Research Article

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Ultrasound-assisted extraction *versus* traditional Soxhlet apparatus for the obtention of polyphenols, carotenoids and tocopherols from *Tagetes erecta* L. flowers

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Abstract

BACKGROUND: *Tagetes erecta* L., commonly known as American marigold, serves as a food plant used for the extraction of carotenoids such as lutein, employed both as culinary ingredient in certain dishes and for its ornamental and medicinal applications. Two extraction techniques, Soxhlet and ultrasound-assisted extraction (UAE), were used on two cultivars (yellow and orange) of *T. erecta*. Polyphenols were quantified using HPLC-tandem mass spectrometry, whereas carotenoids and tocopherols were determined using HPLC-diode array detection. Biological activity for antioxidant and antiglycation properties was carried out.

RESULTS: The best extraction yield was obtained for UAE (7.51% and 6.83% for yellow and orange flowers), corresponding with the largest amounts of polyphenols quantified. The highest content of tocopherols was obtained in the yellow cultivar extracted by Soxhlet (6499.3 $\pm$ 21.2 and 4671.0 $\pm$ 92.9 mg kg^{-1} dry extract for α- and γ-tocopherol). The antioxidant potential resulted higher in the orange Soxhlet extract, whereas the yellow Soxhlet extract displayed the best antiglycation activity (median 50% inhibitory concentration of 25.3 $\pm$ 3.3 µg mL^{-1}).

CONCLUSION: Both extraction techniques showed interesting results in terms of bioactivity and compounds obtention.
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Keywords: edible flowers; green extraction; natural pigment; Soxhlet extraction; ultrasound-assisted extraction

INTRODUCTION

The obtention of plant extracts has traditionally been made by different extraction methods such as maceration, decoction, infusion or percolation. The improvement and modernization of these techniques around the 18th century resulted in a new method called Soxhlet.[1] Usually, conventional extraction methods require long extraction times and the use of elevated temperatures that can damage or modify the final composition.[2] Although, currently, those earlier methods are still being used, there is a need for new greener techniques. The need for new environmentally friendly, less consuming and faster processes has resulted in the invention of advanced non-conventional techniques such as ultrasound-assisted extraction, microwave-assisted extraction, pressurized liquid extraction or supercritical fluid extraction. Ultrasound-assisted extraction (UAE) is an innovative technique that has been gaining attention and usage during the last years. UAE has many advantages, such as short extraction time, less solvent consumption and high extraction rate.[3,4] These two extraction methods exert their mechanism of action through different physicochemical procedures.[5] UAE works through a mechanism called acoustic cavitation that entails the formation, growing and violent collapsing of microbubbles in a sonication liquid as a result of fluctuations in

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pressure.[6] It makes the removal of solutes from cells easier and faster by intermolecular diffusion, resulting in complete release of intracellular active ingredients.[7] UAE has better efficacy at higher intensity, which could be explained by the more violent collapsing of bubbles compared to lower intensity, works easier in diluted solutions than viscous ones, and is more effective at lower temperatures.[8] UAE is also considered a no-thermal technology because it does not rise the temperature of the treated liquid, reducing the risk of thermal degradation.[9] Meanwhile Soxhlet's extraction mechanism works through a combination of maceration and percolation: percolation because the condensed solvent starts to drop from the top to the dry sample and maceration because the solvent stays in contact with the sample until it reaches the siphon level and descends to the bottom round flask (Fig. 1). The temperatures reached during the process are quite elevated, around 80–85 °C.

Edible flowers have been consumed along the world and throughout the centuries as ingredients of dishes and salads or for the extraction of food colorants and additives.[10,11] Edible flowers comprise a wide range of species; recently, these flowers have been gaining attention because of their complex composition and their interesting properties that make them suitable as nutraceuticals or healthy ingredients.[12-14] *Tagetes erecta* L., also known as American marigold, is an edible flower from the Asteraceae or Compositae family. It has been widely used in ornamentation and landscaping.[15] Noteworthy, *T. erecta* is considered as one of the richest sources of lutein and has been used for its extraction as well as for obtention of other carotenes such as zeaxanthin. Approximately 10% of the carotenoids present in *T. erecta* are lutein and zeaxanthin.[16] However, the composition of this edible flower is more complex because it is rich in other bioactive compounds that can be of interest.[17,18]

Sedentary lifestyle and the consumption of processed foods have been increasing for the last decades, especially in developed countries.[19,20] These behaviours have led the population to suffer from numerous health problems by-product of nutrition deficiencies. Overweight individuals are more prone to suffer from micronutrients deficiency such as vitamins.[21] Fat soluble vitamins such as vitamin A and E are required for the proper functioning of many body mechanisms. The vitamin E family is composed of four tocopherols (α, β, γ and λ) and four tocotrienols (α, β, γ and λ), and they can be found in vegetables, seeds and cereals. Vitamin E is a potent antioxidant that also plays an important role in the regulation of inflammation and immune response.[22] Carotenoids can be found in nature, and some of them are dietary substances such as α-carotene, β-carotene, lutein, zeaxanthin and lycopene. These compounds serve in the organism as major precursors for vitamin A.[23] Vitamin A deficiency is a global health problem; the lack of this hydrophilic vitamin is the main cause of child blindness, bone fragility, and increased risk of infections and death in developing countries.[24-26] Lutein can be found in green vegetables and cannot be produced by our organism, and thus there is a need for its ingestion.[27] Along with zeaxanthin, these carotenoids are referred to as macular pigments because of their presence in the centre of the retina. The ingestion of natural products rich in compounds such as lutein can be beneficial in the prevention of diseases and health problems, specifically related to the sight, such as macular degeneration, cataracts and atherosclerosis.[28]

Food colourants are used to make food more attractive and eye-catching because it is recognized that appearance is the most important parameter when judging the flavour of food.[29] The main purpose of food colouring is generally to restore colours lost

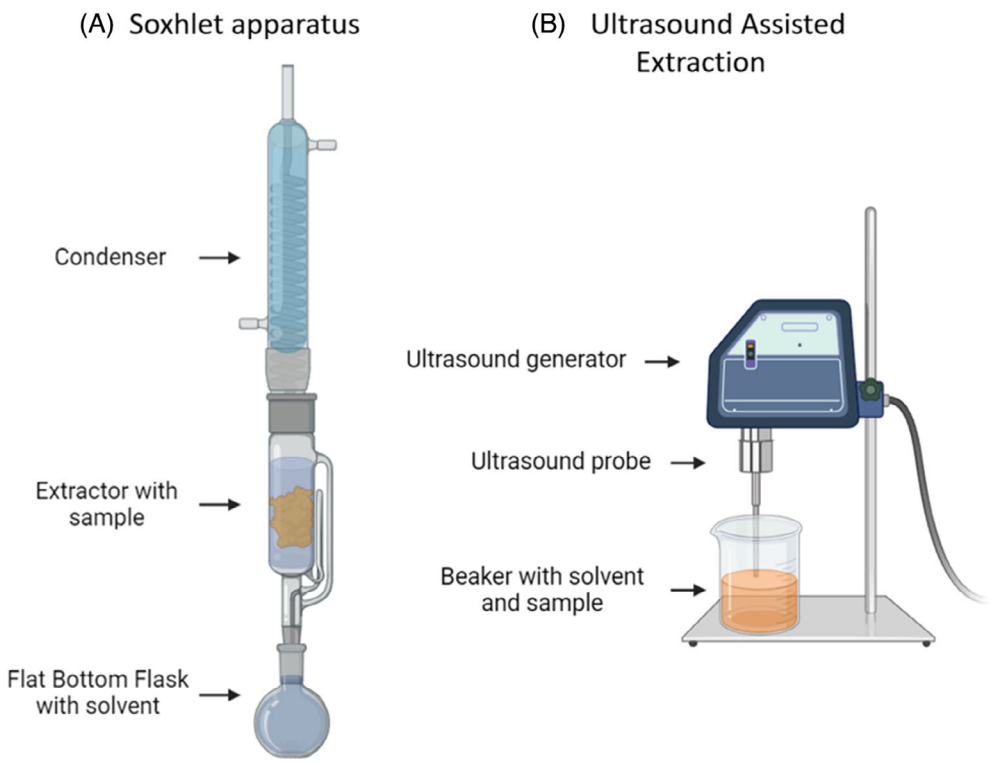

Figure 1. Different extraction methods diagrams.

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during processing, storage or transportation, make food look more appealing, or reinforce previously existing colours. Normally, these compounds do not have a nutritious purpose. Based on the precedence of food colourants, we can classify them as natural or artificial, also called synthetic. With the development of food science and technology, the use of synthetic colorants increased exponentially. They can be produced by chemical procedures or modifying natural precursor compounds.[30] Recently, the awareness of health and environmental problems derived from the consumption of synthetic dyes and colourants made natural pigments the focal point.[31,32] These extracts from natural sources may suppose an interesting and complete source of certain nutrients, as well as be beneficial for health, as a result of their numerous properties and benefits.

The present study aimed to compare the differences in extraction yield, composition profile and bioactivity of different *T. erecta* extracts using two extraction techniques: one traditional and the other non-conventional.

MATERIALS AND METHODS

Reagents

Ethanol, xanthine oxidase from bovine milk, nitro blue tetrazolium chloride (NBT), xanthine, α-diphenyl-β-picrylhydrazyl (DPPH), gallic acid, α-glucosidase from *Saccharomyces cerevisiae*, 4-nitrophenyl α-D-glucopyranoside (pNPG), aminoguanidine bicarbonate (AMG), pancreatic lipase, 4-nitrophenyl butyrate (NPB), were purchased from Sigma Aldrich Co. (St Louis, MO, USA). Na_2HPO_4, NaH_2PO_4, Na_2CO_3, Tris–HCl, $CaCl_2$ and KH_2PO_4 were purchased from Panreac Quimica (Castellar del Vallès, Barcelona, Spain). Methanol was purchased from Fisher Scientific (Loughborough, UK). Bovine serum albumin was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Fresh flowers and extraction methods

Capitula from two cultivars of *T. erecta* with yellow and orange flowers were purchased from Innoflower SL (Zaragoza, Spain). Whole fresh inflorescences were cut into small pieces and extracts were prepared following two different methods:

- Soxhlet extraction: this conventional extraction was performed using a Soxhlet apparatus and ethanol at an extraction temperature between 80 and 85 °C for 4 h.[33] The flower pieces were introduced in a filter paper sachet that allowed solvent to flow and extract the bioactive compounds.
- Ultrasound-assisted extraction (UAE): this non-conventional extraction was carried out using an ultrasonic extractor (US2020; Albrigi, Stallavena-lugo VR, Italy) and ethanol at room temperature (22–25 °C). The cut flowers were introduced in direct contact with the solvent for four cycles of 35 min up to 2.3 h.

The solvent was removed with a rotatory evaporator and resulted extracts were stored in the dark at −20 °C. Each cultivar was put under both extraction methods, obtaining four final extracts: yellow *Tagetes* Soxhlet (YTS), orange *Tagetes* Soxhlet (OTS), yellow *Tagetes* UAE (YTU) and orange *Tagetes* UAE (OTU).

Polyphenolic composition study by HPLC-tandem mass spectrometry (MS/MS)

The quantification of 38 bioactive molecules (37 of them are phenolic compounds) was carried out using a modified version of a previously described method.[34] The extracts were re-dissolved at a concentration 5 mg mL^{-1} with ethanol. The HPLC-MS/MS investigations were carried out with an Agilent 1290 Infinity series and a Triple Quadrupole 6420 (Agilent Technology, Santa Clara, CA, USA), linked to an electrospray ionization source that operated in negative and positive ionization modes. The bioactive compounds were separated in gradient elution mode on a Synergi Polar–RP C18 column (Phenomenex, Torrance, CA, USA) (250 × 4.6 mm, 4 μm) using a mixture of water and methanol as solvents A and B, respectively, both with 0.1%, formic acid. For column protection, a Polar RP security guard cartridge (Phenomenex) preceded the column (4 × 3 mm inner diameter). The mobile phase composition was made up of the following components: 0–1 min, isocratic condition, 20% B; 1–25 min, 20–85% B; 25–26 min, isocratic condition, 85% B; 26–32 min, 85–20% B. The injection volume was 2 μL and the flow rate was kept at 0.8 mL min^{-1}. The temperature of the column was set to 30 °C, and the drying gas temperature in the ionization source was set to 350 °C. The flow rate of the gas was set to 12 L min^{-1}, the capillary voltage was 4000 V and the nebulizer pressure was 55 psi. The peak areas were integrated for quantitation after detection in the dynamic-multiple reaction monitoring mode. Each analyte's most abundant product ion was employed for quantification, whereas the other ions were used for qualitative analysis. The results were expressed as mg/kg of extract.

Carotenoids and tocopherols quantification by HPLC-diode array detection

Carotenoids (lutein and β-carotene) and tocopherols (α-tocopherol and γ-tocopherol) concentrations were determined using an HPLC Agilent 1260 Infinity II series (Agilent Technology) equipped with a quaternary pump and two detector systems in series: diode array detector and fluorescence detector. Briefly, the separation of analytes was achieved by a Chromolith RP-18e analytical column (100 × 3 mm inner diameter, macropore size 2 μm, mesopore size 13 nm) from Merck (Darmstadt, Germany) thermostated at 40 °C. The mobile phase consisted of MilliQ (Merck Millipore, Burlington, MA, USA) water (A), acetonitrile (B) and 2-propanol (C), at a flow rate of 0.8 mL min^{-1}. Elution was performed in gradient mode with mobile phase varying as follows: 0–2 min, 70% B and 0% C; 2–3 min, 100% B and 0% C; 3–5 min, 100% B and 0% C; 5–11 min, 80% B and 20% C; 11–12 min, 100% B and 0% C; 12–14 min, 70% B and 0% C; 14–18 min, 70% B and 0% C. The injection volume was 10 μL. A diode array detector was used for the detection of carotenoids with a wavelength of 450 nm for lutein and β-carotene. α-Tocopherol and γ-tocopherol were quantified using a fluorescence detector at 295 and 330 nm as excitation and emission wavelength, respectively. Calibration curves used in the quantification process: lutein ($y = 27.698x–20.561$, $r^2 = 0.9993$), β-carotene ($y = 57.097x–36.291$, $r^2 = 0.9994$), α-tocopherol ($y = 6.0271x–0.927$, $r^2 = 1$) and γ-tocopherol ($y = 16.916x + 44.917$, $r^2 = 0.987$). The results were expressed as mg g^{-1} extract.

In vitro antioxidant and antiradical activity

The antioxidant activity and capacity of the extracts to scavenge free radicals was performed through two methods, DPPH and by the xanthine/xanthine oxidase assay, both using gallic acid as reference compound.[35,36] The antioxidant activity of the extracts was expressed as median 50% inhibitory concentration (IC_{50}) (μg mL^{-1}), the concentration of extracts required to scavenge 50% of DPPH or X/XO radicals. Percentage calculated using:

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$$\text{Inhibition}\,(\%) = \left[\frac{(\text{Abs control} - \text{Abs sample})}{\text{Abs control}}\right] \times 100 \qquad (1)$$

DPPH scavenging activity

In total, 150 μL of DPPH methanolic solution (0.04 mg mL^{-1}) was added to 150 μL of the sample at different concentrations dissolved in methanol (6.25, 12.5, 25, 50 and 100 μg mL^{-1}). The plate was measured at 517 nm after an incubation time of 30 min under dark conditions.

Xanthine/xanthine oxidase system (X/XO)

A mixture of 16 mM Na_2CO_3, 22.8 μM NBT and 90 μM xanthine were dissolved in phosphate buffer pH 7 to reach a volume of 240 μL. Then, 30 μL of each sample (concentrations of 6.25, 12.5, 25, 50 and 100 μg mL^{-1}) and 30 μL of xanthine oxidase (168 U L^{-1}) were added to start the reaction. After an incubation time of 3 min at 37 °C, the plate was measured at 560 nm.

In vitro antidiabetic and anti-obesity activity

α-Glucosidase inhibition

α-Glucosidase inhibition was performed in a 96-well microplate reader at 405 nm.[37] Each well contained 50 μL of sample (concentrations 62.5, 125, 250, 500 and 1000 μg mL^{-1}) and 100 μL of enzyme (1 U mL^{-1}). After 10 min, 50 μL of pNPG was added and incubated at 37 °C for 20 min. Control wells contained 50 μL of solvent (buffer 12.5 mM Na_2HPO_4, 3.3 mm NaH_2PO_4; pH 6.9). Absorbance was read and inhibition was calculated using Eqn (1).

Lipase inhibition

The ability of the extracts to inhibit pancreatic lipase was measured in 96 well plates.[38] The enzyme was diluted at a concentration of 2.5 mg mL^{-1} in buffer 0.1 M TRIS base buffer with 5 mM $CaCl_2$ (pH 7.0) and centrifugated at 2000 × *g* (Nahita Multibas 2750, swing out rotor) at room temperature for 7 min. Next, 40 μL of extract solution (concentrations 62.5, 125, 250, 500 and 1000 μg mL^{-1}), 40 μL of enzyme and 20 μL of substrate solution (10 mM p-NPB) were mixed and incubated for 15 min at 37 °C. Absorbance was read at 405 nm and enzyme inhibition was calculated in percentage using Eqn (1).

Advanced glycation end products formation inhibition

Advanced glycation end-products (AGEs) inhibition was measured in 96-black well-plates.[39] Here, 50 μL of bovine serum albumin solution 10 mg mL^{-1} was mixed with 80 μL of 0.1 M phosphate buffer (containing sodium azide 3 mM, pH 7.4), 50 μL of 0.5 M fructose solution and 20 μL of extracts (concentrations 6.25, 12.5, 25, 50 and 100 μg mL^{-1}). After 24 h incubation at 37 °C in the dark, fluorescence was measured (355 nm excitation wavelength and 460 nm emission wavelength) and inhibition calculated using Eqn (1) but using fluorescence instead of absorbance.

Statistical analysis

Three independent experiments were performed for the cited assays. Statistical analysis was carried out using Prism, version 6.0 (GraphPad Software Inc., San Diego, CA, USA). The results were expressed as the mean ± SEM. The IC_{50} was estimated by means of a linear regression equation. Comparisons and *P* value calculations were made between same cultivar flower extracts obtained by different methods using Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Composition profile of the *T. erecta* flower extracts

The variations in conditions used to obtain the different extracts and yield rates are reported in Table 1. The lowest ratio solvent/raw material was obtained by both UAE extracts, particularly the yellow *Tagetes*, whereas the extracts obtained by Soxhlet displayed ratios at least two times higher; meaning that they needed bigger amount of solvent or obtained lower amount of extract in comparison. This result was reinforced by the percentage yield obtained by each cultivar with the two different extraction methods. The Soxhlet procedure gave yield values of 4.89% and 4.03% for the yellow and orange cultivar, respectively, whereas the UAE extracts obtained higher yield percentages of 7.51% for yellow *Tagetes* and 6.83% for orange *Tagetes*.

Regarding the composition of the extracts (Table 2), 26 compounds were detected and quantified using HPLC-MS/MS: 11 phenolic acids, 16 flavonoids and a non-phenolic acid.

The total amount of polyphenols quantified was 16 578.68 mg kg^{-1} dry extract (DE) for YT Soxhlet, 11 935.75 mg kg^{-1} DE for OT Soxhlet, 17 137.05 mg kg^{-1} DE for YT UAE and 18 299.24 mg kg^{-1} DE for OT UAE.

The most abundant compounds were ellagic, gallic and vanillic acid for phenolic acids, and quercetin and isorhamnetin for flavonoids; the structures are reported in Fig. 2. The highest concentration of ellagic acid was detected in OT UAE with 9330.27 ± 0.84 mg kg^{-1} DE, as well as gallic acid with 3208.8585 ± 4.41 mg kg^{-1} DE. Vanillic acid had a higher content in YT Sox with 2245.03 ± 1.79 mg kg^{-1} DE. Other flavonoids and phenolic acids were detected in minor proportions such as kaempferol, chlorogenic acid, hyperoside and quercetin derivates. In general, ultrasound extraction yielded higher quantities of compounds than Soxhlet did for the same flower cultivar.

Regarding the carotenoid and tocopherol content, the respective values obtained are represented in Fig. 3. The tendency observed was higher content of these compounds in the extracts obtained by Soxhlet, specially both tocopherols in the yellow

Table 1. Comparison of conditions used for the extraction of yellow and orange *Tagetes erecta* flowers by conventional method Soxhlet and non-conventional UAE.

Extraction method	Sample size (g)	Temperature (C°)	Time (h)	Solvent volume (mL)	Solvent/raw material ratio	Extraction yield (%, w/w)
Yellow *Tagetes* Soxhlet	150	80–85	4	1200	8	4.89
Yellow *Tagetes* UAE	395	Room temperature	2.3	1000	2.53	7.51
Orange *Tagetes* Soxhlet	154	80–85	4	1100	7.14	4.03
Orange *Tagetes* UAE	300	Room temperature	2.3	1000	3.33	6.83

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Table 2. Content of bioactive compounds in yellow and orange *Tagetes erecta* extracts obtained by different methods expressed as mg kg^{-1} DE, with the extracts analyzed by HPLC-MS/MS.

No.	Compound	Yellow *T. erecta* Soxhlet	Yellow *T. erecta* UAE	Orange *T. erecta* Soxhlet	Orange *T. erecta* UAE
Phenolic acids					
1	Gallic acid	788.92 ± 2.72	2314.92 ± 3.53****	2164.61 ± 5.25	3208.85 ± 4.41****
2	Neochlorogenic acid	5.83 ± 3.54	43.10 ± 1.13	3.76 ± 6.17	9.28 ± 2.67
3	Chlorogenic acid	82.32 ± 4.19	85.18 ± 6.91**	41.43 ± 2.44	64.33 ± 3.96
4	p-Hydroxybenzoic acid	263.32 ± 4.76	511.69 ± 5.21	279.48 ± 4.22	375.59 ± 5.64
5	Caffeic acid	74.35 ± 4.29	61.48 ± 3.54	42.91 ± 5.25	32.50 ± 2.99
6	Vanillic acid	2245.03 ± 1.79	2202.27 ± 4.23	2073.98 ± 5.87	2138.12 ± 2.43
7	Syringic acid	154.58 ± 4.89	233.97 ± 3.65	283.15 ± 1.98	340.07 ± 2.88*
8	p-Coumaric acid	53.11 ± 3.33	81.56 ± 5.15	101.34 ± 1.21	85.53 ± 1.83
9	Ferulic acid	33.36 ± 2.23	10.16 ± 1.65	33.36 ± 3.68	15.46 ± 5.43
10	3,5-Dicaffeoylquinic acid	4.56 ± 5.26	1.84 ± 4.53	2.23 ± 6.11	4.62 ± 4.62
11	Ellagic acid	8510.22 ± 4.97	8523.80 ± 5.07	4971.29 ± 2.66	9330.27 ± 0.84****
Flavonoids					
(A) Anthocyanins					
12	Delphinidin 3,5 diglucoside	101.52 ± 3.54	114.01 ± 1.34	39.76 ± 6.05	78.33 ± 1.74
13	Cyanidin-3-glucoside	ND	ND	1.29 ± 0.61	28.60 ± 1.07
(B) Flavonols					
14	Rutin	5.66 ± 4.96	5.31 ± 2.77	2.94 ± 3.34	6.06 ± 5.08
15	Isoquercitrin	124.16 ± 5.59	143.22 ± 1.55	54.38 ± 4.29	93.83 ± 3.02
16	Quercitrin	18.26 ± 1.34	29.19 ± 2.54	7.46 ± 2.45	13.09 ± 3.09
17	Kaempferol-3-glucoside	10.27 ± 1.69	16.88 ± 2.87	4.42 ± 1.90	23.06 ± 3.22
18	Quercetin	2365.77 ± 2.76	1516.11 ± 4.55****	723.81 ± 5.05	1025.50 ± 3.43***
19	Isorhamnetin	1472.02 ± 5.04	938.24 ± 2.38**	970.01 ± 6.70	1239.0 2 ± 3.83**
20	Hyperoside	109.47 ± 2.94	139.46 ± 5.88	39.80 ± 5.66	75.93 ± 3.39
21	Kaempferol	85.48 ± 3.75	85.34 ± 1.57	46.17 ± 1.46	48.86 ± 4.91
(C) Flavan-3-ols					
22	Catechin	0.71 ± 2.86	1.06 ± 1.67	0.18 ± 3.11	0.35 ± 2.07
23	Procyanidin B2	3.33 ± 2.38	9.99 ± 3.44	0.83 ± 3.93	2.22 ± 1.77
24	Procyanidin A2	27.76 ± 3.22	30.34 ± 1.91	22.35 ± 3.33	34.94 ± 1.59
(D) Dihydrochalcones					
25	Phloridzin	10.28 ± 3.11	6.25 ± 2.31	6.62 ± 4.15	7.64 ± 1.29
26	Phloretin	0.27 ± 4.04	0.33 ± 3.03	0.17 ± 3.85	0.37 ± 2.98
(E) Flavanones					
27	Hesperidin	28.14 ± 4.22	31.35 ± 3.77	18.02 ± 2.11	16.79 ± 5.65
Non-phenolic acids					
28	Trans-cinnamic acid	1.58 ± 2.24	68.13 ± 4.34	59.99 ± 1.49	100.92 ± 3.74
Total phenolic content		16 578.68	17 137.05	11 935.75	18 299.24

Relative standard deviation (RSD%) for all compounds ranged from 1.13% to 6.91%. *$P < 0.005$, **$P < 0.001$, ***$P < 0.0005$ and ****$P < 0.0001$ with respect to statistical differences between the same cultivars (Student's *t*-test).
ND, not detected.

Soxhlet extract with values of 6499.3 ± 21.2 and 4671.0 ± 92.9 mg kg^{-1} DE for α and γ-tocopherol, respectively, with clear differences compared to the UAE extract that obtained values of 1860.0 ± 120 and 1182.5 ± 81.5 mg kg^{-1} DE for the α and γ-tocopherol. For the orange flower, there were no differences found in the α-tocopherol content (3487.0 ± 477 for Soxhlet and 2815.5 ± 79.5 mg kg^{-1} DE for UAE) but did in the γ-tocopherol (789.4 ± 4.7 for Soxhlet and 406.3 ± 26.0 mg kg^{-1} DE for UAE).

Carotenoids compounds were found in lower concentrations in yellow extracts compared to orange flowers, especially β-carotene (377.3 ± 46.5 mg kg^{-1} DE in YT Sox, 2701.4 ± 21.9 mg kg^{-1} DE in OT Sox and 1611.1 ± 1.8 mg kg^{-1} DE in OT UAE), which could not be detected in the UAE extract. The presence of lutein is well known in the flower of *T. erecta*, which in turn has been used as a source for its extraction. In our extracts, the quantities detected showed differences depending on the two extraction methods, 1213.9 ± 8.7 mg kg^{-1} DE in YT Sox and 907.1 ± 15.2 mg kg^{-1} DE in YT UAE ($P < 0.005$), and 1368.5 ± 11.6 mg kg^{-1} DE in OT Sox and 1172.8 ± 9.4 mg kg^{-1} DE in OT UAE ($P < 0.01$).

In vitro antioxidant and antiradical activity

Two different antioxidant methods were used to assess the reducing capacity of these four extracts, results are given in Fig. 4. The radical scavenging activity was determined firstly by the DPPH assay, obtaining inhibition values of 26.49 ± 1.17 and 25.34 ± 1.41 μg mL^{-1} for yellow Soxhlet and UAE extracts, respectively, and 17.96 ± 0.88 and 22.64 ± 0.089 μg mL^{-1} for the orange Soxhlet and ultrasound extracts, respectively. In the yellow cultivar

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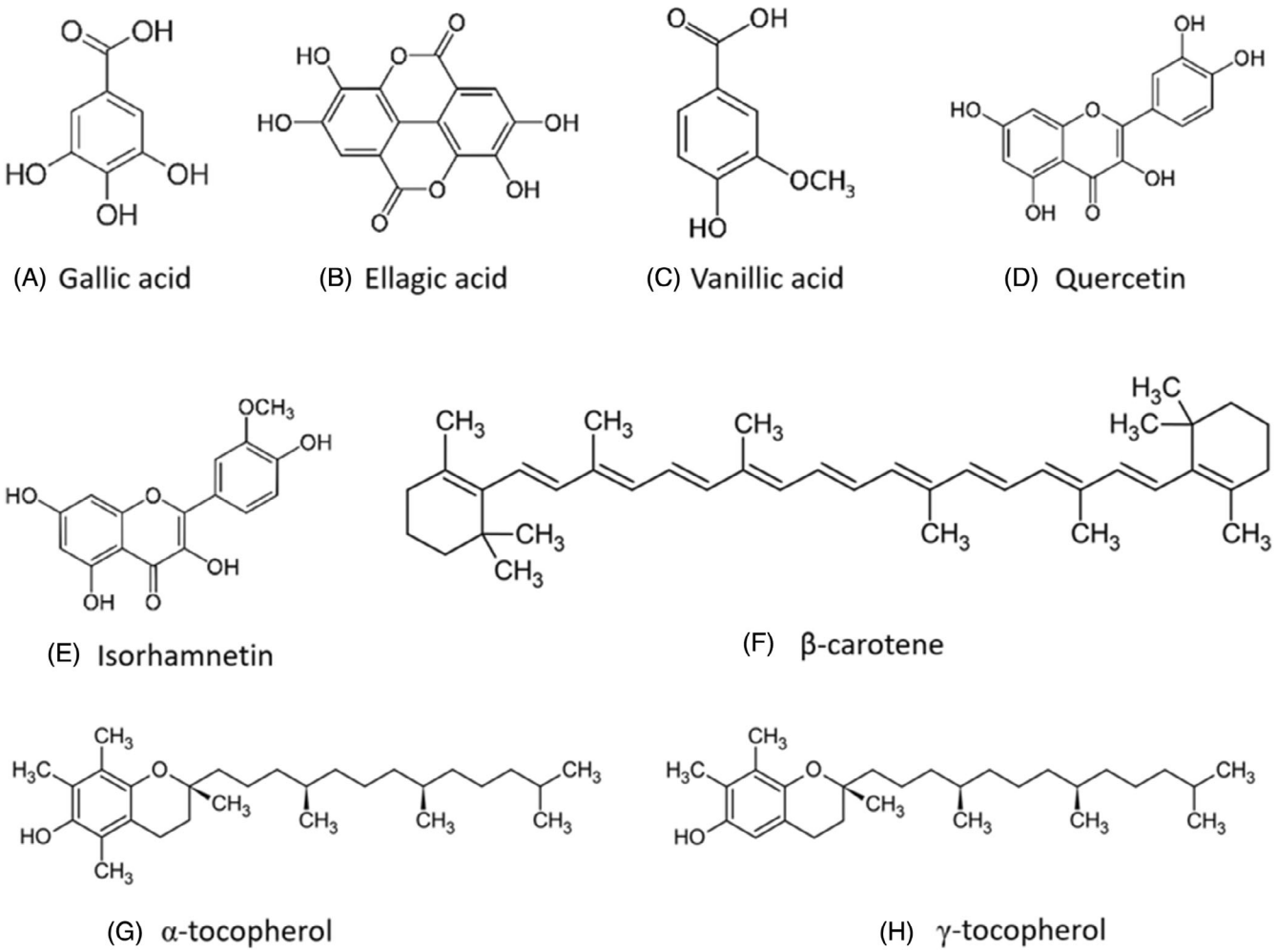

Figure 2. Structures of the main compounds found in the extracts: phenolic acids, flavonoids, carotenes and tocopherols.

both extracts obtained similar values, with UAE being slightly lower, whereas, for the orange flowers, Soxhlet extract showed a lower IC_{50} ($P < 0.05$).

This behaviour of the extracts is maintained in the xanthine/xanthine oxidase assay, where both yellow extracts showed similar activity (UAE slightly more active), whereas orange extracts displayed statistical differences ($P < 0.05$) with Soxhlet also having the lower IC_{50}. The values obtained in the X/XO assay were 19.96 ± 1.85 and 18.3 ± 1.71 μg mL^{-1} for yellow Soxhlet and UAE, respectively, and 15.11 ± 0.49 and 21.69 ± 1.83 μg mL^{-1} for orange Soxhlet and UAE, respectively.

In vitro antidiabetic and anti-obesity activity

The antilipemic potential of the different extracts of *T. erecta* was evaluated through the *in vitro* inhibition of pancreatic lipase. The obtained IC_{50} values can be observed in Fig. 5(A), where no differences were found among the same cultivar extracts (YT Sox 397.1 ± 30.1 μg mL^{-1}, YT UAE 334.1 ± 39.5 μg mL^{-1}, OT Sox 319.1 ± 51.7 μg mL^{-1} and OT UAE 373.6 ± 40.8 μg mL^{-1}), although there were significant differences compared to reference drug orlistat 27.7 ± 15.3 μg mL^{-1} ($P < 0.01$). For the glucosidase inhibition (Fig. 5B), the values obtained revealed no differences among the extracts (YT Sox 391.7 ± 31.6 μg mL^{-1}, YT UAE 292.3 ± 34.3 μg mL^{-1}, OT Sox 342.5 ± 36.6 μg mL^{-1} and OT UAE 288.7 ± 19.5 μg mL^{-1}), nor with the reference drug acarbose (297.9 ± 41.3 μg mL^{-1}).

To evaluate whether the extracts were able to avoid the formation of AGEs (Fig. 5C), their activity was compared to reference drug aminoguanidine (AMG), obtaining much higher activity with statistical differences.[40] AMG obtained an IC_{50} of 73.9 ± 7.7 μg mL^{-1}, whereas the extracts showed 25.3 ± 3.3 μg mL^{-1} for YT Sox, 36.7 ± 2.4 μg mL^{-1} for YT UAE, 29.8 ± 4.9 μg mL^{-1} for OT Sox and 32.4 ± 3.9 μg mL^{-1} for OT UAE. No differences were found between the orange cultivar extracts but there were differences between the yellow ones ($P < 0.05$).

DISCUSSION

The first step for the investigation of any plant or natural product in terms of bioactive compounds is extraction and, depending on the technique used, the sample extracted will be different. The obtention of natural extracts has changed through the years, but Soxhlet has remained one of the main methods, comprising a well-established model for the comparison of new extraction alternatives.[41] The most common factors affecting the outcome of the extracts are the own properties of the plant, the solvent used, the temperature reached, the pressure employed and the time spent. Inflorescences of the same species may have different flower colouring, but these changes can affect their organoleptic

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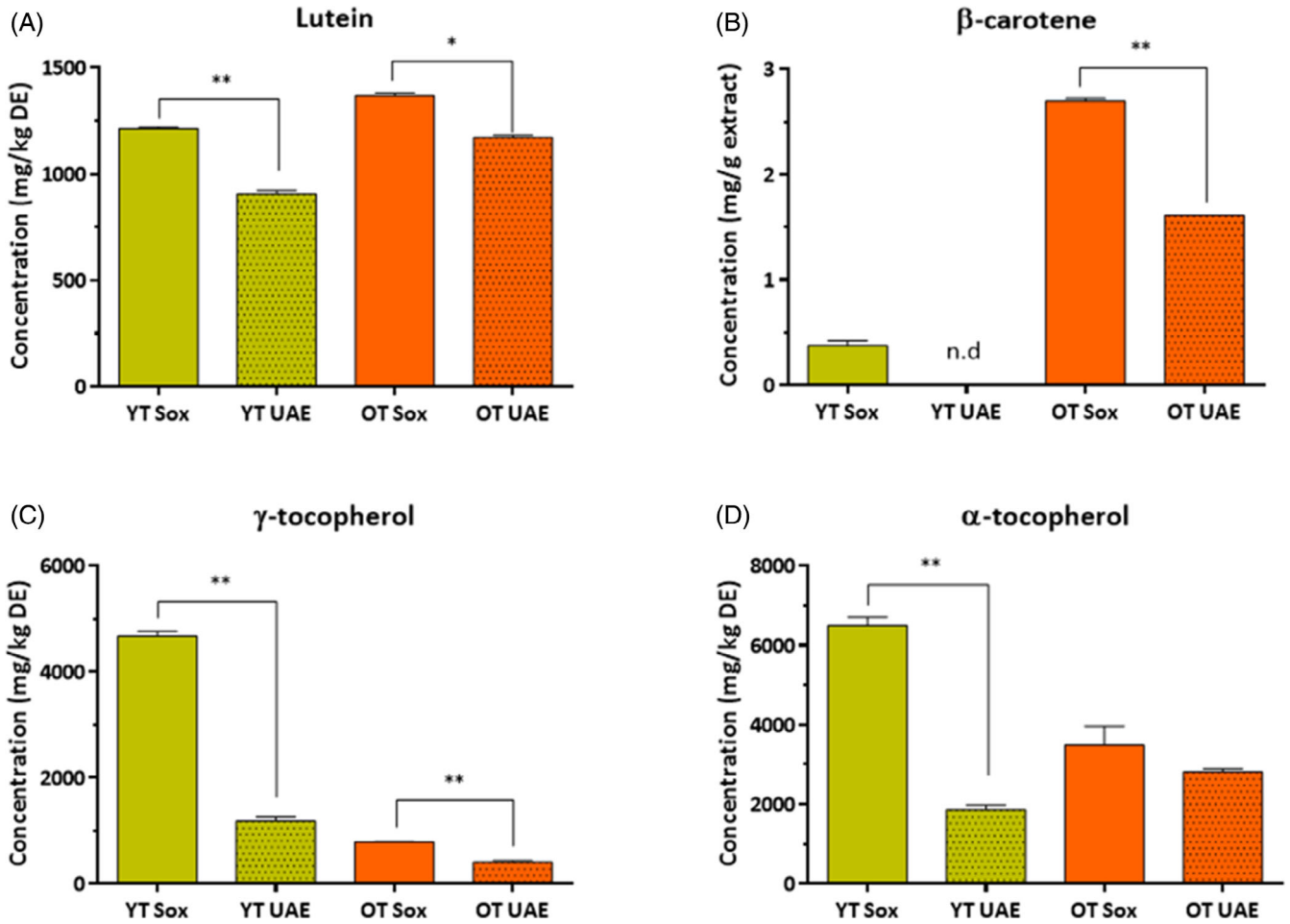

Figure 3. Carotenoid and tocopherols content in the flower extracts expressed as mg kg^{-1} dry extract (DE) (mean ± SEM). (A) Lutein, (B) β-carotene, (C) γ-tocopherol and (D) α-tocopherol. Statistic differences between extracts of same flower obtained by different methods calculated with *t*-student, * $P < 0.05$, ** $P < 0.005$. n.d, not detected.

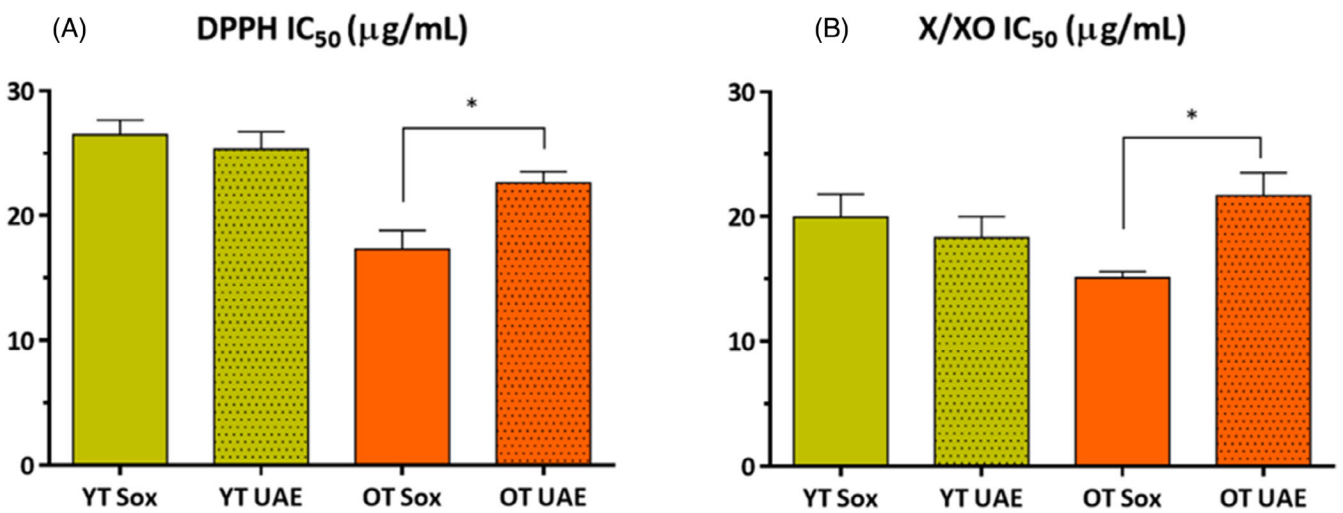

Figure 4. Antioxidant and antiradical scavenging activity of the different flower extracts expressed as IC_{50} value (μg mL^{-1}) (mean ± SEM of three independent replicates). (A) DPPH and (B) xanthine/xanthine oxidase assay. The reference drug used was gallic acid for both assays; IC_{50} values not shown. (A) 0.471 ± 0.079 μg mL^{-1} and (B) 0.0036 ± 0.008 μg mL^{-1}. Differences between extraction methods for the same flower were calculated by Student's *t*-test. * $P < 0.05$. YT, yellow *Tagetes*; OT, orange *Tagetes*.

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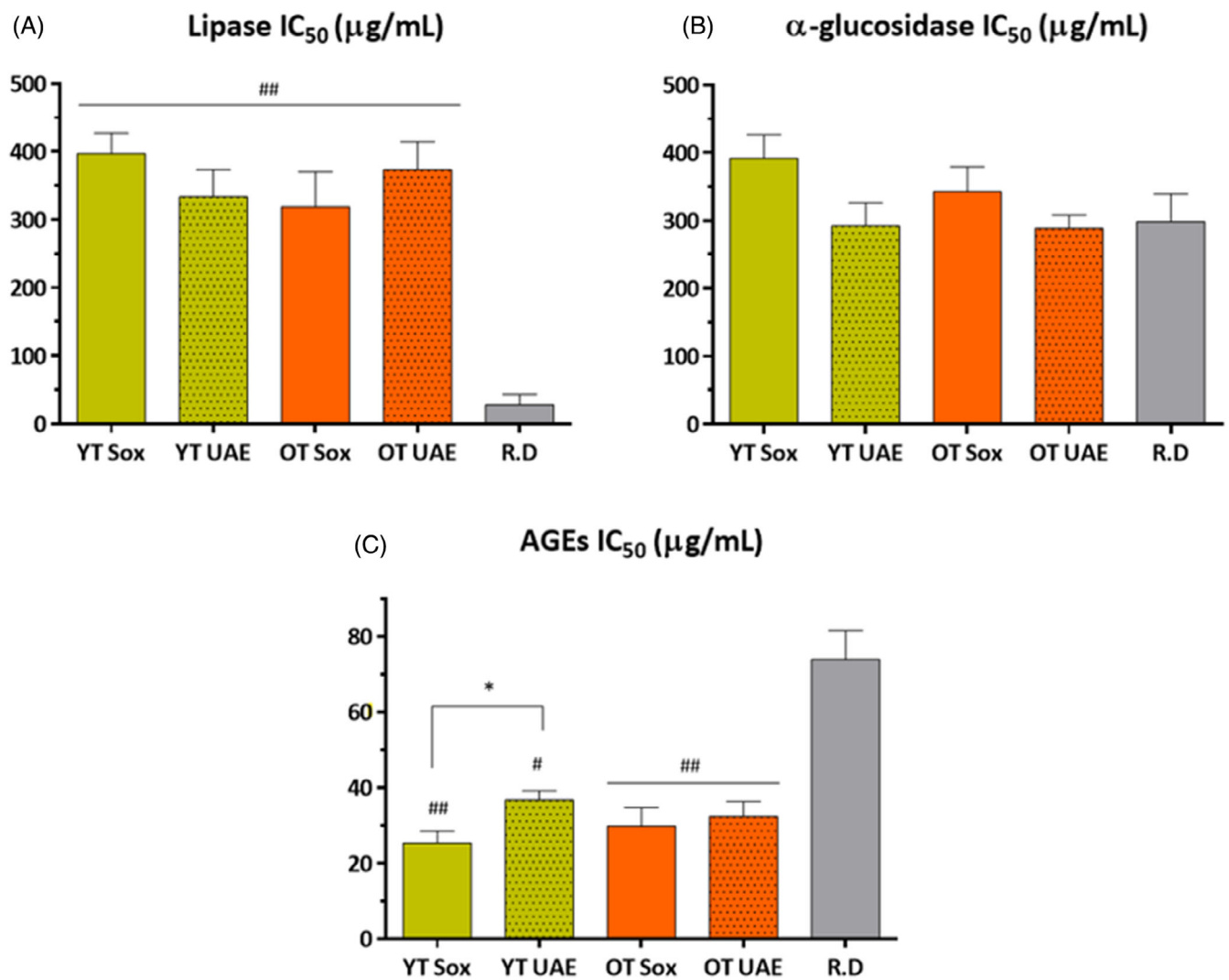

Figure 5. Antidiabetic and anti-obesity activity of the different flower extracts expressed as IC_{50} value (µg mL^{-1}) (mean ± SEM of three independent replicates). *In vitro* inhibition of (A) pancreatic lipase, (B) α-glucosidase and (C) advanced glycation end products. Reference drug (R.D) used as control: (A) orlistat, (B) acarbose and (C) aminoguanidine. Differences between extraction methods for the same flower cultivar were calculated by Student's *t*-test. $^{*}P < 0.05$ between cultivars; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* Reference Drug. YT, yellow *Tagetes*; OT, orange *Tagetes*.

and biological properties. Therefore, because the solvent used for the obtention of the extracts is the same, the differences in the final matrix's properties lie in the time spent, the temperature reached and the physicochemical procedure.

During ultrasound extraction, the solvent is constantly in contact with the flower as opposed to Soxhlet extraction, where the solvent is in contact for a short period depending on the evaporation speed and the number of cycles reached. At high temperatures, the degradation of some compounds such as polyphenols might take place entailing the extraction of polyphenol derivates or different quantities, as we have seen in the HPLC-MS/MS study, where UAE obtained higher values of these compounds compared to the same cultivar in Soxhlet. For all these reasons, the yield of extraction and the polyphenol content present in the extracts are higher for those obtained by UAE than by Soxhlet.

On the other side, the quantities of lipophilic pigment compounds such as carotenes and tocopherols were higher in the Soxhlet extraction. Compounds such as pigments (carotenes and tocopherols) are lipophilic and, depending on the solvent used for the obtention of the extract and other parameters such as temperature, the yield of obtention can vary considerably.[23] The obtention of the extracts was made using ethanol as solvent, comprising a polar solution with limited solubility for lipid molecules that can be increased at higher temperatures.[42] The differences in carotenoid content between the two cultivars are a result of the coloring of the petals, with yellow flowers presenting a lower concentration of pigments such as β-carotene.[28]

The studied antioxidant activities show results similar to previous studies of *T. erecta*.[33,43] However, when compared with other Soxhlet extractions of marigold flower, these extracts showed higher antioxidant power.[44] Regarding the antilipolytic and antidiabetic activity, the results of lipase and α-glucosidase inhibitions are consistent with previous studies.[45-47] Regarding AGEs inhibition, this particular property of *T. erecta* was recently reported and the extracts showed higher inhibitory activity.[46] More recent works with *T. erecta* flowers reveal the potential of these flowers as anti-inflammatory agents, in which these bioactive compounds, and particularly polyphenols, might be involved in the mechanism of action.[48,49]

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Observing the differences between same cultivars, yellow flowers showed similar bioactivity, although there were some differences in the compounds quantified by the two extraction methods. Meanwhile, in the orange cultivar, the best antioxidant and antiradical activity was presented by the OT Soxhlet extract, potentially a result of the high presence of carotenoids (lutein and β-carotene), which are well-known as antioxidants.[18]

Vitamin deficiency, mainly vitamin A, is a major issue not only in lower income countries, but also in developed countries where the population has increased the prevalence of unhealthy practices such as fast-food ingestion and a sedentary lifestyle.[21,50] Vitamin A is an essential nutrient required for good vision, growth, healthy bones, immunity and correct cellular functioning.[25,51] Another important nutrient group is vitamin E, which is involved in a wide range of physiological processes such as immune function, inflammation control and cognitive performance.[22] Furthermore, low values of vitamin E and carotenes in blood are associated with obesity and metabolic disorders.[52] Because daily vitamin A consumption in developed countries does not reach the 70% of retinol equivalents, the ingestion of natural products rich in vitamin A precursors such as carotenoids present in *T. erecta* could help to reach these recommended levels, thus preventing health problems.

A very interesting way to incorporate these phytochemicals with important biological and nutritional applications would be the use of *T. erecta* as a natural colorant. Pigments obtained from fruits and vegetable sources are not only safe for the organism, but also may confer health benefits.[53] The presence of anthocyanins and carotenoids in the flower extracts endows them with certain potential therapeutical properties.[54]

CONCLUSIONS

Both extraction methods, Soxhlet and UAE, yielded bioactive compounds from edible yellow and orange marigold flowers, showing interesting properties as antioxidant, anti-lipolytic and antidiabetic agents. In terms of yield, UAE obtained the best results, as well as for polyphenol content. However, Soxhlet remains as an interesting technique for the extraction of lipophilic pigments such as carotenoids and vitamin E precursors (tocopherols).

Edible flowers of *T. erecta* could present a dual utility, serving as natural colorant additives that give interesting colours to foods at the same time as enhancing their nutritional profile because of their rich composition. Alternatively, they may also be consumed by themselves as healthy ingredients.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

AUTHOR CONTRIBUTIONS

S. Núñez and AM Mustafa were responsible for investigations. S. Núñez was responsible for writing the original draft. MS Valero and C Gómez-Rincón were responsible for validation. MS Valero, C Gómez-Rincón and V López were responsible for reviewing and editing. AM Mustafa was responsible for methodology. G Caprioli, F Maggi, C Gómez-Rincón and V López were responsible for supervision. F Maggi and V López were responsible for conceptualization.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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