin equivalents/g (mg QEs/g extract).

2.3.3. LC-ESI-MS/MS analysis

The phytochemical analysis of the different aqueous extracts of *C. phelypaea* roots was carried out by liquid chromatography - electrospray ionization - (tandem) mass spectrometry (LC-ESI-MS and MS/MS). Freeze-dried powders were dissolved in formic acid (FA) 0.1% (v/v) and filtered onto a sterilized PVDF hydrophilic membrane with pores of 0.45 μm (Millipore ®). After dilution, the samples were analyzed by an Agilent Technologies®1200 series capillary pump coupled with a dual ESI source on a 6520 Q-TOF mass spectrometer. Briefly, LC runs were performed on a reverse-phase ZORBAX Eclipse XDB-C18 column (Rapid Resolution HT, 2.1 × 50 mm, 1.8 μm, Agilent

Technologies®) in acidic conditions (FA 0.1%, v/v) applying a 43 min linear gradient from 5 to 55% (v/v) of acetonitrile with a flow rate of 150 μL min^{-1}. The analysis was performed in both negative and positive acquisition modes. The ESI source was set at 350°C and at 3500 V and 3000 V in positive and negative modes, respectively. Data acquisition was performed within a range from mass to charge (m/z) of 125 to 1500. Chromatographic peak interpretation was performed using the MassHunter Workstation software (version B.03.01, Agilent Technologies®). The compound assignments in negative mode were verified by targeted MS/MS analyses with an isolation width of m/z 4 and fixed collision energy of 30 or 40 V. The MS and MS/MS spectra were interpreted according to literature. The relative quantification of identified compounds was done on MS analyses by extracting the individual EIC (Extracted Ion Current, ± 20 ppm) in negative and positive mode. The amount of each compound was expressed as the relative percentage abundance with respect to the average value among all of the samples. Three samples were analyzed for each type of aqueous extraction (n = 3).

2.4. In vitro antioxidant activity

2.4.1. DPPH radical scavenging assay

A solution of DPPH (0.004%, w/v) radicals was freshly prepared and 1 mL of this solution was added to 1 mL of various concentrations of the extracts (62.5-1000 μg/mL). The mixtures were shaken and left to stand for 30 min in the dark. After that the absorbance was measured at 517 nm and the radical-scavenging activity (RSA) (DPPH discoloration) was calculated using the equation: RSA (%) = [(ADPPH - Asample)/ADPPH] x 100 (Gurnani et al., 2016). Results were expressed as IC_{50} values, calculated from the graph of RSA percentage against extract concentration. The former correspond to the extract concentration providing 50% of radical-scavenging activity. Ascorbic acid was used as the standard.

2.4.2. Ferric-reducing antioxidant power assay

The ferric reducing power assay (FRAP) was performed according to the method described by Gavamukulya et al. (2014) and Liao et al. (2015). 0.2 mL aliquots were taken from each extract in the range of 15.62 to 1000 μg/mL, and then mixed with 500 μL of sodium phosphate buffer (200 mM, pH 6.6) and 500 μL of potassium ferricyanide solution (1%, w/v). Mixtures were incubated at 50°C in a water bath for 20 min, and 500 μL of trichloroacetic solution (10%, w/v) were added. After that, mixtures were centrifuged at 3000g for 16 min and 700 μL of distilled water were added to 700 μL of the obtained supernatants, then 140 μL of ferric chloride (0.1%, v/v) were added and 10 min later the absorbance was read at 700 nm. The extract concentration that gave 0.5 absorbance (EC_{50}) was calculated from linear regression analysis. Ascorbic acid was used as the standard.

2.4.3. Total antioxidant activity

The total antioxidant activity (TAA) of the aqueous extracts was determined by phosphomolybdenum assay according to the modified method of Do et al. (2014). In brief, an aliquot (0.2 mL) of plant extracts was added to 1.8 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The samples tubes were incubated in a water bath at 90°C for 90 min, then cooled at room temperature and the absorbance was measured at 695nm. A calibration curve was expressed as mg ascorbic acid equivalents/g of extract (mg AAEs/g extract).

2.5. Statistical analysis

All the studies were conducted in three independent experiments using triplicate samples (n=3) and the values were averaged and expressed as mean ± standard error of mean (SEM). The statistical significance ($p < 0.05$) was analyzed using a one-way analysis of variance (ANOVA) followed by the Tukey pairwise multiple comparison test. Statistical analyses were performed using GraphPad Prism v. 8.0 (GraphPad software, Inc., La Jolla, CA, USA).

3. Results and discussion

3.1. Phytochemical studies

3.1.1. Total phenolic content

The total phenolic content (TPC) of the different aqueous extracts as determined by the Folin-Ciocalteu method and reported as gallic acid equivalents (GAEs, Table 1) showed that the DEC process allowed a significantly higher amount of total phenolics (92.45 ± 0.73 mg GAEs/g extract) to be obtained. There was no significant difference between INF (79.12 ± 1.27 mg GAEs/g extract) and CMA (78.59 ± 4.56 mg GAEs/g extract) procedures suggesting that, in our case, the short heat treatment applied during infusion did not enhance directly the release of phenolic compounds. On the other hand, the contact time of heating might be a predominant factor allowing a greater and better extraction, as exhibited by the larger amount of highly stable phenolic compounds extracted by DEC procedure, in which a longer boiling time is maintained than INF. To the best of our knowledge there are no studies available on TPC of *C. phelypaea.*

3.1.2. Total flavonoid content

The results of total flavonoid content (TFC, Table 1) revealed that DEC and INF extracts had similar amounts of flavonoids (14.26 ± 0.54 and 14.71 ± 0.30 mg QEs/g extract) while CMA had the lowest content (9.85 ± 0.55 mg QEs/g extract). Higher levels of TFC were obtained when extracts were prepared with hot water compared to those obtained at room temperature. This can be related to the high solubility of flavonoids at high temperatures.

In contrast to TPC, it seems that the time of heating did not influence the yield extraction of flavonoids. Moreover, considering that the TFC in INF was found to be higher than that in CMA, it seems that increasing the time of extraction at room temperature did not compensate for the absence of heating. This result does not correspond to that of TPC, since similar amounts of TPC were found in these two extracts, suggesting that other kinds of phenolic compounds might be more extractable by the CMA process. To our knowledge there is no report concerning the flavonoid composition of *C. phelypaea,* that is why there is a particular interest in the study of flavonoids.

3.1.3. LC-ESI-MS/MS analysis

The phytochemical investigation of different aqueous extracts from *C. phelypaea* roots was carried out by LC-ESI-MS, in both negative and positive acquisition modes. The compounds detected in the chromatographic profiles were assigned according to literature (Table 2). Quantifications of the characterized compounds were expressed as the relative percentage abundance for each compound

Table 1
Total phenolic (TPC) and flavonoid (TFC) contents in aqueous roots extracts from *C. phelypaea.*

Extract	TPC (mg GAEs/g extract)	TFC (mg QEs/g extract)
Decoction	92.45±0.73^{a}	14.26±0.54^{a}
Infusion	79.12±1.27^{b}	14.71±0.30^{a}
Cold maceration	78.59±4.56^{b}	9.85±0.55^{b}

Values represent the mean ± SEM of three separate experiments using triplicate samples in each (n=3). Different superscripted letters in the same column indicate significant differences ($p<0.05$). GAEs, gallic acid equivalents; QEs, quercetin equivalents.

Table 2
Compounds identified in aqueous extracts from roots of *C. phelypaea* by LC-ESI-MS.

N.	Compound	Formula	Formation mode	RT (min)	m/z	References
1	8-epiloganic acid (isomer A)	$C_{16}H_{24}O_{10}$	$[M-H]^-$	4.69 ± 0.10	375.13	Yoshizawa et al., 1990
2	6-deoxycatalpol	$C_{15}H_{22}O_9$	$[MNH_4]^+$	4.76 ± 0.10	364.16	Yoshizawa et al., 1990
			$[MNa]^+$		369.11	
			$[MK]^+$		385.09	
			$[2MNa]^+$		715.24	
3	Salidroside	$C_{14}H_{20}O_7$	$[MNH_4]^+$	5.36 ± 0.08	318.15	Yuejie et al., 2017
			$[MNa]^+$		323.11	
			$[MK]^+$		339.08	
4	8-epiloganic acid (isomer B)	$C_{26}H_{24}O_{10}$	$[M-H]^-$	5.88 ± 0.12	375.13	Yoshizawa et al., 1990
5	Bartsioside	$C_{15}H_{22}O_8$	$[M+H]^+$	7.24 ± 0.08	331.14	Yuejie et al., 2017
			$[MNH_4]^+$		348.16	
			$[MNa]^+$		353.12	
			$[MK]^+$		369.09	
			$[2MNa]^+$		683.25	
6	Cistanoside F (isomer A)	$C_{21}H_{28}O_{13}$	$[M-H]^-$	8.01 ± 0.17	487.15	Kobayashi et al., 1985
7	Gluroside	$C_{15}H_{24}O_8$	$[MNH_4]^+$	8.06 ± 0.13	350.18	Yuejie et al., 2017
			$[MNa]^+$		355.14	
			$[MK]^+$		371.11	
			$[2MNa]^+$		687.28	
8	Cistanoside F (isomer B)	$C_{21}H_{28}O_{13}$	$[M-H]^-$	8.35 ± 0.15	487.15	Tao el al., 2018 Quirantes-piné et al., 2009
9	Syringin	$C_{17}H_{24}O_9$	$[MNH_4]^+$	11.10 ± 0.06	390.17	Yuejie et al., 2017
			$[MNa]^+$		395.13	
			$[MK]^+$		411.10	
			$[2MNa]^+$		767.27	
10	Cistantubuloside C_1/C_2	$C_{35}H_{46}O_{21}$	$[MNH_4]^+$	15.68 ± 0.11	820.28	Yuejie et al., 2017
			$[MNa]^+$		825.24	
11	Acteoside	$C_{29}H_{36}O_{15}$	$[M-H]^-$	19.74 ± 0.10	623.20	Kobayashi et al., 1985
12	Isoacteoside	$C_{29}H_{36}O_{15}$	$[M-H]^-$	20.85 ± 0.09	623.20	Kobayashi et al., 1985
13	Campneoside II (isomer A)	$C_{29}H_{36}O_{16}$	$[M-H]^-$	16.97 ± 0.13	639.19	Imakura et al., 1985
14	Campneoside II (isomer B)	$C_{29}H_{36}O_{16}$	$[M-H]^-$	17.15 ± 0.13	639.19	Imakura et al., 1985
15	2'-acetylacteoside/Tubuloside B	$C_{31}H_{38}O_{16}$	$[M-H]^-$	22.58 ± 0.10	665.21	Li et al., 2016b
16	2'-acetylacteoside/Tubuloside B	$C_{31}H_{38}O_{16}$	$[M-H]^-$	24.09 ± 0.12	665.21	Li et al., 2016b
17	Echinacoside	$C_{35}H_{46}O_{20}$	$[M-H]^-$	17.41 ± 0.13	785.25	Kobayashi et al., 1985
18	Pheliposide	$C_{36}H_{46}O_{20}$	$[M-H]^-$	21.85 ± 0.10	797.25	Jedrejek et al., 2020
19	Kankanoside H_1/H_2	$C_{37}H_{48}O_{20}$	$[M-H]^-$	21.25 ± 0.08	811.27	Morikawa et al., 2010
20	Tubuloside A	$C_{37}H_{48}O_{21}$	$[M-H]^-$	19.93 ± 0.10	827.26	Tao el al., 2018 Chen et al., 2018

N.: chromatographic peak number. Formation mode: ions selected in negative ($^-$) or positive ($^+$) acquisition mode. RT: retention time (min). m/z: mass to charge ratio of the selected ion.

in the three different aqueous extracts, detected in negative and positive acquisition modes (Fig. 1 and 2). These results highlighted some quantitative variations in the chemical profiles of the three different aqueous extracts, DEC, INF and CMA.

In our study, the mass spectrometry analysis in negative mode revealed a complex pattern with 14 compounds, whereas in positive mode 6 compounds were detected. From a qualitative point of view, the analyses showed similar compositions of PhGs and iridoid

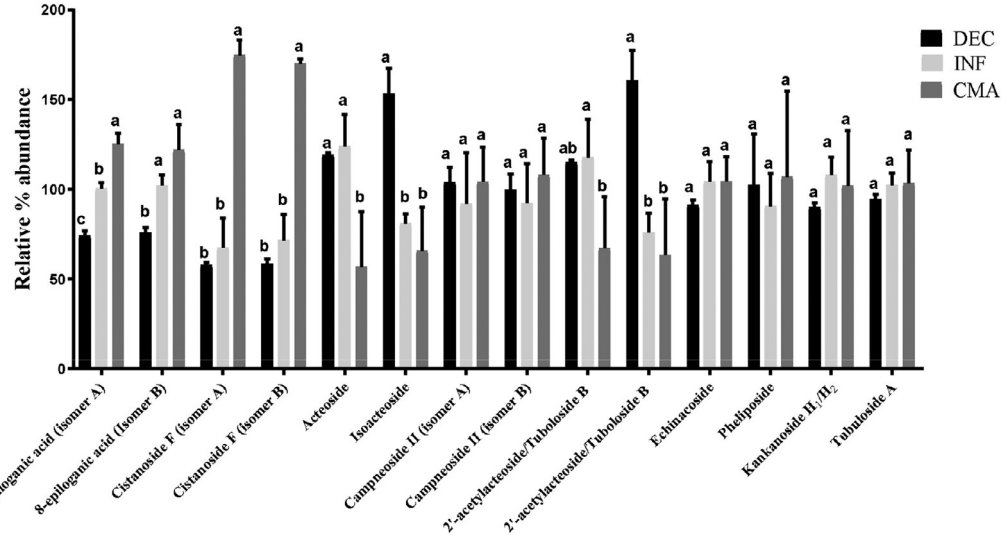

Fig. 1. Relative percent abundance of the identified compounds in different aqueous extracts from *C. phelypaea* roots by LC-ESI-MS analysis in negative acquisition mode.
DEC: decoction; INF: infusion; CMA: cold maceration. Values are the mean ± SEM (n = 3). Different letters indicated significant differences assigned according to one-way ANOVA and Tukey's *post-hoc* test ($p < 0.05$).

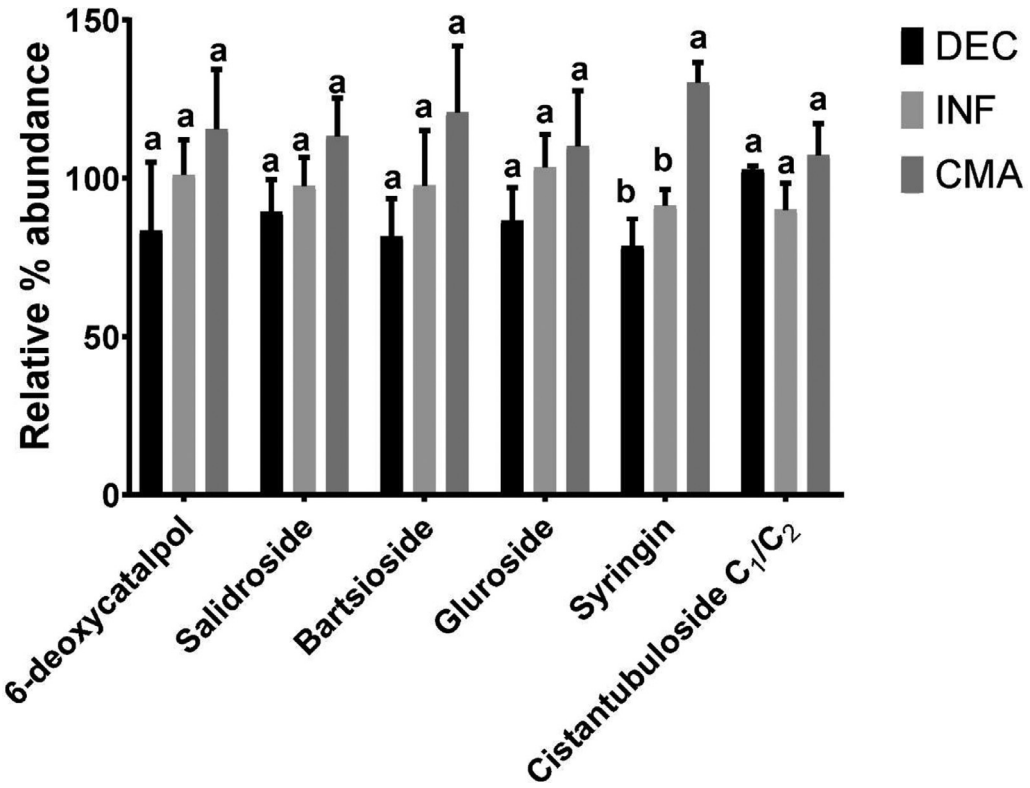

Fig. 2. Relative percent abundance of the identified compounds in different aqueous extracts from *C. phelypaea* roots by LC-ESI-MS analysis in positive acquisition mode.
DEC: decoction; INF: infusion; CMA: cold maceration. Values are the mean ± SEM (n = 3). Different letters indicated significant differences assigned according to one-way ANOVA and Tukey's *post-hoc* test ($p < 0.05$).

glycosides for the three different aqueous extracts. This observation is consistent with the previous phytochemical analysis of Cistanches herba, reporting that these compounds together with lignans, alditols, oligosaccharides and polysaccharides are the major phytochemical constituents of this genus (Jiang and Tu, 2009; Liu et al., 2013; Bougandoura et al., 2016; Song et al., 2016; Ahn et al., 2017; Yan et al., 2017; Fu et al., 2018).

The MS spectra of PhGs and iridoid glycosides showed that the analytes were detectable in positive mode as adducts with inorganic ions, such as $[MNH_4]^+$, $[MNa]^+$ and $[MK]^+$ (Table 2), as already highlighted for PhGs by other authors (Jiang et al., 2009). Probably, this behavior may have been further reinforced by the water-based extraction procedures adopted in our study. Conversely, in negative mode the prominent signals were observed as $[M-H]^-$ (Table 2), as previously reported (Song et al., 2019).

The chemical profiles obtained in negative ionization mode revealed the presence of compounds with the same molecular ions but different retention times (RTs, Table 2). In order to obtain additional information about these profiles, we performed an LC-ESI-MS/MS analysis (Table 3). In particular, it was possible to detect 5 pairs of isomers (compounds 1-4, 6-8, 11-12, 13-14 and 15-16), each of those couples having the same molecular ion and fragmentation profile. The LC-ESI-MS/MS analysis did not reveal any structural differences between isomers, indicating that other strategies, such as multiple MS analyses, NMR or the use of specific standards will be required for further characterization.

PhGs are water soluble compounds belonging to the polyphenols with a chemical structure characterized by a phenethyl alcohol (C_6-C_2) moiety linked to β-glucopyranose/β-allopyranose. In addition, substituents like aromatic acids and sugars are often linked to the core structure by ester or glycosidic linkages (Xue and Yang, 2016). Generally, PhGs of Cistanches herba are found as monosaccharide, disaccharide or trisaccharide glycosides. Moreover, a peculiar characteristic in this genus is that the PhG disaccharide form generally comprises a Glucose (3→1) Rhamnose linkage, where the glucose linked to the aglycone is usually substituted at C_4 or C_6 by a caffeoyl or coumaroyl moiety, while at the C_6 position, an additional glucose, or rhamnose, appears in the case of the trisaccharide glycosides (Jiang and Tu, 2009). Until now, 69 PhGs have been identified by HPLC-LTQ-orbitrap-MS through the analysis of three different species (Fu et al., 2018).

To specify, we were able to identify two monoglycosides in the positive mode analysis, corresponding to the PhG salidroside (compound 3) and syringin (compound 9) (Table 2).

In addition, 4 pairs of caffeoylated PhGs disaccharides were tentatively identified in the negative mode, each of them corresponding to a pair of isomers (compound 6-8, 11-12, 13-14 and 15-16). The MS/MS profile of these compounds is marked by the presence of at least one of the fragments at 179, 161 and 135 m/z, which were derived from the cleavage of a caffeic acid moiety (CA), and its subsequent dehydration and decarboxylation, respectively (Table 3, Wang et al., 2009). Consequently, the compounds 6 and 8 were identified as cistanoside F and its isomer. Interestingly, we were able to discriminate among the second pair of isomers, assigning the compound 11 to acteoside and the compound 12 to isoacteoside on the basis of their RTs (Table 3) and relative abundance (unpublished results). Indeed, according to what was proposed by different authors through the use of authentic standards, in reverse phase chromatography the former elutes first and is one of the major PhGs in the *Cistanche* species (Han et al., 2012; Shi et al., 2013; Cui et al., 2016; Li et al., 2016a). Moreover, the pair 13-14 was assigned to campneoside II and its isomer. This molecule is a β-hydroxylated form of acteoside that follows the typical fragmentation mode of the acteoside-type PhGs, with the exception of a distinctive ion at 151 m/z corresponding to a dehydrophenethanol moiety (Shi et al., 2013). Finally, compounds 15-16 were assigned to 2'-acetylacteoside/tubuloside B. According to the study of Li et al. (2016b), on the basis of the different RTs it is possible to speculate that the first chromatographic peak corresponds to 2'-

Table 3
Compounds identified in aqueous extracts from roots of *C. phelypaea* by LC-ESI-MS/MS analysis in negative mode.

N.	Compound	RT (min)	$[M-H]^-$ m/z	CE(V)	Fragment ions (m/z)	References
1	8-epiloganic acid (isomer A)	4.69 ± 0.10	375.13	30	213.07 - 169.08 - 151.07	Li et al., 2016b Song et al., 2016
4	8-epiloganic acid (isomer B)	5.88 ± 0.12	375.13	30	213.07 - 169.08 - 151.07	Li et al., 2016b Song et al., 2016
6	Cistanoside F (isomer A)	8.01 ± 0.17	487.15	30	179.03 - 161.02 - 135.04	Li et al., 2016b
8	Cistanoside F (isomer B)	8.35 ± 0.15	487.15	30	179.03 - 161.02 - 135.04	Li et al., 2016b
11	Acteoside	19.74 ±0.10	623.20	40	461.16 - 161.02 - 135.04	Cui et al., 2016 Li et al., 2016a
12	Isoacteoside	20.85 ±0.09	623.20	40	461.16 - 161.02 - 135.04	Cui et al., 2016 Li et al., 2016a
13	Campneoside II (isomer A)	16.97 ±0.13	639.19	30	621.17 - 529.14 - 459.14 179.03 - 161.02 - 151.03	Song et al., 2016 Shi et al., 2013
14	Campneoside II (isomer B)	17.15 ±0.13	639.19	30	621.17 - 529.14 - 459.14 179.03 - 161.02 - 151.03	Song et al., 2016 Shi et al., 2013
15	2'-acetylacteoside/ Tubuloside B	22.58 ±0.10	665.21	30	503.16 - 461.16 - 161.02	Li et al., 2016b Shi et al., 2013
16	2'-acetylacteoside/ Tubuloside B	24.09 ±0.12	665.21	30	503.16 - 461.16 - 161.02	Li et al., 2016b Shi et al., 2013
17	Echinacoside	17.41 ±0.13	785.25	40	623.21 - 161.02	Li et al., 2016b
18	Pheliposide	21.85 ±0.10	797.25	40	635.21 - 593.20 - 161.02	Jedrejek et al., 2020
19	Kankanoside H_1/H_2	21.25 ±0.08	811.27	40	769.25 - 665.22 - 647.21 623.21 - 605.20 - 477.16 145.03	Zhang et al., 2015
20	Tubuloside A	19.93 ±0.10	827.26	40	665.22 - 623.21 - 161.02	Li et al., 2016b Song et al., 2016

RT: retention time (min). $[M-H]^-$: mass to charge ratio (m/z) of the precursor ion. CE: collision energy (V).

acetylacteoside and the second one to tubuloside B. However, further studies are needed to verify this hypothesis.

Four PhGs trisaccharides were also identified in negative mode (Table 3). The first was echinacoside (compound 17), another major PhG along with acteoside in *Cistanche* species, while compound 20 was assigned to tubuloside A, an acetylated derivative of echinacoside. Compound 19 was assigned to kankanoside H_1/H_2 on the basis of an acetylated substitution and a coumaroyl moiety indicated in the MS/MS profile by the sequential losses of -42 m/z and by the diagnostic ion at 145 m/z, respectively (Table 3, Morikawa et al., 2010; Li et al., 2014). At the same time, compound 18 was identified as pheliposide. This molecule differs with respect to tubuloside A in being substituted by xylose rather than glucose (Andary et al., 1985). Based on the classical fragmentation mechanism of PhGs, we can suppose that pheliposide during fragmentation produced the ion at 635 m/z by losing its CA moiety, and the ion 593 m/z by the concurrent losses of CA and acetyl at C_2 position (Table 3). Finally, a caffeoylated trisaccharide PhG was identified as cistantubuloside C_1/C_2 (compound 10) in positive mode (Table 2).

In addition to PhGs, our results also allowed the characterization of five iridoids. This family is considered to be one of the most numerous cyclopentanoid monoterpene derivatives, and it occurs in the glycosides and aglycosides forms comprised in the *Cistanche* species (Bianco, 1994; Jiang and Tu, 2009). Three iridoid glycosides were found in positive mode by the LC/MS analysis, corresponding to 6-deoxycatalpol (compound 2), bartsioside (compound 5) and gluroside (compound 7) (Table 2). Moreover, one iridoid and its isomer were characterized in negative mode LC-ESI-MS/MS as 8-epiloganic acid (compounds 1-4) due to the neutral losses of glucose residue (213 m/z) and CO_2 (169 m/z, Table 3).

The majority of the phytochemical studies of Cistanches herba have focused on the *C. tubulosa, C. deserticola* and *C. sinensis* species but only a few reports about the *phelypaea* species are available. Our results are consistent with surveys showing mainly the same compounds (Melek et al., 1993; Deyama et al., 1995; Trampetti et al., 2019). In addition, cistanoside F, campneoside II, kankanoside H_1/H_2, cistantubuloside C_1/C_2 and 8-epiloganic acid are reported herein for the first time in this species. Until now, and to the best of our knowledge, pheliposide was reported only in the species *C. phelypaea* (Melek et al., 1993). Nonetheless, Deyama et al. (1995) as well as Trampetti et al. (2019), who studied the aerial parts from Qatar and the whole flowering plant from Portugal respectively, did not report its identification in *C. phelypaea*. However, these studies employed different periods of collection, as well as different solvents and methods of extraction and analysis. Overall, considering the fact that despite the large number of phytochemical studies pheliposide was not isolated or identified in other *Cistanche* species (Fu et al., 2018), it is possible to propose the use of this molecule as a typical chemotaxonomical marker of *C. phelypaea* within the *Cistanche* genus, at least for the root organ.

At the same time, in our study we could not identify the compounds tubuloside E and ajugol, previously described by Deyama et al. (1995); this can be due to several factors, including solvent extraction, plant organ, and/or the period of collection. In fact, these two compounds have been identified in the methanolic extract from the aerial parts of *C. phelypaea* collected in March. Interestingly, this difference suggests a possible variability in the synthesis of these secondary metabolites, according to the organ and stage of plant growth. In support of this hypothesis, Trampetti et al. (2019) have reported a variation in the secondary metabolite composition of the water extracts from different organs of *C. phelypaea*, showing that PhGs, dominated by echinacoside, were found mainly in roots while iridoids, i.e., bartsioside, ajugol and gluroside, were more abundant in flowers. They also mentioned that the *C. phelypaea* stems contained both PhGs (essentially tubuloside A and 2'-acetylacteoside) and iridoids. Recently, four new PhGs, trisaccharide glycosides carrying coumaroyl substituents have been isolated from a butanolic extract of the aerial part of *C. phelypaea,* growing in the southwest of Algeria (Beladjila et al., 2018).

As we have described, in our study the same qualitative chromatographic pattern was found for all the three kinds of extraction. However, an obvious difference was observed in the relative abundance for some compounds. These compounds include 8-epiloganic acid and its isomer, cistanoside F and its isomer, as well as acteoside, isoacteoside, two isomers of 2'-acetylacteoside/tubuloside B and syringin (Fig. 1 and 2).

The compounds acteoside, isoacteoside and 2'-acetylacteoside/ tubuloside B were significantly higher in abundance in DEC and/or INF than in CMA (Fig. 1). This can be attributed to their high solubility in hot water. In addition, isoacteoside and one of the two isomers of

2'-acetylacteoside/tubuloside B were extracted more efficiently by DEC than INF (Fig. 1). These results suggest the requirement of a longer contact time in water at boiling temperature for a better extraction of these PhGs disaccharides. Interestingly, in this case, all of these compounds, having an identical behavior in modified temperature extraction procedures, are structurally homologous. Their chemical structures are similar, since they all consist of three chemical moieties: CA, hydroxytyrosol (3.4 dihydroxyphenethyl alcohol) phenylethanoid aglycone, and rhamnose.

However, the CMA procedure showed more efficiency in the extraction of 8-epiloganic acid and cistanoside F, with their respective isomers (Fig. 1) and syringin (Fig. 2). These results suggest that the 8-epiloganic acid, cistanoside F and syringin are extractable mainly at an ambient temperature because of their thermal instability.

3.2. In vitro antioxidant activities

Several pathological alterations such as cancer, diabetes, degenerative disorders and inflammation are strongly related to oxidative stress, which is the result of an imbalance between endogenous body antioxidant systems and free radical production, leading to the cellular damage of macromolecules (Kohen and Nyska, 2002). Thus, exogenous antioxidant compounds taken as food and as extracts from medicinal plants may support the endogenous body antioxidant systems to fight oxidative damage and, consequently, can be used as chemopreventive and reducing agents against oxidative stress-induced alterations (Costamagna et al., 2013).

The evaluation of the broad antioxidant activities of plant extracts needs several antioxidant test systems because of the phytochemical complexity of bioactive compounds that respond in a different manner to the variable assay mechanisms. For this purpose, three *in vitro* antioxidant methods were used to evaluate both the scavenging and reducing ability of the aqueous extracts from *C. phelypaea* roots.

3.2.1. DPPH radical scavenging activity

Assessment of the scavenging potential was performed by the widespread DPPH system. This standard and easy colorimetric method reflects the scavenging property of an antioxidant molecule, through its ability to transfer both the hydrogen atom or electron to the stable nitrogen radical $DPPH^{\cdot}$ (purple) which is reduced to $DPPH_2$ (yellow), resulting thus in absorbance change. The measurement of the amount of unreacted $DPPH^{\cdot}$ is correlated to the antioxidant's capacity.

Our results showed that the DPPH assay revealed a marked scavenging potential of the different aqueous extracts, as increased concentration improved the percentage inhibition of the DPPH radical in a dose-dependent manner (unpublished results). Estimation and comparison of sample scavenging activities were carried out by IC_{50} values. As shown in Table 4, no difference between them indicates that aqueous roots extracts of *C. phelypaea* exhibited the same scavenging potential in the DPPH system. Our results are in agreement with previous studies which reported high radical scavenging activities towards the DPPH radical for different extracts of Cistanches herba (Elkamali and Hamed, 2015; Peng et al., 2016; Wang et al., 2017).

Comparative solvent extraction studies for *C. phelypaea* have shown that the best DPPH radical scavenging potential is exerted by the water extracts of different parts as well as of the whole plant (Aboul-Enein et al., 2012; Trampetti et al., 2019). In addition, studies performed by Trampetti et al. (2019) regarding the scavenging activity of stems, roots and flowers of *C. phelypaea* towards different radicals revealed that the water extracts from roots showed the highest DPPH scavenging potential with an IC_{50} value of 0.37mg/mL, which is higher than our IC_{50} for the three aqueous extracts found in the current study.

Compounds identified in our study, mainly PhGs, have been reported to exert from moderate to potent scavenging activity when isolated from the *Cistanche* genus and other plants, as some of them have shown equal or lower IC_{50} values than standards like α-tocopherol and BHT (Xiong et al., 1996; Si et al., 2013). Furthermore, the sequence of the strength of the scavenging potential for these compounds was variable. For instance, Xiong et al. (1996) have isolated 9 PhGs from *C. deserticola*, among which tubuloside B was shown to have the strongest DPPH scavenging potential. At the same time, 2'-acetylacteoside has been reported to exhibit the best scavenging potential among 6 PhGs isolated from *C. salsa* (Yang et al., 2009). In these studies, the scavenging potential of molecules has been related to the presence of catechol groups, the number and position of hydroxyl groups, the presence of 2-acetyl in the middle glucopyranose, and steric hindrance. Jedrejek et al. (2020) also reported that PhGs with the caffeoyl moiety exhibited stronger scavenging activities than those with feruloyl and *p*-coumaroyl ones.

Iridoids have also been reported to exert a DPPH scavenging potential, which is increased by dihydroxybenzoyl and caffeoyl substitutions (Jensen et al., 2010).

3.2.2. Ferric-reducing antioxidant power assay

The FRAP assay was carried out in order to evaluate the ability of the different aqueous extracts to reduce the ferric ion (Fe^{+3}) to ferrous (Fe^{+2}). Similarly to the DPPH system assay, the reducing activity increased by increasing sample concentration (unpublished results) indicating a good reducing power of the analytes.

As we can see from the EC_{50} values displayed in Table 4, no significant difference was observed in the EC_{50} values of DEC and INF, indicating similar reducing potentials. However, CMA extract exhibited a significantly lower reducing power among the samples, suggesting that some antioxidant molecules might be extracted better by hot water. Earlier studies revealed a good reducing power by the FRAP assay for different Cistanches herba extracts (Xiong et al., 2013; Peng et al., 2016; Piwowarczyk et al., 2020). Nevertheless, the data from these studies cannot be compared to ours, because the values were expressed differently. So far, to the best of our knowledge, no studies have been conducted on the potency reduction of the *phelypaea* species. The reducing capacity of plant extracts is related to the oxidizability of the chemical compounds that are able to transfer

Table 4
Antioxidant activities of different aqueous extracts from *C. phelypaea* roots.

Extract	DPPH scavenging activity IC_{50} (μg/mL)	FRAP assay EC_{50} (μg/mL)	TAA assay mg AAEs/g extract
Decoction	19.545 ± 0.993[a]	321.6± 6.87 [b]	319.93 ± 3.26[a]
Infusion	19.061 ± 0.211[a]	327.8± 16.01[b]	326.92 ± 10.64[a]
Cold maceration	22.748 ± 1.498[a]	458.6± 20.56 [a]	324.21 ± 5.78[a]
Ascorbic acid	02.20 ± 0.068[b]	100± 01.05 [c]	–

Values represent the mean ± SEM of three separate experiments using triplicate samples (n=3). Different superscripted letters in the same column indicate significant differences ($p<0.05$). DPPH, 2,2-diphenyl-1-picrylhydrazil; FRAP, ferric-reducing antioxidant power; TAA, total antioxidant activity; IC_{50}, inhibition concentration 50%; EC_{50}, Effective concentration at which the absorbance was 0.5; AAEs, ascorbic acid equivalents.

electrons, and it was reported that the most efficient reducing capacity is obtained by compounds extracted with water from different plants (Wong et al., 2006; Akhtar et al., 2018).

3.2.3. Total antioxidant activity

TAA was evaluated by the phosphomolybdenum method, based on the reduction of Mo (VI) to Mo (V) by the extract at acidic pH. All the tested extracts showed a great antioxidant capacity with no significant difference in their values (Table 4). To the best of our knowledge, no TAA values have previously been reported for *C. phelypaea.*

Our results show a similar trend of TAA and DPPH tests. However, despite the fact that FRAP and TAA are based on the reduction properties of compounds different trends were obtained in this study, indicating that different reaction conditions, as well as different metal reducible ions, involve different antioxidant molecules contained in the extracts.

Generally, our results suggest that the quantitative differences in some PhG and iridoid abundances determined by LC-ESI-MS (Fig. 1 and 2) as well as in the amounts of TPC and TFC (Table 1) in the three water extracts did not influence their antioxidant potential, except for the FRAP assay. Overall, several authors reported that phenolic compounds are not permanently associated with the antioxidant potential of a sample and that other compounds are also good antioxidants. Wang et al. (2017) showed a positive correlation between total PhG content and antioxidant activity of cultivated *C. deserticola*. However, certain samples with high PhG content, such as inflorescence, axis and corolla extracts, did not show a high antioxidant potential.

Despite the quantitative differences for some compounds highlighted by LC-ESI-MS (8-epiloganic acid and isomer, isoacteoside, and 2'-acetylacteoside or isomer tubuloside B, Fig. 1) and in the TPC (Table 1), similar behaviors were observed in all tested antioxidant systems for hot extraction methods (DEC and INF), suggesting that the time of heating-extraction had no effect on the antioxidant capacity. However, when we considered the temperature parameter for water extraction, we were able to find that CMA had less extractive ability than hot processes for some molecules (i.e. acteoside, isoacteoside, 2'-acetylacteoside/tuboloside B and TPC/TFC, Table 1 and Fig. 1). CMA also exhibited lower ferric reducing capacity, a result probably indicating that the higher antioxidant activity of extracts obtained with hot water can be mainly attributed to those compounds. Nevertheless, CMA showed the same potential as hot procedures in scavenging the DPPH radical as well as in the TAA tests. These results most likely reflect that the antioxidant compounds of the extract act in a complementary and synergistic manner. In fact, although CMA is not as effective as hot methods in the extraction of the previously mentioned compounds, it seems that other compounds (8-epiloganic acid isomers, cistanoside F isomers and syringin) are more extractable by this method, and, interestingly, all of them have been described as good DPPH scavengers (Pan and Hori, 1996; Us et al., 2015).

Finally, it is possible that other bioactive compounds contained in the crude extracts, that have not been identified in our analysis and that contribute to antioxidant capacity, were differentially extracted, during the three tested procedures. In fact, the extraction of polysaccharides known as major constituents of Cistanches herba is influenced by temperature. Zhang et al. (2016) demonstrated that the increase in temperature between 30 to 50 °C during 40 min increases polysaccharide yield from *C. tubulosa* stems. Additionally, it was found that these polysaccharides exert a considerable antioxidant activity.

4. Conclusion

In this study, the obtained results revealed that aqueous extracts from *C. phelypaea* roots prepared by decoction, infusion and cold maceration methods showed the same qualitative PhG and iridoid phytochemical profiles when analyzed by the LC-ESI-MS analysis. Nonetheless, quantitative differences in the abundance of some individual compounds as well as in phenolic and flavonoid contents were observed. Interestingly, the influence of temperature and the extraction time was not related to significant variations in antioxidant capacity, except for the cold macerate which showed the weakest ferric reducing power. Altogether, our findings suggest a synergistic antioxidant effect of the phytochemical components of these complex mixtures, although further studies should be performed to identify other antioxidant compounds of interest from these extracts. Decoction and infusion preparations can be used as good alternative dietary supplements, and along with maceration, these procedures can provide pharmaceutical, nutritional and cosmetic products.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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