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Research article

Impact of ultra-high dilutions from Iranian endemic and commercial *calendula* on the germination and growth quality of *Oryza sativa* L.

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ABSTRACT

Purpose: This article deals with producing ultra-diluted compounds (UHDs) prepared from Iranian calendula's characteristic and endemic species. It compares their chemical, biological and biochemical characteristics with the commercial sample of calendula species (grown in the Alps). In the following, these UHDs have been used to improve the quality of germination and growth and reduce contamination of rice (*Oryza sativa*) seeds in the laboratory environment.
Methods: High-performance thin-layer chromatography (HPTLC) is used to isolate the active compounds. On the separated results, antioxidant and antibacterial were identified directly on the plate (Bio-autographic method). Direct on the plate)DESI mass spectrometry was used to identify the active compounds.
Results: The HPTLC reveals that the chromatogram of native *C. percica* and *C. officinalis* extract is the most similar to the commercial compounds. The highest antioxidant activity is related to *C. officinalis*. The best antibacterial activity of the extracts against *Staphylococcus aureus* and *Escherichia coli* belongs to *C. officinalis* and *C. tripterocarpa*. Rutin, quercitrin, β-campstrole and di-*o*-caffeoylquinic acid, which are among the flavonoid and terpenoid categories were identified as active compounds. The prepared UHDs from native calendula are biologically more effective than the commercial ones in increasing seed germination efficiency, improving rooting quality and reducing contamination.
Conclusion: Using UHDs increases the production of photosynthetic pigments the root length and the number of lateral roots. Also, the amount of protein, gibberellic acid and abscisic acid in seedlings treated using native UHDs of *C. officinalis* (native or commercial) is higher than the others.

1. Introduction

Rice is the most important food source, employment and livelihood for more than one billion households in Asia, Africa and America. To fulfill the need of growing population further research is required to increase production, improve efficiency and reduce

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production costs. Rice has minerals, vitamins and fibre and provides 15 percent protein and 21 percent global energy. Recently, rice sprouts have gained attention as a healthy and affordable source of proteins, carbohydrates, minerals and vitamins [1]. Due to their high nutritional value, people consume them as a nutritious food globally.

The FAO (Food and Agriculture Organization of the United Nations) report says climate change, modernity and urbanization are reducing the quality and productivity of arable land. There is also a lack of natural resources. These factors could lead to a food shortage [2,3]. To solve the problem of not enough food, the amount of production can increase [4,5]. The heavy use of water, soil and energy in agriculture affects the environment and cannot be fixed. To maintain a healthy ecosystem and achieve long-term economic production, it is crucial to improve balance, reduce waste and enhance soil potential [6,7]. The production of healthy and high-quality seedlings is the key to guaranteeing the final production. Scientists believe that increasing the rate of seed germination and plant growth can meet the world's food needs.

Plants grow well when germinating and are not affected by temperature, oxygen, water, soil, or microorganisms. They need to be healthy, fertile and strong. According to Corbineau et al. [8], the quality of seed growth improves during germination and early seedling growth. Seed and soil contamination by bacteria, microorganisms and fungi can reduce germination recovery, [8]. To ensure good germination, researchers use chemicals like fungicides or insecticides to protect seeds [9–11].

Rice can be affected by various harmful organisms, such as *Magnaporthe oryzae*, *Rhizoctonia solani*, *Sarocladium oryzae*, *Burkholderia glumae* and *Xanthomonas oryzae*. These pathogens can cause a decrease in rice quality and yield by over 70 percent [12]. Traditional and chemical methods used to handle these pathogens contaminate water and soil. They harm beneficial bacteria in the soil and reduce long-term production efficiency. Non-organic products like chemicals, synthetics and GMOs are also problematic [13]. Researchers, officials and farmers are very interested in new methods and believe they are necessary. Researchers have found a new effective method called ultra-high diluted compounds (UHDs). The UHDs usage improve germination and recover pathogens damage [14–16]. Researchers have used these compounds to make *Pisum sativum* L. stems longer and help wheat seedlings grow and survive. They have also made wheat (*Triticum aestivum* L.) bacteria-resistant and controlled *Sclerotinia sclerotiorum* infection in *Phaseolus* vulgaris. Additionally, the compounds have reduced the number of *Meloidogyne incognita* in *Solanum lycopersicum* and boosted the activity of defense enzymes in the same plant. They have also stopped the growth of *Alternaria solani* fungus on *Solanum lycopersicum* L. and improved the germination and characteristics of *T. aestivum* [10,17].

One of the most useful UHDs in this field is made of the aqueous-alcoholic extract of *Calendula officinalis*. The *C. officinalis* 3000X dilution increases in the speed and quality of seedling growth, rooting and resistance to pathogens. Researchers found that the UHD of *C. officinalis* affects growth stages, boosts seed germination, improves root quality and reduces damage during transplantation. Commercial *C. officinalis* UHD increases chlorophyll production, shoot height strength and root length and number [18,19]. *Calendula contains* different substances like flavonoids, terpenoids, alkaloids and glycosides. The levels of these substances vary depending on the plant part and the region in which it grows [19]. The commercial *C. officinalis* is made from a plant in the Alps mountains. It is imported to Iran at a high price, which is not affordable for Iranian farmers and applicants. However, Iran has over 8000 endemic plants and various species of *calendula* [20,21].

The research aimed to compare the phytochemical and biological characteristics of the aqueous-alcoholic extract of Iranian calendula species with the commercial extract. Further, the impact of prepared UHDs on rice seed growth was studied. Also, their effects on seed germination, quality, contamination levels and early seedling were established.

2. Materials and methods

2.1. Materials

Methanol, Chloroform, toluene, ethyl acetate, ethanol, formic acid, acetic acid and HPTLC plates (on pre-coated bare silica 5 μm gel aluminum plate 60F254) were purchased from Merck Co. (Germany). Rice-specific hydroponic growth cultivation media: Yoshida, DPPH, BHT, Na_2HPO_4 and NaH_2PO_4 were purchased from Sigma Aldrich Co. (Germany). Placebo (as a control material), was obtained in the same size of granules, from Boiron Co., (USA). Bradford assay kit and Gibberellic acid/Abscisic acid assay kit were provided by BioRad Co., (USA) and MyBioSource, Inc. (Canada), respectively.

Three species of *Calendula* were collected from the mountains of Hamedan (*C. tripterocarpa* Rupr., 34°42′35.3″N 48°31′01.6″E, Herbarium code: MPH-2947), Gilan (*C. persica* C.A.Mey., 37°00′02.1″N 50°14′03.8″E, Herbarium code: MPH-2944) and Fars province (*C. officinalis*, 29°40′14.0″N 52°24′13.3″E, Herbarium code: MPH-2946) in May and June 2021 (Fig. 1). Samples were collected from aerial parts, including stems, leaves and flowers. Sampling was done so that each plant base was not completely removed from the ground, was not damaged and could grow again. Experts from Shahid Beheshti University's Medicinal Plants and Drugs Research Institute identified and coded the genus and species of plants (Flora Iranica collection, identification, and herbarium procedures protocol). The dried samples were powdered and subjected to extraction and study.

Oryza sativa L. (cv. IR651: Hashmi variety) seeds were obtained from Sari University of Agricultural Sciences and Natural Resources, Agricultural Genetics and Biotechnology Research Institute of Tabarstan (Mazandaran, Iran). The bacterial strains *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 6538 were obtained from the Iranian Research Organization for Science and Technology (IROST).

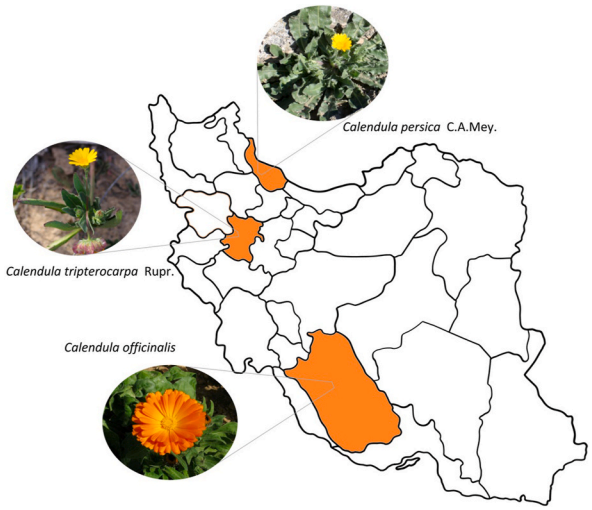

Fig. 1. Geographical map of the studied *calendula* species.

2.2. Instrumentations and methods

2.2.1. Extraction and primary chemical study (UV–Vis and IR) of calendula species

Plant extraction was performed exactly according to the Homeopathy pharmacopeia using a water-alcohol solution for 24 h using the maceration technique. Then, the phytochemical characteristics of the prepared extracts and commercial *C. officinalis* (at the same concentration of 2.5 mg/mL) were analyzed. Based on pharmacopeia and for spectroscopic analysis, to study the ultraviolet–visible (UV–Vis) absorption pattern of the extracts, UV-2501PC absorption device equipped with tungsten and deuterium lamp, UV-Probe software manufactured by the company (Shimadzu Scientific Instrument Co., Japan) and 1 cm quartz sample container were used. For the infrared absorption analysis of the extracts FTIR-Tensor 27 device (Bruker Co., Germany) was used.

2.2.2. HPTLC separation of calendula species

Sample solutions for HPTLC analyses were spotted (20 μL) in the form of bands of 8 mm width with a CAMAG 100 mL syringe on an HPTLC plate and a Linomat V (CAMAG Co., Switzerland) sample applicator with the help of nitrogen gas pressure. A constant application rate of 200 nl/s was employed and the space between the two bands was 1 cm. The spots of the plant extracts were measured using different solvent systems. The plates were monitored under UV–Vis detection at 365 nm. The best mobile phase system for methanol extract separation is composed of chloroform, toluene, ethyl acetate, ethanol, water and formic acid at a ratio of 11:7:56:22:3:1. These developed plates were used to investigate direct on-plate antioxidant and antibacterial bio-autography.

2.2.3. Direct on plate anti-oxidant (DPPH) bio-autography analysis of calendula species

The DPPH radical scavenging capacity of each extract was determined basically according to the method of Brand-Williams with some modifications [22–24]. The DPPH antioxidant inhibition study method was used directly on the developed HPTLC plate to determine the antioxidant activity. In detail, the developed plate was soaked for 2 s in a fresh $DPPH^{\bullet}$ solution in methanol (6×10^{-5} M) and placed in a dark place for 10 min. The plate was then dried at 40 °C. The spots on the plates turned purple to the yellow and white, indicating medium and high antioxidant activity, respectively.

2.2.4. Direct on plate anti-bacterial bio-autography analysis of calendula species

In vitro antimicrobial activity of extracts was assessed against the *S. aureus* (PTCC1431) and *E. coli* (PTCC1399). Micro-broth dilution susceptibility tests were used according to the standard protocols of NCCLS with some modifications [25]. The inoculants of the microbial strains were prepared from freshly cultured bacteria that were adjusted to 0.5 McFarland standard turbidity using sterile normal saline. They were then further diluted (1:100) by adding sterile Mueller-Hinton before adding them to the trays. In this method, the developed HPTLC plate was placed in not warm nutrient agar at 37 °C for 12 h. Then 100 μL of the bacterial suspension was placed on the agar and the plate was incubated for 18 h at 37 °C. The results were checked using 0.22 μm filtered resazurin aqueous solution (10 percent w/v) as cell viability assays for bacteria as the pink to blue color variations were monitored [26–28].

2.2.5. DESI mass spectrometry analysis of active compounds

In this study, the desorption electrospray ionization source, suitable for a time-of-flight mass spectrometer (LCT Waters Scientific, Milford, MA), was used. The desorption electrospray system was designed domestically, with standard capabilities and equipped with adjustable and changeable probe angles. The distance to the surface and the spectrometer's entrance, along with a digital microscope with a resolution of X 500. A probe nozzle with an inner diameter of 400 μm and a silica fuse capillary with an inner diameter of 160 μm

and an outer diameter of 360 μm was used. The inlet of the spectrometer was a stainless-steel tube with an inner diameter of 10 mm and an outer diameter of 30 mm, along with a thermal cover with the ability to control heat up to 350 °C, as well as a potential difference source with a variable power of 1–11 kV were used in this study.

2.2.6. Iranian calendula species ultra-high dilution preparation

Centesimal method (based on Korsakov dilution methodology), which is one percent dilution, was used to prepare ultra-high diluted compounds (UHDs) [29]. In detail, 1 mg of extracts powder diluted in 1 mL of alcoholic water (75 percent). This solution is diluted 100 times using ultrapure water, which is repeated up to 30, 100 times or more. The resulting dilution (1/100th of the concentration) is the first power 10^{-2}. Each higher power should have 1/100th of the concentration of the power below it. In this research 10^{-60} were prepared. To prepare the UHDs based on the pharmacopeia, the process was performed in a glass container, away from metal and electronic devices and in a dark place. 10 μL of each prepared extract (1mg/mL) was mixed with 990 μL of pure water and the solution was tap and shaken 100 times. Then, 10 μL of that was mixed with 990 μL of water again and repeated 30 times to prepare UHD Calendula C30 (10^{-60} dilution). The prepared UHDs container was covered with foil and kept in a dark place at room temperature.

2.2.7. Oryza sativa cultivation and treatment

A conventional laboratory culture was performed in a sterile process. The autoclave was applied at 121 °C at a pressure of 1.1 bar for 20 min. To sterilize rice seeds, they were soaked in 70 percent ethanol aqueous solution for 30 s and then washed 3 times with sterile water for 5 min each time. Seedlings (7 seeds) were placed in Petri dishes (ID: 7 cm) containing 7 mL of water/UHDs [18,30]. Petri dishes were kept at 27 ± 0.5 °C in a dark place for four days. On the fifth day for the seedlings' growth and chlorophyll production, the dishes were transferred into light and kept for at least three days (second stage of growth). At the third stage of growth and for continuing the study on the growth of the seedlings (5 per pot), they were transferred into a bottle (1L) containing 100 mL of agar solidified rice-specific hydroponic growth cultivation media, Yoshida. The root may incur damage during the transfer process; during this step, 2 mL of UHD was applied. Seedlings were cultivated under constant and controlled conditions in a phytotron, with a temperature of 27 ± 0.5 °C, a photoperiod of 16 h, and a photon flux density of 220 mM/m^2/s for a duration of 20 days. Then, the seedlings were collected and after the physiological information was recorded, they were used for chemical and material studies.

2.2.8. Oryza sativa physiological and growth quality analysis

To analyze the total carbohydrate content, 100 mg of dried root/shoot powder was extracted using 80 percent ethanol for 60 min. Five mL of HCl 1.1 percent was added into the supernatant and the mixtures were heated in a water bath (97 °C) for 30 min. The samples (1 mL) were mixed with 5 mL of ice-cold anthrone reagent (72 percent sulfuric acid containing 0.2 percent anthrone) and reheated (11 min). The solution's absorbance was measured at 630 nm [29]. The total protein content of 0.1 g tissues was extracted over the TRIzol reagent protocol. Thereafter, the protein concentration was determined according to the Bradford assay kit comparing to BSA as the standard [30]. For all photosynthetic pigments, 20 mg of plant powder was extracted using dimethylsulfoxide (DMSO) at 70 °C for 60 min. The absorbance of extract at 470, 646 and 663 nm was measured. The chlorophyll "a" (Chl a) and "b" (Chl b) contents, and the carotenoid content were determined using equations (1)–(4), where DW is the initial dry weight.

$$Cl_a = \frac{[(13.36 \times A_{663}) - (5.19\, A_{646})] \times 8.1}{DW} \quad (1)$$

$$Cl_b = \frac{[(27.43 \times A_{646}) - (8.12\, A_{663})] \times 8.1}{DW} \quad (2)$$

$$Cl_{Total} = \frac{[(5.24 \times A_{663}) + (22.24\, A_{646})] \times 8.1}{DW} \quad (3)$$

$$Pigment = \frac{[(4.785 \times A_{470}) + (3.657\, A_{663}) - (12.76\, A_{646}] \times 8.1}{DW} \quad (4)$$

2.2.9. Oryza sativa growth hormone analysis

Gibberellic Acid (GA) Elisa Kit and Abscisic acid (ABA) Elisa Kit were used to perform this test. These kits are based on enzyme-linked competitive immunosorbent assay technology. An antibody is pre-coated on a 96-well plate. Standards, test samples and reagents conjugated with biotin are added to the wells and incubated at 37 °C for 30 min. A competitive inhibition reaction between biotin-labeled ABA and unlabeled ABA, as well as biotin-labeled GA and unlabeled GA, was performed on the pre-coated antibody. Then the HRP conjugate reagent was added and the entire plate was incubated again. Unbound conjugates were removed using a wash buffer at each step. 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate was used to quantify the enzymatic reaction of HRP. After adding the TMB substrate, only the wells containing sufficient ABA and GA produce a blue-colored product, which changes to yellow after adding an acidic stop solution. The intensity of the yellow color was inversely proportional to the amount of ABA and GA bound on the plate. The optical density of OD was measured by spectrophotometry at a wavelength of 450 nm in a power wave device [31].

3. Results

3.1. Phytochemical analysis of calendula species

The UV absorption pattern of Iranian calendula extracts (3000 ppm) was analyzed in comparison to the commercial case. In Fig. 2A, the native sample exhibits the most similar UV absorption pattern to the commercial sample, with C. persica displaying the lowest absorption and *C. tripterocarpa* the highest. Notably, all three native plant extracts exhibit absorption at 298 nm, akin to the commercial ones (Fig. 2A).

Subsequently, we delved into the native extracts and commercial samples in the infrared (IR), as depicted in Fig. 2B. Across all samples, peaks within the 1600–1640 cm^{-1} range indicate carbonyl groups, ketones, aldehydes, acids, and esters. Peaks in the 3500–13300 cm^{-1} range correspond to possible bands of alcoholic and phenolic OHs. Additionally, prominent peaks at 2900 are likely associated with acidic OH. Moreover, *C. officinalis* and *C. persica* extracts generally exhibit the greatest similarity to the commercial sample. Vibrations in the wave number range of 3000–3700, representing OH and NH single bonds, closely resemble those of the commercial sample. Similarly, a likeness is observed in the wave numbers 2800 to 2900, corresponding to stretching vibrations of single CH bonds. Within the wave number range from 1500 to 1700, similarities emerge between the commercial sample and all three native ones, indicating double bonds of carbon, oxygen, and nitrogen. Vibrations of single bonds in carbon, oxygen, nitrogen, and carbon within the range of 1000–1500 also display similarities, particularly between *C. persica* and commercial samples, as well as between *C. officinalis* and commercial samples. In the fingerprint range of the IR spectrum pertaining to wave numbers below 1000, *C. officinalis* and commercial samples exhibit the highest resemblance.

For the bio-autography study, we utilized the HPTLC method to isolate chemical compounds from extracts, emphasizing the critical role of the mobile phase in thin-layer chromatography optimization. Solvents selected for chromatography should possess low

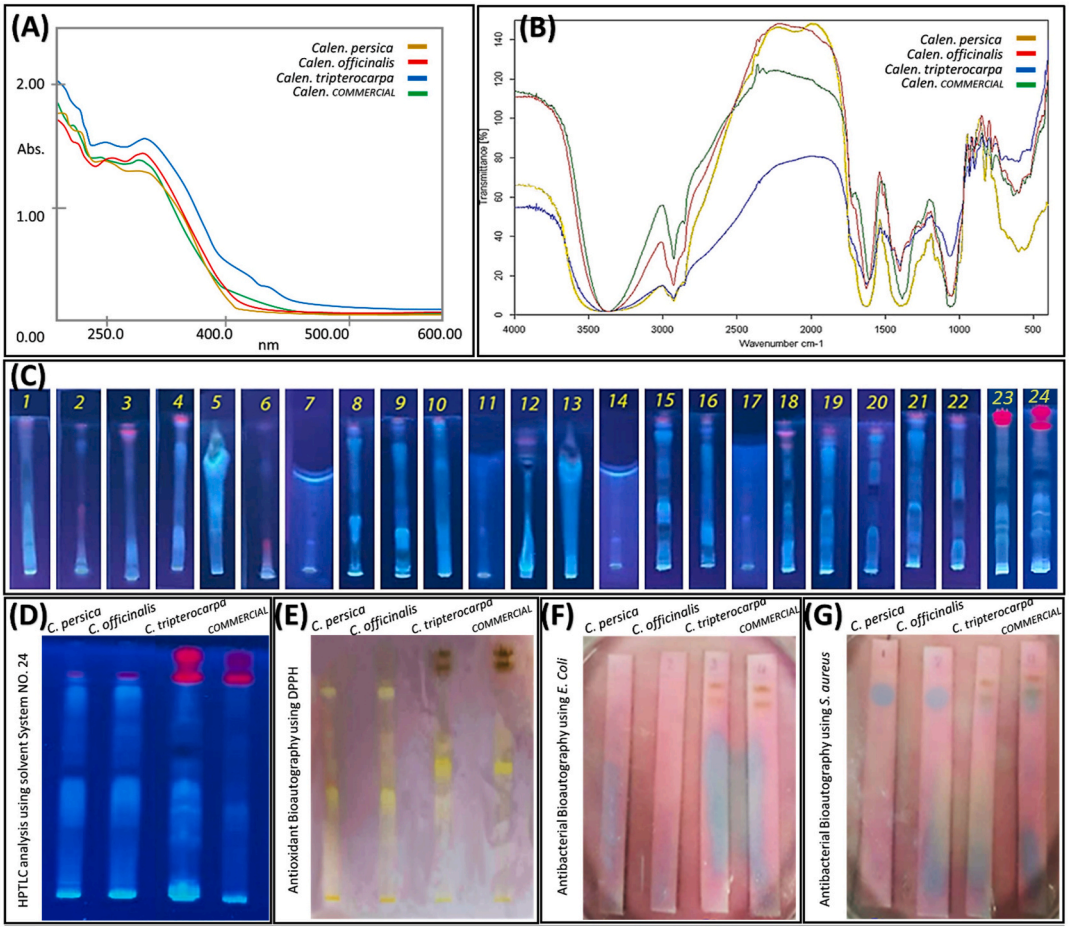

Fig. 2. Ultraviolet–visible (a) and infrared (b) absorption patterns of the examined *Calendula* species. The separation compounds of *C. tripterocarpa* extract at a wavelength of 366 nm using mobile phases mentioned in Table 1, that were used to optimize the separation (C) and the optimal result of separation of native Iranian and commercial *Calendula* species extract with the optimal mobile phase (No. 24) (D). Antioxidant bioautographic result of isolated samples using DPPH indicator (E) and antibacterial bioautographic result of isolated compounds against *Escherichia coli* (F) and *Staphylococcus aureus* (J).

Table 1
Examined developing solvent system.

Plate number	Volume of each Developing Solvent System (mL)																								
Developing Solvent System	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Acetone	70	29	28	28		30				80										10					
Acetic acid			1				9		6		8			9		7	3			2		1			
Chloroform												18						20	12	10	18	17	17		11
Dichloromethane									17				10			17									
Ethanol	30	3	2	2															1	0	23	22	22	24	22
Ethyl acetate		68	69	69		70	9	84	65		30	46		9	84	66	31	49	23	21	58	57	57	59	57
Formic acid				1	2			4	6	5					4	7			5	3	1		1	1	1
Methanol					98			6		10		10	90		6			27							
Toluene							82			5	63			82			66		59	54				12	6
Water								6	7			25			6	3		5			1	3	3	5	3

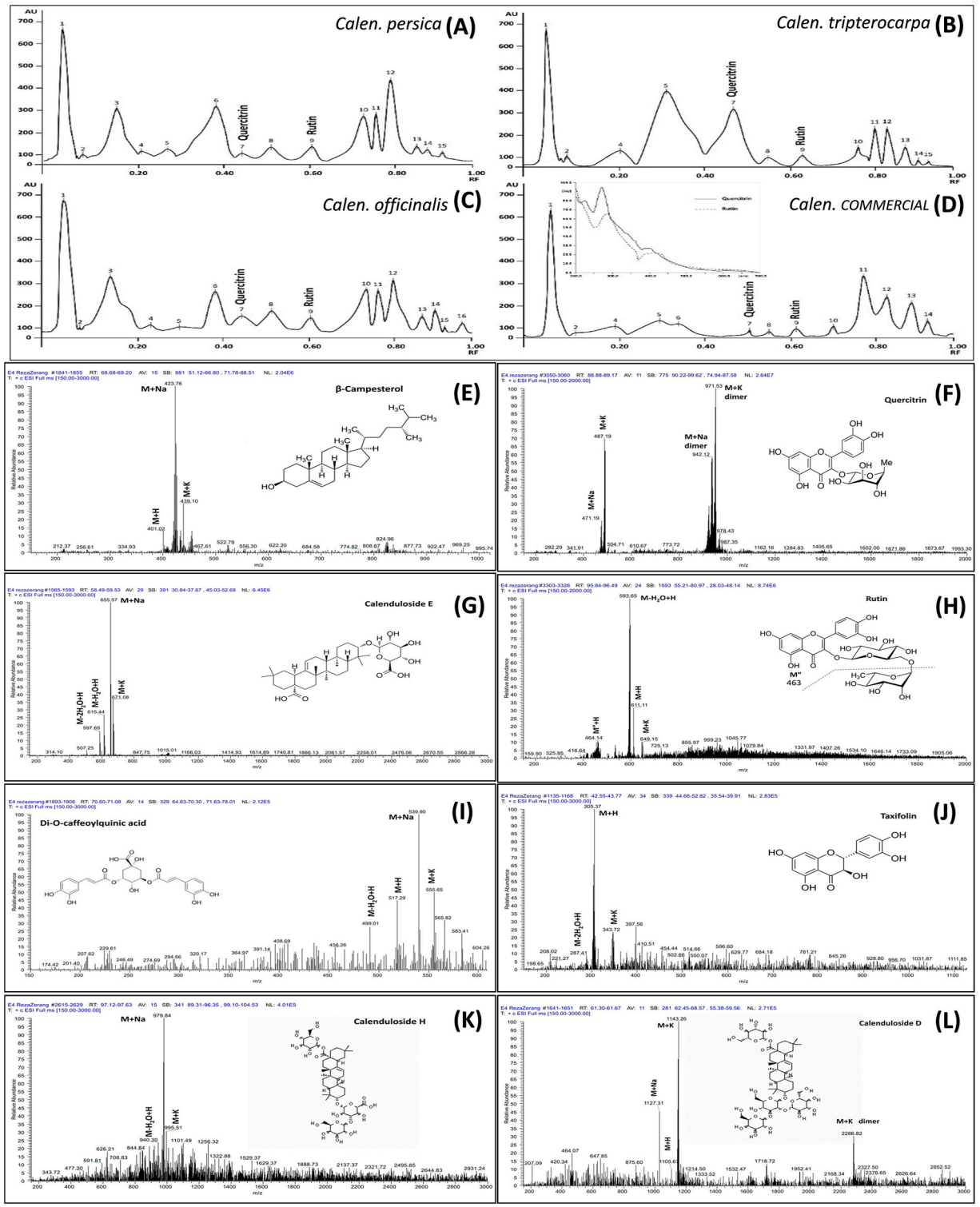

Fig. 3. HPTLC separation spectrum of *C. persica* (A), *C. tripterocarpa* (B), *C. officinalis* (C) and commercial sample (D) using the optimal mobile phase (Table 1, No. 24) and Mass spectrum of identified compounds (E to L).

viscosity, non-toxic properties, inertness towards samples or the system, dissolution capability for sample components, and compatibility with the system identifier. Initially, a single solvent system was employed for component separation, followed by a solvent mixture (Fig. 2C). Table 1 outlines the order of solvent systems used for optimization based on Fig. 2C. The optimal mobile phase for separation, determined as chloroform, toluene, ethyl acetate, ethanol, water, and formic acid in specific proportions (11:6:57:22:3:1), was the 25th developing solvent system for all calendula species separation (Fig. 2D). Imaging of TLC plates was conducted at a wavelength of 366 nm.

Subsequently, we prepared and developed the HPTLC screen with a specialized solvent system. Antioxidant and antibacterial properties of compounds in the extracts were studied, where yellow or white areas in Fig. 2E indicated antioxidants, and Resazurin turning blue (Fig. 2F and G) signified areas with antibacterial compounds inhibiting bacterial growth. Notably, quercetin and rutin emerged as key active ingredients of *calendula*, with *C. tripterocarpa* samples displaying the highest quercetin content, and *C. persica* samples showing the lowest rutin amount, while the commercial sample exhibited the lowest overall. Mass spectrometry was employed to identify active compounds in regions with significant antioxidant and antibacterial properties.

Fig. 3 shows the separation chromatogram of native and commercial extracts (3A - 3D). It also shows the active compounds (3E - 3L) identified by mass spectrometry. We mark the most important detected ions, such as $[M+H]^{+\bullet}$, $[M+K]^{+\bullet}$, $[M\text{-}2H2O + H]^{+\bullet}$, $[M\text{-}H2O + H]^{+\bullet}$ and $[M+Na]^{+\bullet}$, on the spectrum. Quercetin and rutin are the main compounds identified in the extracts. Besides, we found some active compounds in the Clanduloside family. In case of β-Campesterol identified ions were 401.02 m/z: $[M+H]^{+\bullet}$, 432.76 m/z: $[M+Na]^{+\bullet}$ and 439.10 m/z: $[M+K]^{+\bullet}$. Regarding Quercetin identified ions were 471.19 m/z: $[M+Na]^{+\bullet}$, 187.19 m/z: $[M+K]^{+\bullet}$, 942.12 m/z: $[M+Na]^{+\bullet}$Dimer and 971.53 m/z: $[M+K]^{+\bullet}$ Dimer. Calenduloside E, identified over the adducts of 671.08 m/z: $[M+K]^{+\bullet}$, 597.65 m/z: $[M\text{-}2H_2O + H]^{+\bullet}$, 615.44 m/z: $[M\text{-}H_2O + H]^{+\bullet}$ and 655.57 m/z: $[M+Na]^{+\bullet}$. About Rutin a fragment (464.14) and three adduct ions including 649.15 m/z: $[M+K]^{+\bullet}$, 593.65 m/z: $[M\text{-}H_2O + H]^{+\bullet}$ and 611.11 m/z: $[M+H]^{+\bullet}$ were identified. In case of di-*o*-caffeoylquinic acid identified ions were 499.01 m/z: $[M\text{-}H_2O + H]^{+\bullet}$, 517.29 m/z: $[M+H]^{+\bullet}$, 539.90 m/z: $[M+Na]^{+\bullet}$, and 555.65 m/z: $[M+K]^{+\bullet}$. Taxifolin was identified over the adducts of 287.41 m/z: $[M\text{-}2H_2O + H]^{+\bullet}$, 305.37 m/z: $[M+H]^{+\bullet}$ and 343.72 m/z: $[M+K]^{+\bullet}$. About Calenduloside H, adduct ions including 995.51: *m/z* $[M+K]^{+\bullet}$, 940.30: $[M\text{-}H_2O + H]^{+\bullet}$ and 979.84 m/z: $[M+H]^{+\bullet}$ were identified. Regarding Calenduloside D identified ions were 1127.31 m/z: $[M+Na]^{+\bullet}$, 1143.26 m/z: $[M+K]^{+\bullet}$, 1105.67 m/z: $[M+H]^{+\bullet}$ and 2286.82 m/z: $[M+K]^{+\bullet}$ Dimer.

3.2. *Oryza sativa* cultivation and growth analysis

Moving beyond chemical properties, we delved into the biological effects of calendula extract UHDs on rice growth, comparing it to the commercial sample and referencing previous research. Rice, a vital food source in Iran, faces growth challenges due to pests and diseases. The study focused on rice seed germination, contamination, and root development (Fig. 4A). Commercial calendula extract-treated samples exhibited the most uniform growth and germination in Fig. 4B.

You can see the metabolic characteristics, health and non-contamination of seeds and the growth of seedlings in Fig. 4(C–H). After completing the treatment steps in the dark, we exposed the percentage of healthy seeds in each sample to light. The researchers observed that the seeds treated with native *C. officinalis* UHDs had the highest percentage of healthy seedlings. The infected seedlings have a different metabolism and production materials. We checked the percentage of infected and healthy seeds in Petri dishes. We discarded any Petri dishes containing infected seeds. The seeds treated with native *C. officinalis* UHDs had the highest weight for aerial parts and roots. It is important to mention that the seeds treated with commercial *C. officinalis* UHDs had fewer healthy seeds. However, these seeds had a higher dry weight than the other samples. It indicates that the germination and growth of these seeds are more efficient than the others. So, we can say that commercial UHDs have a more influential effect on the germination and growth of seeds. Using native *C. officinalis* UHDs shows a better influence on seedling health and growth. The lowest dry weight of seedlings treated using native *C. officinalis* UHDs related to *C. persica.*

Total protein production, a crucial biochemical factor linked to metabolism and growth rate, was higher in samples treated with native and commercial *C. officinalis* UHDs compared to other native UHDs. Among controls, placebo-treated samples in non-sterile conditions had the lowest protein levels. The active metabolism and health in samples treated with *C. officinalis* UHDs were evident, making them the most effective among *Calendula* UHD-treated groups.

4. Discussion

The application of ultra-high dilution (UHD) in agriculture represents a cutting-edge approach to enhance crop productivity, seed germination and overall plant health. UHD involves the dilution of plant extracts to extremely low concentrations, unlocking their inherent biological potential in a nuanced and sustainable manner. UHD treatments derived from indigenous plants, such as *Calendula officinalis*, have demonstrated superior efficiency in promoting seed germination. This process is fundamental for establishing robust plant populations and ensuring optimal yield. Numerous studies have delved into the application of homeopathy for enhancing crop growth. In yerba mate (*Ilex paraguariensis*) forests subjected to severe pruning, *Arnica Montana* 30CH and *Calendula officinalis* 30CH have demonstrated efficacy in promoting robust regrowth and increasing fresh mass weight. The replacement of pesticides with homeopathic treatments has proven beneficial for rice plants, resulting in a notable increase in grain yield (≥2000 kg/ha). They also observed enhanced germination in lettuce seeds treated with Arsenicum album 7CH [16]. Additionally, Verdi et al. achieved longer branches and a remarkable 79 percent multiplication success in the root cuttings of the plant known in Brazil as erva-de-touro (*Poiretia latifolia*) through the application of Calcarea phosphorica 20CH [32]. Sampaio et al., showed that the application of Arsenicum UHDs (30CH) with positive interference reached germination 166.7 percent higher than the control. Also the best average germination time

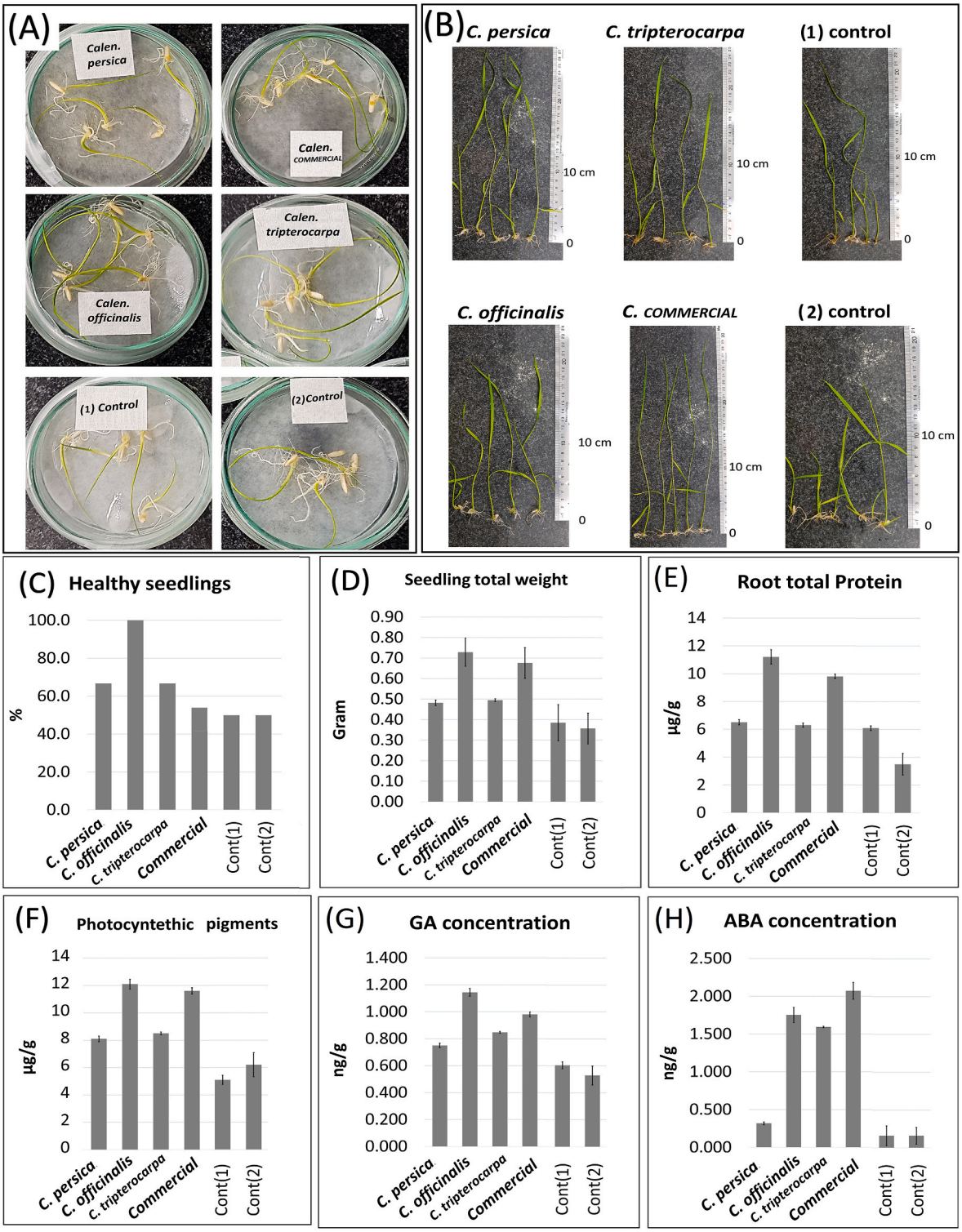

Fig. 4. (A) The variations of the seedlings grown in the plate six days after cultivation under the UHDs treatment compared to the control conditions and the growth and physiological variations of seedlings 20 days after cultivation in specific hydroponic growth cultivation medium, Yoshida under the UHDs treatment compared to the control conditions (B). Number of healthy seedlings (C) and their weight (D), the amount of root total protein (E), photosynthetic pigments (F), gibberellic acid (G) and abscisic acid (H) in control and treated seedlings. The control condition 1 is placebo and control 2 is without any treatment.

was 3.7 days at 20–30 °C with 30CH dilution. In another study on the effect of UHDs on germination and initial growth of *Salicornia bigelovii* (Torr.) revealed that three UHDs treatments, Natrum muriaticum 7CH, Phosphoricum Acidum 13CH and Natrum muriaticum 31CH increase the percentage and germination rate (up to 44 percent), stem and radicle length (35 percent above the control) and fresh and dry biomass of aerial and radicle parts. They confirmed the positive effect of homeopathic medicines on germination and initial growth of *S. bigelovii*, revealing that agricultural homeopathy, particularly NaM-7CH, PhA-13CH and NaM-31CH treatments, is a viable alternative to optimize the cultivation of species since it increases germination percentage and stimulates initial growth [33]. In the case of *Hypericum perforatum* under high dilutions (Kali carbonicum, Natrum muriaticum, Phosphorus and Silicea 12 cH) the percentage of germination (63 percent), average germination time (50 percent) and seedlings health (85 percent) improved. Here, the study of the super diluted effect of calendula on *Oryza sativa* seeds showed an increase in the germination rate (up to 70 percent) and their health and non-contamination rate (up to 95 percent). It also resulted in UHDs contributing to the development of a healthy and extensive root system. This enhances nutrient absorption and fortifies plants against environmental stresses, fostering resilience in various growing conditions.

The use of UHDs has shown promising results in minimizing contamination, a critical factor in ensuring the purity of crops. This is particularly valuable in sustainable and organic farming practices. Exploration into the application of homeopathy extends to pest management in tomato cultivation under field conditions [17]. Notably, Deboni et al. demonstrated effective control of the bean weevil [34]. In orchards, the inclusion of *Ac. tannicum* 30CH in insect traps led to a 20 percent increase in the attraction of *Anastrepha fraterculus* compared to the control, aiding in fruit fly reduction—an approach applicable to organic orchards [16]. Giesel et al. successfully diminished the foraging activity of *Acromyrmex* species (leafcutter ants) *A. laticeps* and *A. heyeri* using a high dilution (nosode) of *Acromyrmex* species and Belladonna 30CH, without inducing colony collapse or re-colonization elsewhere [35]. In this research, the amount of contamination in the samples treated with UHDs to the control was controlled and reduced by 95 percent. Everything that happens in the growth and health of seeds and seedlings is caused by changes in the plant's primary and secondary metabolites. Therefore, in this study, the changes of these metabolites and hormones were investigated to improve seedlings' growth. It found that UHD treatments positively impact the production of photosynthetic pigments, essential for efficient photosynthesis. This, in turn, boosts overall plant vitality and contributes to increased crop yields.

5. Conclusion

In the pursuit of unraveling the metabolic intricacies and biological effects of *Calendula officinalis*, our comprehensive study embarked on two primary objectives. Firstly, we delved into the metabolic profiles and effects of extracts from three indigenous *C. officinalis* plants in Iran, drawing comparisons with a commercially procured *C. officinalis* extract. Subsequently, we scrutinized the influence of ultra-high diluted compounds (UHDs) derived from these Calendula plants on the growth of rice seeds, providing a comparative analysis against commercial UHDs. The culmination of our research has yielded significant insights:

1. Metabolic Profile Identification: Utilizing thin layer chromatography (TLC), we successfully separated and identified compounds in the extracts, revealing the presence of Rutin and Quercitrin. Mass spectrometry further elucidated the flavonoid and terpenoid categories, including β-Campstrole and di-*o*-Caffeoylquinic acid.
2. Chromatogram Analysis: High-performance thin liquid chromatography (HPTLC) comparisons between native and commercial extracts unveiled chromatogram similarities, emphasizing flavonoid and terpenoid compounds. DESI mass spectrometry analysis identified these active compounds, contributing to the antioxidant and antibacterial activities.
3. Biological Activity: Commercial extract exhibited the highest antioxidant activity, closely followed by native Iranian *C. officinalis.* Flavonoid compounds like Quercitrin, Rutin, Taxifolin and di-*o*-caffeoylquinic acid were pivotal in antioxidant effects. Distinct antibacterial activities against *Staphylococcus aureus* and *Escherichia coli* were observed. Terpenoid compounds, including β-Campesterol and Calendulosids E, D, H, contributed significantly to antibacterial effects.
4. UHDs' Impact on Seed Germination: Native Calendula UHDs surpassed their commercial counterparts in enhancing seed germination efficiency, rooting quality and contamination reduction. Moreover, UHDs positively influenced photosynthetic pigment production, root length and lateral root count.
5. Biological Parameters: Seedlings treated with native *C. officinalis* UHDs, whether native or commercial, exhibited higher levels of protein, gibberellic acid and abscisic acid compared to other treatments, emphasizing the biological efficacy of these UHDs.

In conclusion, this multifaceted exploration advances our understanding of Calendula officinalis and accentuates the potential of native UHDs in revolutionizing agricultural practices. The intricate interplay of phytochemicals, coupled with the biological responses observed, opens avenues for sustainable and effective applications of *Calendula* extracts in agriculture.

Future implications

This research provides valuable insights into sustainable agricultural practices, the efficacy of UHDs and the potential of native Calendula species, paving the way for future studies and applications in enhancing crop yield and quality.

Ethics approval and consent to participate

Not applicable.

Consent for publication and competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Availability of data and materials

Data will be made available on request.

CRediT authorship contribution statement

Fateme Mirzajani: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Conceptualization. **Hassan Rezadoost:** Writing – review & editing, Resources, Funding acquisition. **Reza Zerang:** Visualization, Investigation, Formal analysis, Data curation. **Ali Sonboli:** Methodology, Formal analysis, Data curation.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Hassan Rezadoost reports financial support was provided by Iran National Science Foundation (INSF) [Project No. 99012038]. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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